INSTITUTO DE QUÍMICA

UNIVERSIDADE DE SÃO PAULO

QUIMILUMINESCÊNCIA:

SISTEMAS GERADORES, MECANISMOS E APLICAÇÕES

LUIZ HENRIQUE CATALANI

TESE DE LIVRE-DOCÊNCIA

SÃO PAULO

1996

Dedicatória

Dedico esta tese aos meus dois amores,
Célia e Felipe.

Agradecimentos

Para não cometer injustiças, não vou citar nominalmente as pessoas que colaboraram no desenvolvimento de minha carreira e, assim, desta tese, pois certamente esqueceria de alguém. Originadas de São Paulo, Würzburg e Boston, hoje elas estão espalhadas em vários cantos do mundo. Espero poder ter retribuído de alguma forma suas mais diversas contribuições.

Agradeço também às agências que financiaram meu trabalho que foram: FAPESP, CNPq, CAPES, FINEP, BID-USP e Fundação Alexander von Humboldt.

Apresentação

Compreendo que uma Tese de Livre-Docência, como é formulada nos dias atuais, deve sumariar para leitor o trabalho desenvolvido pelo candidato dentro do contexto do seu campo de pesquisa. Logo, o texto aqui apresentado não tem a pretensão de ser uma revisão do assunto, ainda que apresente uma visão ampliada. Assim, as referências indicadas tem o intuito de ajudar o leitor a dirimir dúvidas sobre pontos que mereceriam uma maior reflexão.

Não querendo ressaltar o óbvio, esta tese apresenta resultados de três linhas de pesquisa distintas, com pouca ou nenhuma inter-relação. Assim, os assuntos foram apresentados em três capítulos separados. Resolvi intitular a tese pelo primeiro capítulo por razões de volume e até sentimentais, já que iniciei de minha carreira nesta área, e não reflete, necessariamente, uma preferência atual.

ÏNDICE

Capítulo 1: QUIMILUMINESCÊNCIA: SISTEMAS GERADORES, MECANISMOS E APLICAÇÕES

Introdução

Etimologicamente a composição da palavra quimiluminescência pode parecer antagônica: o prefixo ***quimi*** teria origem no termo egípcio ***chem*** que significaria negro[1] (daí alquimia ser conhecida como *Arte Negra*), enquanto ***luminescência*** é originário do latim ***lumen***, que ilumina, luz. Este termo foi originalmente cunhado por Wiedmann[2] para descrever processos luminosos resultantes de reações químicas. Recentemente, foi publicado uma excelente revisão destacando a classificação moderna dos diversos tipos de quimiluminescências[3].

De uma maneira simples, quimiluminescência é a conversão da exotermicidade (ΔG) de uma reação em energia de excitação eletrônica. Também pode ser vista como o reverso de uma reação fotoquímica. Em ambos os casos, o passo chave é a passagem da superfície do estado fundamental para a superfície do estado excitado (e *vice-versa*).

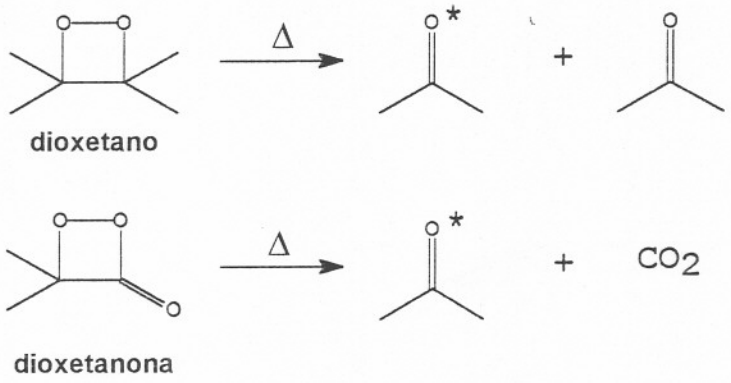
Dentre as reações quimiluminescentes conhecidas, a termólise de dioxetanos tem se destacado por várias razões: (i) os rendimentos de formação de espécies excitadas são altos, chegando a 50%[4]; (ii) dioxetanos são postulados como os intermediários em reações bioluminescentes e em outras responsáveis por "fotobiologia no escuro"[4]; (iii) derivados dioxetânicos tem sido utilizados em diversas aplicações analíticas, revolucionando algumas áreas de análises clínicas como a de imunoensaios[5].

Aspectos químicos de dioxetanos

Cálculos termoquímicos, baseados em valores tabelados de energias de ligação ou em valores de entalpias de formação de grupos atômicos, mostram que a clivagem térmica de compostos dioxetânicos é suficientemente exotérmica para excitação eletrônica de uma das moléculas-produto. Enquanto a exotermicidade da reação (ΔH^{o}_{r})

é da ordem de 80 a 100 kcal/mol, a excitação de aldeídos e cetonas aos respectivos estados singlete ou triplete requer em geral menos de 85 kcal/mol.

Esquema 1



Mecanismo de clivagem de dioxetanos

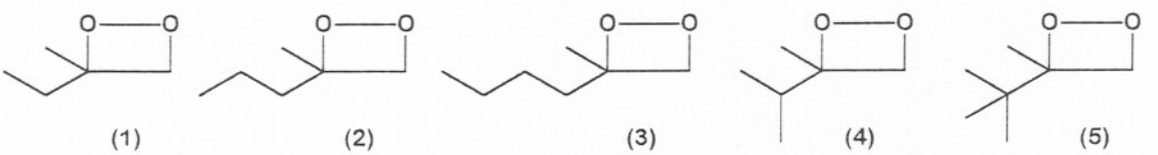
Através do estudo sistemático de como parâmetros de ativação de termólise, rendimentos quânticos e reatividade química são afetados por efeitos estéricos e eletrônicos de substituintes, efeito de solventes e de aceptores fluorescentes adicionados[6], tem-se procurado, nos últimos anos, explicar quais os fatores estruturais que determinam a estabilidade do dioxetano, o seu mecanismo de clivagem e a distribuição de energia de excitação disponível na termólise em energia singlete e triplete dos produtos.

Mesmo antes do isolamento do primeiro dioxetano McCapra[7] já havia postulado que a clivagem concertada de um dioxetano deveria ser quimiluminescente. Segundo este mecanismo, durante a clivagem do dioxetano, a quebra das ligações O-O e C-C ocorrem simultaneamente à formação das carbonilas. Este mecanismo foi proposto com base a diagramas de correlação de simetria de orbitais e de estado, os quais mostraram que a configuração eletrônica do dioxetano no estado fundamental somente poderia ser correlacionado com um estado eletronicamente excitado do produto carbonílico. Os cálculos de Kearns[8] previam ainda que deveria ocorrer formação preferencial de espécies triplete na termólise. Hoje, entretanto, o mecanismo de clivagem que mais satisfaz os valores experimentais de energia de ativação (E_a) determinados para dioxetanos substituídos por grupos alquila, alcoxila, acila e homoarila é o mecanismo em etapas *via* diradical[9]. Este mecanismo explica os efeitos

de substituição e da natureza do solvente nos valores de E_a dos dioxetanos correspondentes e ainda satisfaz a cálculos teóricos e semi-empíricos posteriores aos de Kerns e McCapra.

Uma colaboração nossa nesta área foi a demonstração, através do estudo de uma série de dioxetanos alquílicos 3,3-dissubstituídos (Esquema 2; **ANEXO I**[10]), de que interações estéricas entre substituintes geminais 3,3 aumentam a energia de ativação de termólise sem, contudo, afetar substancialmente o $\Delta S^{\#}$. Isto sugere que interações estéricas são mais importantes no estado de transição, que é consistente com o mecanismo de clivagem via formação de um diradical. Estudos anteriores[11] demonstraram que tais parâmetros são insensíveis a interações de substituintes 3,4, corroborando estas afirmações.

Esquema 2



Quimiluminescência *via* transferência de elétrons

Para o caso de dioxetanonas ou dioxetanos contendo grupos de baixo potencial de oxidação (heteroaromáticos policondensados) ou que reajam com compostos de baixo potencial de oxidação adicionados ao sistema (hidrocarbonetos aromáticos policondensados), McCapra[12] e Schuster[13] propuseram, independentemente, um mecanismo de clivagem iniciado por transferência de elétrons ("Chemically initiated electron exchange luminescence", CIEEL). Nestes casos aqueles autores verificaram que a velocidade e eficiência de emissão de luz aumenta linearmente com o decréscimo do potencial de oxidação do hidrocarboneto ("sensibilizador") adicionado.

Este mecanismo, bastante sedutor, foi recebido com entusiasmo pelos pesquisadores da área e passou a ser aceito como o mecanismo responsável pelo passo de quimiexcitação na grande maioria das bioluminescências. De fato, em reações bioluminescentes as moléculas-substratos invariavelmente contém um grupo de baixo potencial de oxidação atado ou não à ligação peroxídica, provavelmente responsável por um mecanismo CIEEL intramolecular.

Em contrapartida, dioxetanos com substituintes simples, como grupos alquila e arila, não sofrem decomposição por este tipo de mecanismo. Numa revisão de 1985, Wilson[14] destaca as principais generalizações obtidas dentre os exemplos em que tal esquema se aplica e conclui que "ainda é cedo para se esperar que CIEEL seja um mecanismo provado ao invés de ser apenas uma fascinante hipótese".

Um dos pontos fracos apontados por Wilson é o que diz respeito a 'correlações lineares de energia livre'. As projeções de ln k_{CAT} vs E_{ox} (neste caso E_{ox} equivale à diferença de energia livre do sistema) fornece tg = $-\alpha/2.3RT$ onde α é um parâmetro empírico ($0<\alpha<1$). Dentre todos os casos estudados α é próximo a 0.3. Scandola e colab.[15] argumentaram longamente sobre o significado de α e concluiram que o valor 0.3 é compatível com a transferência total de um elétron no passo limitante da reação. Porém, como bem observou Wilson, tais argumentos não conseguem descartar a hipótese de transferência parcial de carga.

Um outro ponto bastante obscuro é o que diz respeito aos rendimentos quânticos envolvidos no passo bimolecular da reação (ϕ_{CIEEL}). A análise dos dados publicados revela que na maioria dos casos os valores de ϕ_{CIEEL} foram calculados em condições onde a decomposição bimolecular corresponde a apenas 20-30% do total do processo, i.e. $k = 4k_{CAT}[ACT]$. Em um trabalho conjunto com Wilson (**ANEXO II**[16]), pudemos constatar que os valores calculados para ϕ_{CIEEL} do sistema difenoilperóxido/ perileno foram inicialmente *superestimados por quatro ordens de grandeza*. Isto pôs por terra o sistema "carro-chefe" de toda a hipótese. Além disso, demonstramos um outro exemplo de decomposição de dioxetano via CIEEL onde os rendimentos de espécie excitada são da ordem de 10^{-4}. Os demais exemplos de CIEEL conhecidos carregam diversas complicações experimentais na determinação de ϕ_{CIEEL}. Com valores tão baixos a proposta de CIEEL intermolecular como mecanismo capaz de gerar quimiluminescência como passo principal perdeu força e deve ser revista.

Para complicar ainda mais o quadro, Adam e colab.[17] recentemente demonstraram a redução de dioxetanos simples aos seus diois, por uma série de compostos (fenotiazinas, tiois, NADH, etc) via SET ("single electron transfer"). Os autores observaram que a clivagem gerando os compostos carbonílicos é uma reação competitiva importante na maioria dos casos. Em um trabalho recente (**ANEXO III**[18]), descartamos definitivamente a possibilidade de decomposição de dioxetanos via SET

para os casos onde o redutor é um sulfeto orgânico, ou seja, a maioria daqueles apontados por Adam.

Quimiluminescência indireta - Transferência de energia eletrônica

Os rendimentos quânticos de produção de espécies singlete ($^1\phi$) e triplete ($^3\phi$) podem ser determinados[19] por (i) medidas de quimiluminescência direta ($^1\phi$, em solução aerada e $^3\phi$, em solução deaerada), (ii) medidas de emissão fluorescente de agentes "sensibilizadores" adequados adicionados á solução, os quais funcionam como aceptores específicos da energia singlete ou triplete das moléculas-produto excitadas, e (ii) "contagem química", baseada na dosagem de fotoprodutos originados de conversões fotoquímicas do produto excitado ou quando originados de reações com aceptores convenientes adicionados ao meio.

A baixa eficiência de emissão direta e as dificuldades experimentais na escolha de contadores químicos popularizaram a utilização do 9,10-difenilantraceno, DPA, e do 9,10-dibromoantraceno, DBA, como "contadores" de singlete e tripletes, respectivamente. A excitação eletrônica destes aceptores dá-se via mecanismo de Dexter (transferência de elétrons), porém, o mecanismo pelo qual DBA gerava singletes a partir de carbonilos triplete sempre foi matéria de polêmica. Muitos autores consideravam uma transferência $triplete_{doador} \rightarrow singlete_{aceptor}$ possível, enquanto outros preferiam a hipótese de uma transferência $triplete_{doador} \rightarrow 2^o\ triplete_{aceptor}$ seguido de cruzamento intersistema interno ao 1º singlete do DBA[20].

Inicialmente verificamos a possibilidade de transferência de energia para níveis energéticos superiores (S_n) utilizando azuleno e xantiona (**ANEXO IV**[21]), uma vez que ambos exibem fluorescência a partir de estados S_2. Assim, a termólise de tetrametildioxetano em presença de azuleno resultou em emissão idêntica a fluorescência $S_2 \rightarrow S_0$ determinada por fotoexcitação. Supressores específicos de acetona triplete não causaram mudança na intensidade de emissão, de onde se concluiu que acetona S_1 formada transferiu energia de excitação diretamente ao S_2 de azuleno. Resultados semelhantes foram obtidos para xantiona.

A seguir, a partir uma série de acetofenonas com níveis de energia triplete próximos do segundo triplete do DBA pudemos provar de forma definitiva que, também

neste caso, a transferência de energia de tripletes se dá inicialmente para um triplete superior, seguido de cruzamento intersistema do aceptor (**ANEXO V**[22]). Utilizando da técnica de fluorescência resolvida no tempo, Wilson[23] desenvolveu uma metodologia simples de obtenção de valores de ϕ_{TS}, que é o parâmetro que correlaciona o total de tripletes obtidos contra o total de espécies suprimidas para um processo de transferência de energia ($\phi_{TS} = k_{TS}/k_{ET}$). Os resultados mostraram uma dependência de ϕ_{TS} com a temperatura que determinava o valor de energia exato do triplete superior que é inicialmente formado, *ca.* 4 kcal/mol acima do S_1.

O sucesso do DBA e DPA como "contadores" de triplete e singlete nos estimulou a desenvolver sondas idênticas para solventes aquosos. Assim, sintetizamos os seus derivados sulfonados, o 9,10-dibromo- e 9,10-difenilantraceno-2-sulfonato de sódio (DBAS e DPAS, respectivamente), determinamos seus parâmetros químicos e fotofísicos, assim como de transferência de energia de acetona excitada para os mesmos (**ANEXO VI**[24]). A partir de então, seu uso foi popularizado, principalmente entre a comunidade que estuda sistemas quimiluminescentes em sistemas biológicos.

A termólise de dioxetanos gera carbonilos triplete e singlete, e portanto, a definição exata dos mecanismos envolvidos nas reações pós-termólise é de importância incontestável. Wilson[23] constatou uma dependência com natureza do solvente utilizado dos valores de ϕ_{TS} de acetona triplete para DBA. Em solventes aromáticos como benzeno e tolueno, a excitação do DBA se dava a partir de um complexo excitado entre a acetona triplete e uma molécula do solvente. Em um trabalho em colaboração (**ANEXO VII**[25]), demonstramos que com a utilização de aromáticos polimetilados, como mesitileno e dureno, a intermediação de um complexo excitado ou do aromático triplete (formado por transferência de energia) era indistinguível, re-posicionando a questão do tratamento da reatividade de carbonilos triplete em solventes aromáticos.

Uma vez que dioxetanos e dioxetanonas são fontes convenientes de carbonilos triplete e, como visto, podem ser facilmente monitorados em solução através do uso de DBA, estes compostos tem sido largamente utilizados no estudo de efeitos das características dos aceptores sobre o mecanismo de transferência de energia operativo[26]. Também contribuímos para esta discussão quando, através da seleção de várias classes de compostos de importância biológica, determinamos os mecanismos de supressão de acetona triplete por estas classes em meio aquoso (**ANEXO VIII**[27]).

Com o intuito inicial de comparar um sistema químico de produção de acetona triplete (a termólise do tetrametildioxetano) contra um sistema enzimático (isubutiraldeído/ O_2/ peroxidase, vide item abaixo sobre "fotobioquímica no escuro"), testamos a ação de (i) pigmentos xantênicos, (ii) quinonas, (iii) derivados do indol e (iv) flavinas como supressores de acetona triplete.

Reatividade de dioxetanos

Estudos da decomposição térmica de dioxetanos levaram a descoberta de vários modos de reação destes compostos. Assim, reações de inserção, redução, decomposição catalisada por íons metálicos e outros compostos foram observadas sem o acompanhamento de produção de estados excitados.

A inserção de compostos de fósforo trivalente na ligação peroxídica é uma das mais estudadas reações deste tipo. Em 1973, Bartlett e colab.[28] observaram a formação de uma fosforana na reação entre trifenilfosfina e o tetrametildioxetano em hexadeuterobenzeno com alto rendimento. Sob aquecimento, a fosforana se decompõe em trifenilfosfinóxido e o epóxido correspondente.

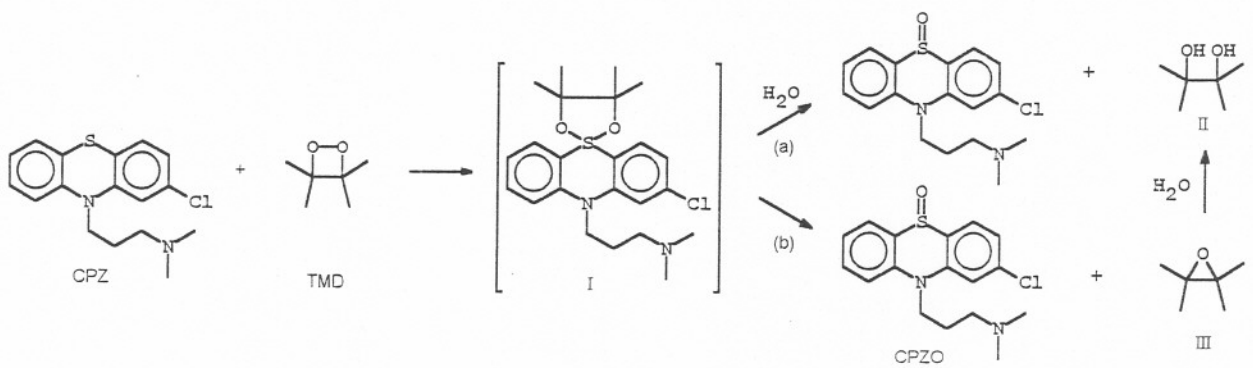
Posteriormente, verificou-se que as reações de tetrametildioxetano com metildifenilfosfito, trifenilfosfito e trietilfosfito também produzem fosforanas com razoáveis rendimentos. Alguns pesquisadores tem se utilizado destas reações como métodos de preparação de fosforanas[5]. As reações entre tetrametildioxetano e trifenilarsênio, trifenilantimônio e trifenilbismuto também foram testadas. As reações com derivados de arsênio e antimônio mostraram a formação de um aduto de inserção do mesmo tipo das fosforanas. A reação com derivado de bismuto apresentou, apenas, a decomposição catalítica do dioxetano à acetona[5].

Reações de dioxetanos com compostos divalentes de enxofre também foram investigadas[29]. A reação de trimetildioxetano com difenilsulfeto produziu, à temperatura ambiente, difenilsulfóxido e o óxido de trimetiletileno, provavelmente *via* formação de uma sulfurana como intermediário.

A reação da clorpromazina, um conhecido antiemético e sedativo, com tetrametildioxetano gera, igualmente, o sulfóxido e epóxido correspondentes. Neste caso, entretanto, há indícios espectrais positivos da formação da sulfurana I (Esquema 3, **ANEXO III**[18]) . Verificamos que em presença de água a formação do diol II é devida

ou a hidrólise da sulfurana ou do epóxido III, cuja formação foi verificada na ausência de água.

Esquema 3



A sensibilidade de dioxetanos à presença de íons metálicos foi notada durante estudos cinéticos de sua decomposição. Os "efeitos de solvente"[30] observados na termólise de dioxetanos em metanol e acetonitrila eram na verdade resultado da decomposição "escura" de dioxetanos catalisada por traços de íons metálicos contaminantes.

Notadamente, a utilização de dioxetanos como modelo de sistemas bioluminescentes e que produzem "fotobioquímica no escuro" encontrou resistência exatamente pela necessidade do uso de sistemas aquosos como solventes. Com o desenvolvimento de sondas fluorescentes solúveis em água, como DBAS e DPAS, pudemos determinar o verdadeiro papel da água na estabilidade do tetrametildioxetano (**ANEXO VI**[24]). Assim, determinamos parâmetros de ativação e rendimentos quânticos de formação de espécies excitadas em água e meio tamponado.

Dioxetanos podem, também, sofrer decomposição induzida por espécies excitadas[5]. A transferência de energia de uma espécie excitada qualquer ou mesmo dos produtos excitados da sua termólise catalisam a decomposição do dioxetano. Assim sendo, este efeito é especialmente favorecido em altas concentrações do dioxetano e baixas concentrações de oxigênio.

Recentes aplicações de dioxetanos em sistemas analíticos

Nos últimos anos, paralelamente ao desenvolvimento de fotômetros cada vez mais sensíveis e a detecção de reações biológicas geradoras de quimiluminescência

de intensidade ultra baixa, a comunidade científica mundial testemunhou um aumento exponencial da aplicação de técnicas que utilizam quimi- ou bioluminescência como sistema monitor. Dentre as áreas que já se beneficiaram de tais avanços podemos citar: imunologia, microbiologia, biologia molecular, biologia celular, biotecnologia, biomassa, etc.[31] Vários métodos analíticos de determinação de Ca^{2+}, ATP, hormônios e enzimas, os quais utilizam medidas de emissão de luz, já são comercializados e tem sua sensibilidade estimada na ordem de attomoles, ultrapassando a de ensaios que utilizam material radioativo. Em decorrência de sua sensibilidade e simplicidade há uma constante procura, por parte de investidores, de novas idéias e aplicações destas técnicas com óbvias possibilidades lucrativas.

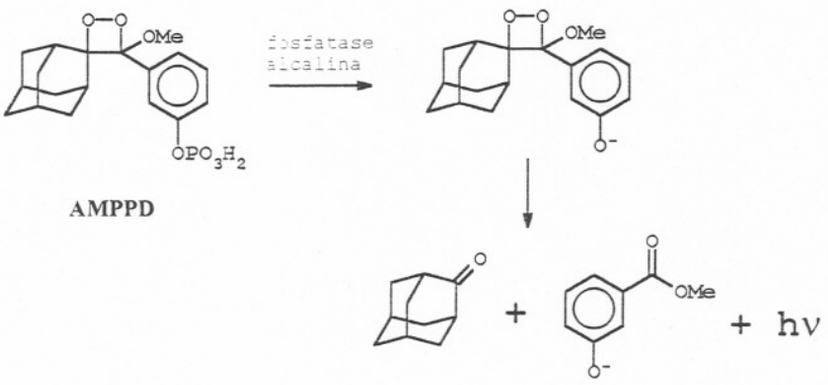
Um número relativamente grande de centros de pesquisa, incluindo diversos laboratórios da iniciativa privada, tem despendido esforços no desenvolvimento de metodologia analítica para a detecção de inúmeras biomoléculas na qual se utilize medida de intensidade de luz como sistema de detecção[32]. A razão é óbvia: facilidade de medição, alta sensibilidade e grande variedade de opções de aplicações. O número de ensaios quimiluminescentes para determinação de nível de enzimas já chega a duas dezenas, enquanto que para os quimiimunoensaios este número chega a meia centena[33].

O maior avanço dos últimos anos na utilização de dioxetanos em aplicações analíticas deu-se através da síntese de uma série de dioxetanos substituídos por grupos fenólicos. Em 1982, Schaap & Gagnon[34] demonstraram que a cinética de decomposição de um dioxetano substituído por um grupo fenólico na presença de base é de *ca.* 10^6 vezes mais rápida que sua forma protonada. A causa deste efeito está no fato de que, enquanto protonado, o dioxetano permanece estável tendo como mecanismo de termólise principal a homólise inicial da ligação O-O com formação de um diradical, como já discutido acima. A desprotonação gera uma carga negativa livre sobre o anel aromático substituinte do dioxetano, em ressonância, portanto, com a carbonila por se formar. Visto de um outro modo, há uma possibilidade de transferência de elétrons do grupo fenolato na direção do anel dioxetânico, promovendo uma decomposição rápida via mecanismo CIEEL intramolecular.

Como conseqüência deste trabalho, anos mais tarde o mesmo grupo produziu uma série de dioxetanos substituídos por fenol protegidos na posição -OH por grupos trialquilsilila[35], acila[36] e fosfatidila[37]. Como esperado, a hidrólise destes grupos dispara

um processo quimiluminescente. Mais interessante ainda foi a demonstração de que a mesma hidrólise pode ser disparada por enzimas hidrolíticas, como a aril-esterase[36] e a fosfatase alcalina[37] como é o caso do 4-methoxi-4-(3-fosfatofenil)spiro[1,2-dioxetano-3,2'-adamantano] (AMPPD; Esquema 4). Abriu-se, assim, imensas possibilidades de aplicação destes sistemas em ensaios analíticos. Muitos outros grupos entraram na corrida do desenvolvimento de novas técnicas interessados nos direitos de propriedade de uso.

Esquema 4



AMPPD

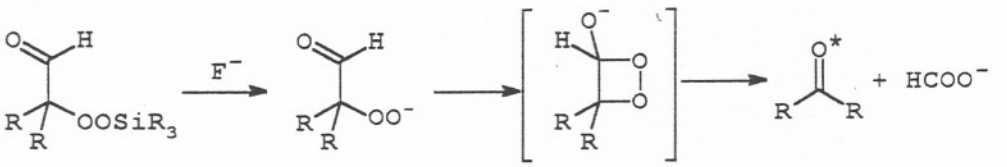
Utilização de α-hidroperoxialdeídos como substratos potencialmente luminescentes

Pode-se observar na literatura[38,39] que considerável atenção tem sido dispendida à reatividade de compostos α-hidroperoxícarbonílicos, que podem agir como precursores de sistemas 1,2-dioxetânicos[40]. α-Hidroperoxicetonas, facilmente obtidas por auto-oxidação das cetonas correspondentes em meio básico, são muitas vezes suficientemente estáveis para serem isoladas. A sua decomposição em meio básico pode conduzir à observação de quimiluminescência via um intermediário 3-hidroxidioxetano[38].

Entretanto, α-hidroperoxialdeídos, considerados intermediários em alguns sistemas quimiluminescentes[38,30] nunca foram isolados, conhecendo-se somente alguns derivados onde a função hidroperóxido está protegida. Em 1989, relatamos a síntese dos primeiros α-sililperóxialdeídos estáveis conhecidos (**ANEXO IX**[41]). Na ocasião, observamos que a hidrólise destes compostos catalisada por fluoreto produzia

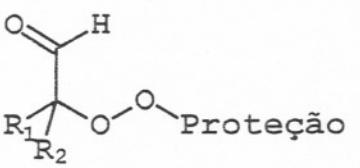
quimiluminescência, revelando-se assim como sistema luminescente disparado por ação hidrolítica externa.

Esquema 5



Aos moldes do bem sucedido sistema do AMPPD, o nosso laboratório está, no momento, empenhado em desenvolver rotas sintéticas de α-hidroperoxialdeídos protegidos por grupos que possam ser removidos por enzimas hidrolíticas de interesse, de forma a tornarem-se alvos de quantificação via medida de luz da reação que se segue.

Esquema 6



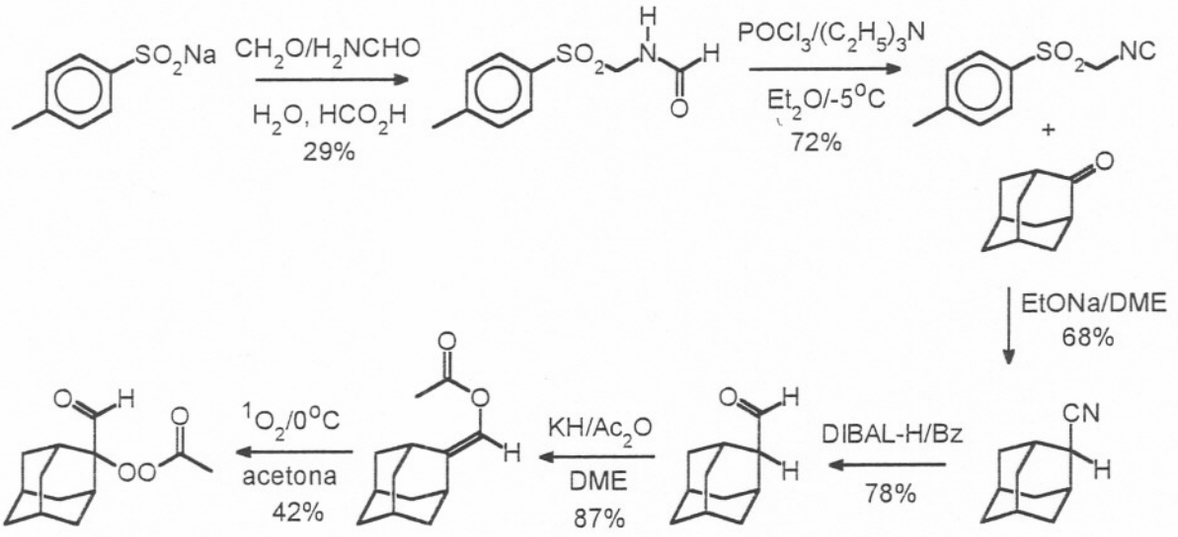
I

Assim, enzimas que hidrolisam ésteres de carbono, como esterases, lipases e muitas outras, poderiam ser quantificadas em testes analíticos pela sua reação com substratos do tipo I (Esquema 6) protegidos por grupos acila, enquanto fosfatases, fosfolipases, etc poderiam igualmente ser analisadas pela reação com substratos protegidos por grupos fosfatidila. Acrescenta-se aqui a vastidão de conjugados de fosfatase alcalina disponíveis no mercado para os mais diversos ensaios, que poderão ter sua sensibilidade muito amplificada pela utilização de medida de luz, atualmente sendo revelados por métodos muito menos sensíveis.

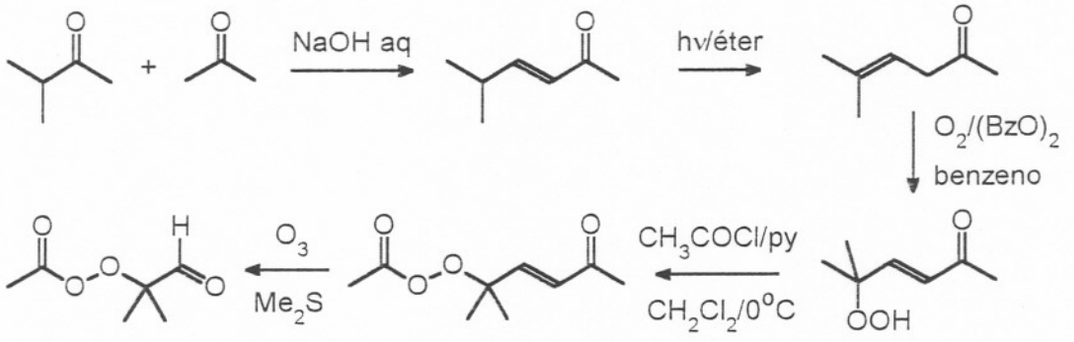
Até o momento, duas rotas distintas foram desenvolvidas com sucesso (resultados não publicados):

Esquema 7

Rota A



Rota B



Em ambas as rotas, a proteção do grupo hidroperóxido é feita por acilação. Assim, pretendemos aplicar tais compostos em sistemas de análise onde o passo de disparo da quimiluminescência é feito por uma esterase. Também vale observar que a rota B tem aplicação genérica, permitindo a síntese de uma ampla gama de estruturas.

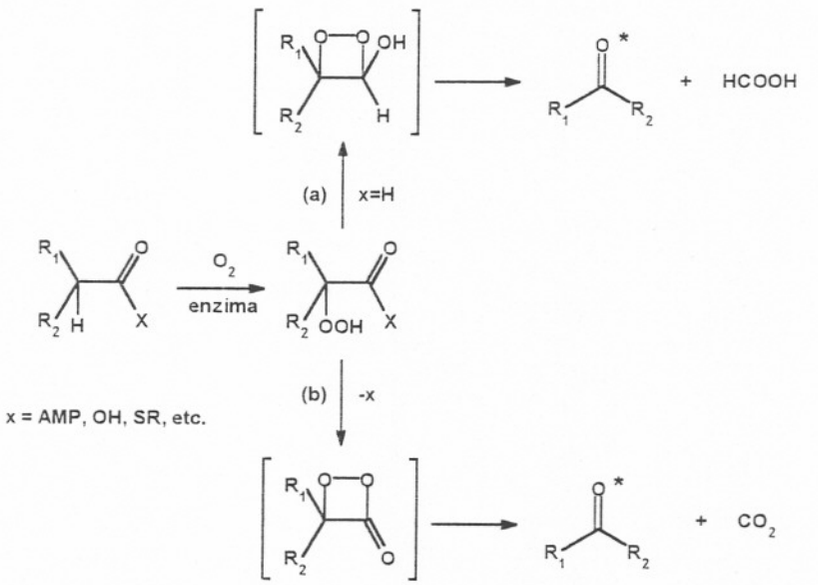
Tanto o desenvolvimento destes caminhos sintéticos quanto sua aplicação em metodologia analítica é de grande interesse econômico e deverão ter seus direitos e previlégios de uso garantidos em sua publicação. Atualmente, este trabalho tornou-se uma das principais linhas de pesquisa em nosso laboratório e é tema da Dissertação de Mestrado do aluno Valdecir F. Ximenes.

Participação de dioxetanos em sistemas bioquímicos

A grande importância dos dioxetanos provém do fato de serem propostos como os intermediários "ricos em energia" de reações bioluminescentes[42,43], de reações responsáveis por "fotobioquímica no escuro"[44], e de algumas reações quimiluminescentes (p. ex., acridinas[45]; peroxioxalatos[46]; aminoetilenos[47]; etc.).

Nas reações bioluminescentes e naquelas que promovem "fotobioquímica no escuro", um substrato contendo um grupo metilênico ou metínico ativado por α-carbonila seria oxidado a um dioxetano ou dioxetanona intermediário através da ação de uma enzima, luciferase ou peroxidase, que atua como dioxigenase interna. Imediatamente, este intermediário dioxetânico seria clivado produzindo dois fragmentos carbonílicos, um deles excitado ao estado singlete (luminescente) ou triplete (em geral, sistemas não emissivos). O mecanismo geral de reação proposto para estes sistemas esta representado no Esquema 8. A via (b) representa genericamente as reações bioluminescentes que ocorrem em vaga-lumes, crustáceos, medusas, anêmonas, etc.[43] Nestes casos, o produto excitado é gerado com alta eficiência no estado singlete (fluorescente), o qual, em seguida decai para o estado fundamental emitindo um fóton.

Esquema 8

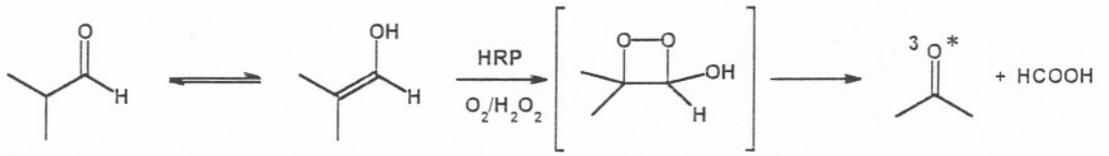


Ambas as vias (a) e (b), podem representar certos processos bioquímicos aeróbicos, catalisados por peroxidases, lipopoxigenases, etc., onde o produto é

gerado, principalmente, no estado triplete. Segundo Cilento[44] e White[49], em virtude da vida média relativamente longa dos estados tripletes e de sua alta reatividade química, tais estados, se produzidos *in vivo*, poderiam promover uma série de conversões tipicamente fotoquímicas em biomoléculas, com conseqüências normais ou lesivas à célula.

A proposta da participação de um intermediário dioxetânico em reações que promovem "fotobioquímica no escuro" parece ser igualmente válida. Por exemplo, a oxidação de isobutanal à acetona e ácido fórmico, catalisada por peroxidase, foi demonstrada por Kenten[50]. Cilento[44] analisou em detalhes este sistema quando catalisado pela peroxidase de raiz forte ("horseradish peroxidase" - HRP), e concluiu que o real substrato desta enzima é a forma enólica do aldeído.

Esquema 9



O espectro de emissão deste sistema mostrou ser o mesmo de acetona fotoexcitada e o da emissão proveniente da termólise de tetrametildioxetano (que produz acetona triplete), ambos em meio deaerado[51]. O caráter triplete da espécie excitada formada foi comprovado, também, por transferência de energia de excitação para aceptores específicos de espécies tripletes, como o íon sorbato e 9,10-dibromoantraceno-2-sulfonato[24,51].

A questão sobre a possível participação de dioxetanos em lipoperoxidação como responsáveis pela quimiluminescência de baixa intensidade que acompanha o processo tem promovido um franco debate na literatura[52]. Uma vez que peroxidação lipídica é responsável por uma série de desordens associadas com estresse oxidativo, todos os processos associados tem imediata implicação patológica. Além disso, tanto a quimiluminescência direta quanto sensitizada tem sido utilizada para monitorar processos de lipoperoxidação[53].

Parece claro que duas espécies excitadas estão envolvidas no processo: oxigênio singlete, que apresenta emissão bimolecular a 634 nm, e carbonilos triplete, que fosforecem na região de 450-550 nm. Oxigênio singlete poderia ser originado

através da aniquilação de dois radicais alquilperoxila, através do mecanismo de Russel[54], ou por supressão de carbonilos triplete eventualmente formados. Por outro lado, carbonilos triplete poderiam ser gerados na termólise de dioxetanos formados por adição 2+2 de oxigênio singlete ao PUFA (polyunsaturated fatty acids)[52].

Esta última hipótese foi por nós descartada quando demonstramos que os únicos produtos da fotooxigenação do ácido linoleico eram hidroperóxidos e endoperóxidos (**ANEXO X**[55]). As evidências mais claras foram: falta de produtos de decomposição de dioxetanos monitorados por cromatografia gasosa e também de absorções características em ressonância magnética nuclear.

A dúvida que resta é de onde vem, então, esta quimiluminescência devida a carbonilos triplete. Recentemente, demonstramos que a oxidação de um elétron de ene-hidroperóxidos gera uma série de espécies radicalares observaveis por "EPR -spin trap" (electron paramagnetic ressonance), sendo que uma delas poderia ser um dioxetano formado pela ciclização de um radical ene-hidroperoxila. Mais ainda, que estes sistemas geram intensa quimiluminescência (**manuscrito em preparação**[56]). Uma vez que radicais ene-hidroperoxilas são gerados na lipoperoxidação por oxidação de um elétron do PUFA, seguido de inserção de oxigênio, acreditamos que o modelo em questão contrapõe um modelo alternativo de formação de carbonilos triplete, corroborando as indicações anteriores de que não há envolvimento de oxigênio singlete na sua produção.

Utilização de éteres e ésteres vinílicos como substrato de HRP

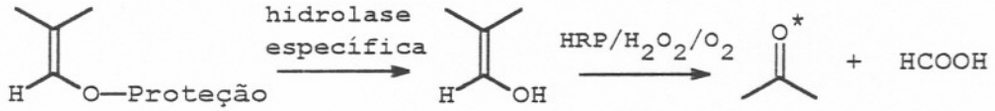
A constatação de que a forma enólica do IBAL é o real substrato da HRP no sistema IBAL/HRP/O_2 gerou uma série de trabalhos mostrando a eficiência de outros substratos como o trimetilsili-enol éter derivado do IBAL[57]. Em condições adequadas, a desproteção deste enol pela reação com íons fluoreto é tão intensa que pode ser observada a olho nú em uma sala escura com a vista adaptada[58]. Entretanto, mesmo na ausência de fluoreto esta reação é quimiluminescente, devido a hidrólise expontânea do trimetilsilil éter.

Historicamente, o primeiros enóis protegidos foram ésteres de fosfato, como o dimetil-isobutenilfosfato e o seu derivado disódico, os quais não produziram quantidade

substancial de luz[59]. Notadamente, tais ésteres tem hidrólise expontânea bastante lenta na ausência de catalizadores.

Aproveitando-se da idéia de desproteção disparada enzimaticamente, propusemos a utilização de enol-éteres e enol-ésteres de estrutura adequada, que sob hidrólise geram o correspondente enol, substrato real da HRP. Assim, um sistema conjugado de HRP + hidrolase ou qualquer outro sistema hidrolítico específico do enol protegido, deverá ser luminescente. Mais ainda, esta luminescência deverá ser proporcional à quantidade do agente hidrolítico ou de HRP, dependendo da relação entre ambos, sendo possível, portanto, desenhar um ensaio analítico quantitativo para um ou para outro.

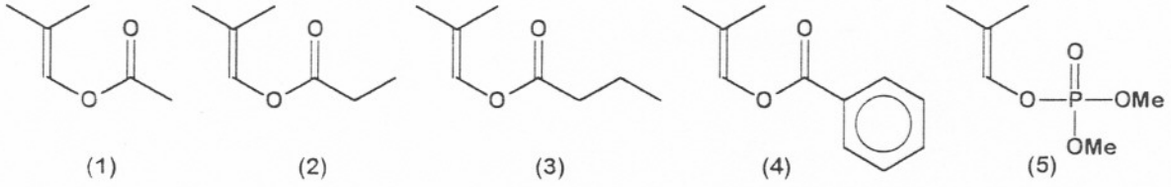
Esquema 10



II

Foram sintetisados os seguintes compostos:

Esquema 11



(1) (2) (3) (4) (5)

Os compostos **1**-**3** (Esquema 11) foram testados em um sistema contendo peroxidase e uma esterase inespecífica (esterase de fígado), além de outros elementos necessários ao sistema. Apesar da baixa intensidade verificada, pudemos demonstrar a funcionalidade do sistema.

Ao testar o benzoato de isobutenila **4**, entretanto, verificamos um aumento significativo de intensidade, comparável aos sistemas anteriormente estudados. Todos os dados indicam que a espécie eletronicamente excitada é acetona triplete, que fosforesce produzindo quimiluminescência com máximo em 430 nm. Também verificamos que o máximo de intensidade de luz do perfil cinético de emissão é

proporcional à quantidade de esterase utilizada, abrindo a possibilidade de utilização destes sistemas em metodologias de quantificação de esterases (**ANEXO XI**[60]).

Neste mesmo trabalho, demonstramos que a HRP poderia ser substituida por conjugados de HRP (HRP ligado a imunoglobulina tipo G de um kit de diagnóstico para HIV-1 EIA recombinante) e que a intensidade máxima também é proporcional a quantidade de conjugado presente. Mais ainda: em ambos os casos a adição de apenas 5 μM de 9,10-dibromoantraceno-2-sulfonato de sódio intensifica a emissão em até 20 vezes !

Estes dados abrem inúmeras possibilidades para aplicações deste sistema. As vantagens também são inumeras: baixo custo (a síntese destes compostos é extremamente simples e barata), possibilidade de variação de "protetores" ampliando a gama de hidrolases passíveis de uso, grande variedade de conjugados comerciais baseados em atividade peroxidásica. No último caso, há possibilidade de aumento da sensibilidade do método, já que medida de intensidade de luz é sempre dependente do grau de sofisticação da aparelhagem utilizada.

Em vista destes resultados, decidimos investir diretamente em metodologia de disagnóstico baseada na determinação de atividade hidrolase. Verificou-se na literatura que nenhum dos métodos descritos até o momento para determinação de lipases, pode ser considerado simples ou sensível. Nestes são utilizados substratos naturais ou sintéticos e a monitoração da hidrólise destes substratos é feita por turbidimetria, potenciometria, espectrofotometria e fluorimetria[61]. As limitações metodológicas muitas vezes inibe a implantação de uma rotina de determinação de lipases até mesmo em laboratórios hospitalares.

Em humanos existem pelo menos duas situações clínicas onde a importância da determinação de atividade de lipase é bem estabelecida. Uma delas é na pancreatite aguda, onde o dano celular libera para o soro grandes quantidades de enzimas intracelulares, sendo a lipase pancreática (LP) um marcador do pâncreas[62]. Esta enzima é a principal responsável pela hidrólise do triglicerídeo da dieta. O ensaio da determinação da LP sérica é o principal marcador para a pancreatite aguda. As alterações observadas nos valores são similares às obtidas para a amilase, entretanto, a elevação de lipase é mais pronunciada, mais prolongada e mais específica. Para fins diagnósticos, avaliações da LP e amilase se complementam. Utilizando ambos os marcadores, a sensibilidade do diagnóstico chega a 98%.

A segunda situação clínica de importância é a determinação da atividade da lipase lipoproteica (LLP) após administração endovenosa de heparina[63,64]. A LLP é amplamente distribuída por vários tecidos, expressando alta atividade no tecido adiposo, coração, músculo esquelético e glândulas mamarias. Em situações onde há suspeita de alteração na síntese ou atividade da LLP; mede-se a atividade de lipase, no soro, antes e após 10 minutos da injeção endovenosa de heparina (75-100 UI/kg peso). A LLP tem alta afinidade à heparina e por isso se libera rapidamente após injeção *in vivo* desta

Numa primeira série de experimentos, verificamos a ação de LP comercial sobre o acetato de isobutenila **1**. Com a utilização de clorofila como sensitizador de acetona triplete, verificamos uma cinética de emissão que depende da presença de todos os componentes do sistema, demonstrando a funcionalidade do método (**ANEXO XII**[65]). Verificamos, também, que a substituição de lipase purificada por soro sanguíneo também dispara uma quimiluminescência bastante intensa. Uma preparação de soro do mesmo indivíduo coletada 10 minutos após administração endovenosa de heparina resultou em um aumento de 60% de emissão. Isto demonstra que é possível definir uma metodologia para determinação de atividade de LLP baseado em medida de quimiluminescência.

No momento, nosso laboratório está empenhado em testar substratos mais específicos como o laurato e o palmitato de isobutenila de forma a definir uma estratégia de aplicação em condições reais, onde a atividade lipasica tem que ser discriminada de outras hidrolases.

Conclusão

Adam e Cilento[4] publicaram, em 1983, um artigo incitando pesquisadores da área de dioxetanos a várias abordagens que os mesmos consideravam, na época, prioritárias. Eles previram um papel fundamental dos dioxetanos na elucidação de fenômenos "fotológicos", onde "fotologia" foi designada como uma ciência que abrange fotoquímica, fotofísica e fotobiologia. De fato, os autores, acertadamente, consideraram que a importância dos mesmos era devida à interdiciplinaridade contida na possível utilização de estados eletronicamente excitados "mascarados" na forma de um

composto sintetizável, ou nas palavras dos mesmos: "equivalentes de estados excitados".

O futuro, entretanto, reservaria a estes compostos um papel ainda mais nobre. Considero que a utilização de dioxetanos em análises clínicas é apenas o início de uma longa carreira de cunho social. Já pode-se contabilizar ganhos significativos com a substituição de radioisótopos por marcadores quimiluminescentes, tanto do ponto de vista ecológico, com a ausência de resíduos radioativos, quanto na diminuição do custo das análises. Há também um aumento da confiabilidade e sensibilidade de vários outros ensaios feitos, no passado, por colorimetria, resultando em uma melhora no diagnóstico de determinadas doenças.

Bibliografia

1. Goldfarb, A.M.A., em Da Alquimia à Química, Editora da USP, 1987, pg. 42.
2. Wiedemann, *Ann. d. Physik u. Chemie*, **34**, 446 (1888).
3. Faria-Oliveira, O.M.M, Brunetti, I.L., Pepato, M.T., Oliveira L.A.A., *Rev. Ciênc. Farm.* **16**, 9 (1995).
4. Adam, W., Cilento, G., *Angew. Chem. Int. Ed. Engl.* **22**, 529 (1983).
5. Catalani, L.H., *Rev. Ciênc. Farm.* **16**, 37 (1995) e ref. Citadas.
6. Adam, W., Zinner, K. em *Chemical and Biological Generation of Excited States*, Adam, W., Cilento, G., eds., Academic Press, New York, 1982, pg. 153.
7. McCapra F., *J. Chem. Soc.. Chem. Comm.*, 155 (1968).
8. Kerns, D.R., *J. Am. Chem. Soc.* **91**, 6554 (1969).
9. Richardson, W.H., O'Neal, H.E., *J. Am. Chem. Soc.* **94**, 1619 (1972).
10. Baumstark, A.L., Dunams, T., Catalani, L.H., Bechara, E.J.H., *J. Org. Chem.* **48**, 3713 (1983).
11. Baumstark, A.L., Dunams, T., *J. Org. Chem.* **47**, 3754 (1982).
12. McCapra F., *J. Chem. Soc.. Chem. Comm.*, 946 (1977).
13. Koo, J.-Y., Schuster, G.B., *J. Am. Chem. Soc.*, **99**, 6107 (1977).
14. Wilson, T., em *Singlet Oxygen*, Frimer, A.A., ed., CRC, Boca Raton, 1985, vol. 2, pg. 37.
15. Scandola, F., Balzani, V., Schuster, G.B., *J. Am. Chem. Soc.* **103**, 2519 (1981).
16. Catalani, L.H., Wilson, T., *J. Am. Chem. Soc.* **111**, 2633 (1989).

17. Adam, W., Vargas, F., Epe, B., Schiffmann, D., Wild, D., *Free Rad. Res. Commun.* **5**, 253, (1989).

18. Bechara, E.J.H., Catalani, L.H., *Free Rad. Biol. Med.* **18**, 731 (1995).

19. Adam, W. em *Chemical and Biological Generation of Excited States*, Adam, W., Cilento, G., eds., Academic Press, New York, 1982, pg. 11.

20. Lim, E.C., Laposa, J.D., Yu, J.M.H., *J. Mol. Spectrosc.* **49**, 412 (1966).

21. Adam, W., Baader, W.J., Catalani, L.H., Cilento, G., Rychla, L. *Photochem. Photobiol.* **42**, 587 (1985).

22. Catalani, L.H., Wilson, T. *J. Am. Chem. Soc.*, **109**, 7458 (1987).

23. Wilson, T., Halpern, A.M. *J. Am. Chem. Soc.*, **102**, 7279 (1980).

24. Catalani, L.H., Wilson, T., Bechara, E.J.H., *Photochem. Photobiol.* **45**, 273 (1987).

25. Indig, G.L., Catalani, L.H., Wilson, T. , *J. Phys. Chem.*, **96**, 8967 (1992).

26. Mirbach, M.F., Ramamurthy, V., Mirbach, M.J., Turro, N.J., Wagner, P.J., *Nouv. J. Chim.* **4**, 471 (1973) e ref. citadas.

27. Catalani, L.H., Bechara, E.J. *Photochem. Photobiol.*, **39**, 823 (1984).

28. Bartlett, P.D., Baumstark, A.L., Landis, M.E., Lerman, C.I., *J. Am. Chem. Soc.* **96**, 5267 (1974).

29. Campbell, B.S., Denney, D.B., Denney, D.Z., Shih, L.S. *J. Am. Chem. Soc.* **97**, 3850 (1975).

30. Turro, N.J., Lecktken, P., *J. Am. Chem. Soc.* **95**, 264 (1973).

31. Scholmerich, J., Andreesen, R., Kapp, A., Ernst, M., Woods, W.G., *Bioluminescence and Chemiluminescence New Perpectives*, 1987, John Wiley X Sons, New York.

32. Van Dyke, K., *Luminescence Immunoassays and Molecular Applications*, 1990, CRC Press, Boca Raton.

33. Beck, S., Koster, H. *Anal.Chem.* **62**, 2258, (1990).

34. Schaap, A.P., Gagnon, S.D., *J. Am. Chem. Soc.* **104**, 3504 (1982).

35. Schaap, A.P.; Sandison, M.D.; Handley, R.S. *Tetrahedron Lett.* **28**, 1159 (1987).

36. Schaap, A.P.; Handley, R.S.; Giri, B.P. *Tetrahedron Lett.* **28**, 935 (1987).

37. Schaap, A.P.; Chen, T.S.; Handley. R.S.; DeSilva, R.; Giri, B.P. *Tetrahedron Lett.* **28**, 1155 (1987).

38. Richardson, N.H.; Hodge, V.F.; Stigall, D.L.; Yelvington, M.B.; Montgomery, F.C. *J. Am. Chem. Soc.* **96**, 6652 (1974).

39. Sawaki, Y.; Ogata Y. *J. Am. Chem. Soc.* **99**, 5412 (1977).

40. Cilento, G.; Adam, W. *Photochem. Photobiol.* **48**, 361 (1988).

41. Adam, W.; Catalani, L.H.; Saha-Möller, C.R.; Will, B. *Synthesis* 121 (1989).

42. McCapra, F.; Chang, Y.C.; François, V.P. *J.Chem.Soc.Chem.Commun.*, 22 (1968).

43. Thomas, A.H.; Seliger, H.H.; White, E.H.; Cass, M.W. *J. Am. Chem. Soc.* **89**, 7148 (1967).

44. Cilento, G. *Pure Appl. Chem.* **56**, 1179 (1984).

45. McCapra, F.; Richardson, D.G. *Tetrahedron Lett.* 3167 (1964).

46. Rauhut, M.M. *Acc. Chem. Res.* **2**, 80 (1969).

47. Urry, W.H.; Sheeto, J. *Photochem. Photobiol.* **4**, 1067 (1965).

48. Hastings, J.W.; Wilson, T. *Photochem. Photobiol.* **23**, 461 (1976).

49. White, E.H.; Miano, J.D.; Watkins, C.J.; Breaux, E.J. *Angew. Chem. Int. Ed. Engl.* **13**, 229 (1974).

50. Kenten, R.H. *Biochem. J.* **55**, 360 (1953).

51. Bechara, E.J.H.; Faria-Oliveira, O.M.M.; Duran, N.; Baptista, R.C.; Cilento, G. *Photochem. Photobiol.* **30**,101 (1979).

52. Cadenas, E. *Ann. Rev. Biochem.* **58**, 79 (1989).

53. Boveris, A.; Cadenas, E.; Chance, B. *Federation Proc.* **40**, 195 (1981).

54. Russel, G.A. *J. Am. Chem. Soc.* **79**, 3871 (1957).

55. Di Mascio, P.; Catalani, L.H.; Bechara, E.J.H. *Free Rad. Biol. Med.* **12**, 471 (1992).

56. Timmins, G.S.; Santos, R.E.; Whitwood, A.C.; Catalani, L.H., Di Mascio, P.; Gilbert, C.G.; Bechara, E.J.H. "The cyclization of an ene-alkylperoxyl radical to form a dioxetane radical intermediate: a model study of the mechanism of chemiluminescence associated with lipid peroxidation, sing heme proteins and peroxynitrite as oxidizing agents", *em preparação*.

57. Adam, W.; Baader, W.J. Cilento. G. *Biochim. Biophys. Acta* **881**, 330 (1986).

58. Baader, W.; Bohne, C.; Cilento, G.; Nassi, L. *Biochem. Educ.* **14**, 190 (1986).

59. Gallardo, H.; Guillo, L.A.; Duran, N.; Cilento, G. *Biochim. Biophys. Acta* **789**, 57 (1984).

60. Yavo, B.; Campa, A.; Catalani, L.H. *Anal. Biochem.* **234**, 215 (1996).

61. Tietz, N.W.;Fiereck, E.A., em *Standart Methods of Clinical Chemistry*, 1972, vol.7, New York, Academic Press, pg. 19.

62. Hultin, M.; Olivercrona,T. Clin.Chem. 32, 50 (1986).

63. Bagibski, M.L. *Method.Enzymol.* **72**, 325 (1981).

64. Wang, C.S.; Hartsuch, J.; McConathy, W.J. *Biochim. Biophys. Acta* **1123**, 1 (1992).

65. Campa, A.; Andrade, A.C.; Catalani, L.H. *Photochem. Photobiol.* **63**, 742 (1996).

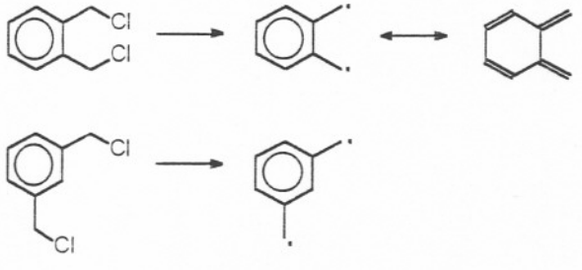
Capítulo 2: ESTUDOS FOTOQUÍMICOS DE HALETOS DE XILILA EM SOLVENTES APOLARES: EM BUSCA DE UM SISTEMA DE FOTOFIXADORES EFICIENTE

Introdução

Desde a publicação do trabalho pioneiro de Zimmerman[1] cerca de 30 anos atrás sobre a fotólise de diversos derivados benzílicos, incluindo haletos e sais de amônio, uma quantidade relativamente grande de trabalho foi acumulada neste campo. Uma atenção especial tem sido dada sobre a competição de processos homolíticos *versus* heterolíticos em solventes polares. A partir destes estudos foram tiradas algumas conclusões gerais sobre o mecanismo de heterólise da ligação carbono-heteroátomo, incluindo a natureza do estado excitado reativo[2]. Por outro lado, a análise dos processos homolíticos envolvidos foram relativamente fracas e superficiais. Além disso, apenas alguns estudos foram feitos em solventes apolares.

Interessantemente, o comportamento de haletos de alquilbenzila (aqui chamados de xilila) sob irradiação de U.V. é praticamente desconhecido. Um dos únicos estudos neste campo é o recente trabalho de Platz e colab.[3], sobre a formação de espécies biradicalares formadas a partir da fotólise de orto e meta isomeros do α,α'-dicloro-xileno. Os autores reportaram a preparação em matriz, respectivamente, de orto- e meta-xilileno a partir destes compostos. Entretanto, apenas alguns experimentos foram feitos em solução com resultados pouco conclusivos.

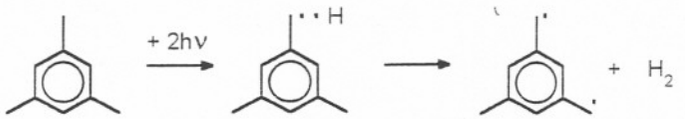
Esquema 1



Platz também mostrou que o biradical mesitilileno é produzido na fotólise de mesitileno em matriz através de um processo bifotônico. Os autores argumentam, entretanto, que os dois fótons são utilizados na produção do mesitileno monoradical primário. O hidrogênio nascente formado reage na gaiola da matriz para formar o

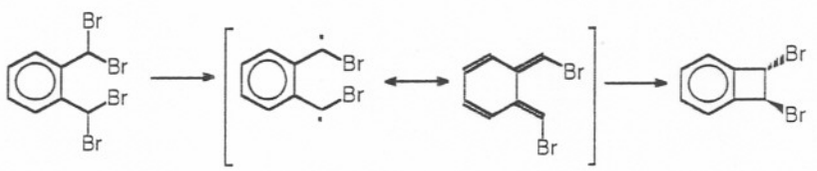
mesitilileno e H_2, muito provavelmente através de ativação térmica. Estes fatos colocam haletos de xilila como bons candidatos na produção monofotônica de xililenos.

Esquema 2



Já em 1957, Cava[4] e colaboradores reconheceram uma estrutura do tipo orto-xilileno como intermediário instável na produção de 1,2-dibromobenzociclobuteno através da redução térmica de α,α,α',α'-tetrabromo-*o*-xileno.

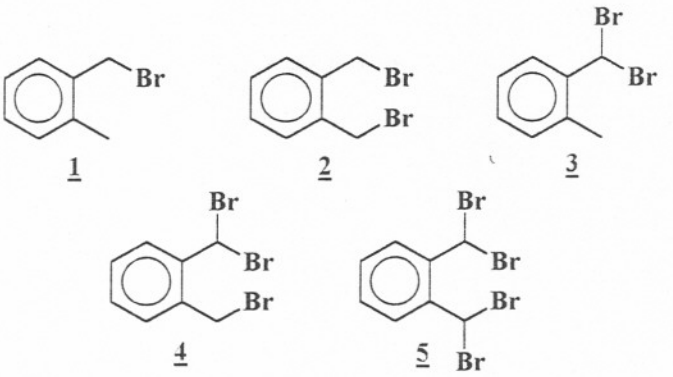
Esquema 3



A importância de orto-xililenos (ou orto-quinodimetanos) em reações de Diels-Alder é atestada pelo grande número de rotas para sua preparação, incluindo métodos fotoquímicos, e de aplicações em reações de cicloadição intramolecular como uma abordagem sintética para sistemas policíclicos[5]. Estas moléculas tem sido observadas através de fotólise relâmpago, também, como intermediários resultante da fotólise de vários compostos[6]. Por outro lado, Adam[7] observou a quebra de duas ligações C-Br na fotólise do 1,8-bis(bromometil)naftaleno por um processo bifotônico, utilizando uma técnica de "laser-jet", gerando um diradical de estrutura semelhante a um quinodimetano.

Dentro desta abordagem mecanística, nosso laboratório publicou (**ANEXO XIII**[8]) um estudo sobre a fotólise de uma série de brometos de xilila, tanto em benzeno quanto em isooctano, utilizando-se lâmpada de mercúrio de baixa pressão (λ_{em} = 254 nm). Este estudo foi tema da Tese de Doutoramento de Daisy de Brito Rezende[9].

Esquema 4



Foi demonstrado que a fotólise de **1**-**5** em solventes apolares gera uma série de produtos que podem ser racionalizados em termos de uma quebra homolítica da ligação C-Br, seguido por reações radicalares termicamente permitidas, sendo que em benzeno a reação é sensitizada pelo solvente. Em todos os casos observou-se um "scrambling" de átomos de bromo em várias novas posições benzílicas indicando abstração de átomos de hidrogênio como reação consecutiva. No caso do isooctano como solvente, observou-se inclusive a formação de 2-bromo-2,4,4-trimetilpentano, um produto de bromação (esperado) do solvente. Como nas reações heterolíticas, a participação do solvente na desativação dos intermediários instáveis determina a distribuição os produtos obtidos. Assim, a habilidade de doar hidrogênios do solvente pode limitar ou até mesmo eliminar o "scrambling" de radicais bromo, como foi observado em misturas benzeno-ciclohexeno. Não observamos produtos formados de um possível o-xilineno intermediário. Mesmo utilizando uma mistura de 20% v/v de ciclohexeno em benzeno na fotólise de **1** e **2**, nenhum aduto de Diels-Alder foi encontrado.

Muitos outros grupos tem questionado os fatores que regulam homólise *versus* heterólise de sistemas benzílicos sob irradiação. O simples fato que tal competição tem sido estudada em solventes nucleofílicos (polares) induz o resultado, devido a polarização da ligação C-X. Baseados em cálculos *ab initio* de ligação de valência para haletos benzílicos, Larson e colab.[2] concluíram que espécies triplete devem dissociar em pares radicais '*a menos que haja um passo alternativo de decaimento*'. Assim, em solventes polares, a formação de pares ionicos seria acompanhada de uma rápida inversão de spin.

Ao examinarem a fotólise de iodetos alquílicos, Kropp e colab[10]. mostraram que a transferência de elétron - produzindo pares iônicos - é um passo de desativação de pares radicalares que compete com a difusão dos mesmos. Esta proposta ficou conhecida como 'hipótese de Kropp'. Vale notar que, até então, não tínhamos nenhuma evidência da formação de pares iônicos nestes solventes, uma vez que, se formados, não teriam outro caminho de desativação a não ser a volta ao produto de partida. A única evidência foi a formação de menos de 6% de α-bromo-α'-fenil-*o*-xileno na fotólise de **2** em benzeno, como produto de substituição eletrofílica em uma molécula do solvente.

Através da técnica de fotólise relâmpago nosso laboratório demonstrou, entretanto, que mesmo em solventes apolares o processo de heterólise compete com homólise, com a formação de carbocátions. Utilizando o 4º harmônico de um laser de Nd-YAG (λ_{exc}=266 nm), a fotólise de **1** e **2** em isooctano mostrou uma curva de decaimento de transiente composta de 2 componentes cinéticos: um com tempo de vida da ordem de microsegundos e outro muito mais lento, com tempo de vida da ordem de milisegundos. A análise da distribuição espectral demonstrou que o primeiro tem máximo de absorção em 320 nm e o segundo, com uma banda de aborsorção muito mais larga, tem máximo em torno de 340-360 nm. Estes transientes foram identificados como o radical e o cátion *o*-metilbenzila, respectivamente. O longo tempo de vida do cátion corrobora a hipótese de que esta espécie, quando formada em solvente não nucleofílico, deve se recombinar de volta ao reagente de partida. Estes dados também fazem parte da Tese de Doutoramento da Daisy de Brito Rezende[9] e estão em fase final de publicação[11].

Um achado interessante deste trabalho diz respeito a produção de HBr. Apesar da homólise da ligação carbono-bromo ocorrer em larga extensão, tanto em benzeno quanto em isooctano, o rendimento de HBr é muito baixo. Uma explicação plausível para a ausência de HBr seria a sua fotólise, já que este composto absorve na faixa de irradiação, devolvendo bromo e hidrogênio atômicos para a solução. Um estudo da fotólise de HBr em xilenos não substituídos está sendo feito, no momento, para testar esta hipótese.

Em busca de um sistema foto-gerador de ácidos

Um dado extremamente interessante foi obtido por Tournier[12] e colaboradores. Aqueles autores demonstraram um alto rendimento quântico de formação de HCl quando cloreto de benzíla é fotolisado em hidrocarbonetos saturados como solventes. Além disso, este rendimento é dependente da viscosidade do solvente. A descoberta de que um cátion benzílico poderia ser formado na fotólise destes compostos em meio totalmente apolar impulsionou uma idéia já antiga de que tais sistemas poderiam ser utilizados como sistemas foto-geradores de ácidos. Idealmente, o cátion benzílico se decomporia produzindo próton.

Tais sistemas tem importância fundamental em aplicações como foto-litografia de circuitos impressos. Atualmente, a tecnologia de produção de circuitos microeletrônicos depende de foto-litografia ótica na redução de dimensões limite de utilização, além de oferecer baixo custo e conveniência. Mais ainda, o emprego de fotofixadores ("*photoresists*") com ação em comprimentos de onde abaixo de 300 nm, através de técnicas de foto-litografia de ultravioleta distante ("*deep UV photoresists*") expandiram tais limites criticos para dimensões abaixo de 0,25 μm, aumentando sensibilidade e resolução[13].

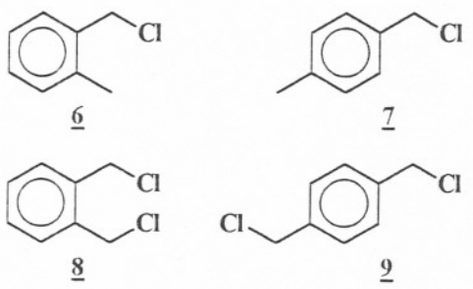
Tipicamente, um fotofixador que atua através da geração de ácidos sofre reação fotolítica sob irradiação e o ácido gerado, numa reação térmica subsequente, promove uma reação ácido-catalizada que modifica a solubilidade de uma resina aplicada a um determinado solvente - revelador- gerando um negativo da área de exposição. Trata-se geralmente de uma resina fenólica bloqueada por grupos *terc*-butil ou *terc*-butoxicarbonil, os quais são facilmente desbloqueados por ação de ácidos.

Por outro lado, cátions benzílicos podem ser utilizados também como iniciadores para polimerização catiônica. Vários destes sistemas são utilizados já a muito tempo[14] e a maioria é baseada na foto- ou termo-decomposição de sais de benzilpiridínio, benzilanilínio ou benzilsulfônio.

A fotólise dos cloretos de xilila **6**-**9** em solventes apolares - benzeno e isooctano - foram analisadas. Os resultados mostraram alto rendimento de produção de HCl, como esperado. Interessantemente, a taxa de recuperação de produtos voláteis (analisados por cromatografia gasosa) varia de no máximo 70% a virtualmente zero, ou seja, há um alto grau de polimerização/oligomerização no sistema. Naturalmente,

cátions benzílicos formados adicionam eletrofilicamente ao próprio reagente de partida muito mais ativado que o solvente aromático - benzeno. A presença de polímeros foi confirmada por técnica de espalhamento de luz, e serão brevemente analisados por cromatografia de exclusão. Este estudo é o tema da Dissertação de Mestrado da aluna Patrícia Ponce.

Esquema 5



6 7 8 9

Além disso, outras aplicações estão em estudo como a adição a sistemas aromáticos ativados, como o 1,4-dimetoxibenzeno, numa reação típica de Friedel-Crafts que dispensaria o uso de ácido de Lewis.

Bibliografia

1. Zimmermann, H.E.; Sandel, V.R. *J. Am. Chem. Soc.*, **85**, 915 (1963).
2. Larson, J.R.; Epiotis, N.D.; McMurchie, L.E. *J .Org. Chem.*, **45**, 1388 (1980).1.
3. Haider, K.; Migirdicyan, E.; Platz, M.S.; Soundararajan, N.; Depress. A. *J. Am. Chem. Soc.* **112**, 733 (1990).
4. Cava, M.P.; Napier, D.R. *J. Am. Chem. Soc.* **79**, 1701 (1957).
5. Oppolzer, W. *Synthesis* 893 (1978).
6. Flynn, C.R.; Michl, J. *J. Am. Soc. Chem.* **96**, 3280 (1974).
7. Adam, W.; Ouchi, A. *Tetrahedron Lett.* **33**, 1875 (1992).
8. Rezende, D.B.; Campos, I.P.A.; Toscano, V.G.; Catalani, L.H. *J. Chem. Soc. Perkin Trans.* 1857 (1995).
9. Rezende, D.B., em "*Fotólise de brometos de orto-xilila: um estudo em solventes apolares*", Tese de Doutoramento, IQUSP, 1994.
10. Kropp, P.J.; Poindexter, G.S.; Pienta, N.J.; Hamilton, D.C. *J. Am. Chem. Soc.* **98**, 8135 (1976).

11. Catalani, L.H.; Arruda Campos, I.P.; Toscano, V.G.; Rezende, D.B. A laser flash-photolysis study of a series of α-Brominated ortho-xylenes: homolysis vs. heterolysis in apolar solvents.

12. Tournier, A.; Deglise, X.; André, J.C. *J. Photochem.* **22**, 223 (1983).

13. Crivello, J.V.; Shim, S.-Y. *J. Polym. Sci. Part A: Polym. Chem.* **33**, 513 (1995).

14. Nakano, S; Endo, T. *J. Polym. Sci. Part A: Polym. Chem.* **33**, 505 (1995).

Capítulo 3: ESTUDO DA TERMO- E FOTO-DEGRADAÇÃO DE COPOLÍMEROS DE ESTIRENO E COMPOSTOS VINILCARBONÍLICOS: PRODUÇÃO DE MATERIAIS COM VELOCIDADE DE DEGRADAÇÃO AMPLIFICADA.

Na medida em que a sociedade atual volta-se para o problema da preservação do meio ambiente, com uma postura ecológica responsável e exigente, a indústria de bens de consumo e, paralelamente, a indústria de captação e tratamento de lixo encontra-se em compasso de espera. Durante décadas a indústria de polímeros tratou de produzir plásticos cada vez mais estáveis e duradouros. Se por um lado isto traduziu-se em bens de consumo de melhor qualidade, por outro houve uma tendência a acumulo de lixo sólido não degradável em proporções descomunais.

Este problema aflige hoje a maioria dos países desenvolvidos e em desenvolvimento, onde os plásticos e seus derivados são largamente utilizados. Uma estatística de 1984 indicou que os E.U.A. produzem ca. 134 milhões de toneladas de lixo sólido por ano, sendo 15 milhões de toneladas em plásticos[1]. Deste total ca. 30% (4.6 milhões de toneladas) é composto de plásticos utilizados em embalagens.

A espera acima citada não refere-se apenas a uma solução tecnológica pura e simples, mas de uma tomada de posição em relação a qual ou quais soluções serão escolhidas. Incineração é uma delas. Apesar de apenas 5% do total do lixo sólido nos EUA serem incinerados (o restante é colocado em aterros), na Europa Ocidental este total chega a 50% e até 70% no Japão, com a utilização do alto poder calórico, principalmente dos plásticos[1]. A pergunta aqui é: quão responsável seria queimar todo o lixo do planeta, resultando obviamente em alta produção de CO_2, um agravante ao efeito estufa?

Duas outras soluções mais viáveis ecologicamente tem sido alvo das atenções: reciclagem e produção de polímeros degradáveis. A reciclagem tem seu lugar garantido no tratamento de diversos tipos de lixo: metais, papeis e vidros. Porém, enquanto papel e vidro são materiais de estreita faixa de variáveis quanto a sua composição, os metais e os plásticos possuem uma grande variedade de tipos. A mistura indiscriminada destes últimos deverá, obviamente, comprometer sua re-utilização. O trabalho extra de classificação e separação dos diversos tipos poderá tornar o preço desta matéria prima inviável em relação à matéria prima original.

A produção de polímeros passíveis de degradação ambiental tem sido o principal foco das atenções nos últimos anos. Do ponto de vista tecnológico os avanços são consideraveis. Abordagens distintas levaram ao desenvolvimento de produtos foto e/ou biodegradaveis[2], desde a copolimerização de etileno/CO (Dow, Dupont, Union Carbide), passando pela adição de amido a um polímero base (St. Laurence) até a produção de polímeros verdadeiramente biodegradáveis como polibutirolactona (ICI). No balanço de custos/benefícios, os benefícios que mais pesam aqui é aplicabilidade do produto e sua real biodegradabilidade enquanto que em custos certamente é a obtenção da tecnologia.

Dentre os modos de degradação ambiental de polímeros, a biodegradação é o que tem maior apelo ecológico, já que o material seria totalmente reciclado pela natureza[3]. A ação biodegradativa é feita por enzimas hidrolíticas e/ou oxidativas secretadas por microorganismos, bactérias e fungos, para posterior digestão. Há porém sérias restrinções quanto a estrutura do polímero para que isto ocorra como: pesos moleculares altos, insensibilidade ao ataque no meio da cadeia, ramificações, hidrofobicidade da superfície e baixa razão área/volume[3]. Dados indicam que hidrocarbonetos de cadeia simples não são biodegradáveis se seu peso molecular ultrapassa 500 u.m.a.[4]

A solução para este problema partiu de um conceito baseado na fotodegradação amplificada de polímeros, principalmente pela adição de grupos carbonílicos na cadeia principal ou próximo a esta[5]. Isto corresponderia a adição de pontos frageis na cadeia polimerica, que pode agora se romper através da clivagem do tipo Norrish I e II. Assim, sob a ação da radiação solar, tais polímeros seriam degradados a pesos moleculares menores até que a ação de microorganismos fosse possível. A partir desta idéia uma gama imensa de novos materiais foi desenvolvida baseada principalmente em: copolimerização com vinil cetônicos ou monóxido de carbono em percentagens variáveis, produção de blendas a partir de homopolímeros contendo grupos carbonílicos, produção de polímeros graftados e de bloco[5].

Portanto, a aplicação de conceitos de fotodegradação a polímeros inicialmente não biodegradaveis pode proporcionar uma redução do tempo de vida deste material quando exposto ao tempo, levando eventualmente a sua completa biodegradação.

Muito mais abrangente do que a degradação foto-oxidativa é o conceito de autoxidação de polímeros. Uma vez que radicais livres podem ser gerados em um

vasto número de reações de iniciação, a subsequente adição de oxigênio molecular aos mesmos é um fenômeno bastante geral. Os modos de iniciação podem ser, térmico, mecânico, químico, além do fotoquímico já descrito. As reações de propagação e terminação são basicamente as mesmas. Por este motivo, raramente são encontrados trabalhos onde a contribuição de cada um destes modos de iniciação é avaliado separadamente. Parece óbvio que uma análise refinada das contribuições individuais dos tipos de iniciação serve muito mais aos propósitos de optimização do processo de degradação, caso desejado, ou de sua proteção.

Parece claro, hoje, que a biodegradação procedida de uma degradação foto-oxidativa pode responder a grande parte dos anseios ecológicos da sociedade atual.

Copolimerização com compostos vinilcetônicos

A introdução de pequenas quantidades (»1%) de monóxido de carbono como co-monômero na produção de diversos polímeros (polietileno, polipropileno, poliacrilonitrila, poliestireno, etc) resultou na geração de novos produtos com tempo de serviço limitado[3]. Esta incorporação seria suficiente para provocar fotodegradação acelerada destes polímeros, levando primariamente a sua decomposição em pequenos fragmentos via reações de Norrish Tipo II. Li e Guillet[6] demonstraram, entretanto, que grupos cetônicos localizados em cadeias laterais, são muito mais reativos fotoquimicamente do que aqueles localizados na cadeia principal. Os autores argumentam que grupos nas cadeias laterais são eficientes tanto em cisões do Tipo II quanto como fontes de radical livre *via* reações do Tipo I.

A copolimerização de metilvinilcetona com diversos polímeros resultou em produtos muito mais susceptíveis à fotólise do que seus homopolímeros originais, mesmo no caso de polímeros já contendo cromóforos fotoreativos como polimetilmetaacrilato[7], poliacrilonitrila[8] e poliestireno[9].

No caso específico do poliestireno, já é conhecida a fotodegradação de seus copolímeros com metilvinilcetona[9], *terc*-butilvinilcetona[10], fenilvinilcetona[9], benzalacetona e benzalacetofenona[11]. Lamentavelmente porém, nunca houve uma comparação direta do desempenho do copolímero frente ao homopolímero em um mesmo laboratório. Ou ainda, não houve a preocupação de comparar os resultados em atmosfera inerte frente a uma atmosfera oxidativa (na presença de ar, por exemplo).

Geuskens e colab.[12] determinaram um rendimento quântico de quebra de cadeia para poliestireno em filmes da ordem de 0,055%[12] em meio aerado, à temperatura ambiente, contra 4,4%[13] determinado de um copolímero de fenilvinilcetona nas mesmas condições e até 30%[13] acima da Tg (±100oC). Percebe-se claramente neste exemplo o efeito drástico da adição de um co-monômero vinilcetônico.

O papel de metais de transição na autoxidação

Em recente artigo de revisão, Miller e colab.[14] sustentam a posição de que a real autoxidação, definida como a oxidação ao ar sem a necessidade de catalisadores, é negligível para a maioria das biomoléculas em seu estado fundamental singlete. E que, assim sendo, a grande maioria dos processos de autoxidação naturais conhecidos necessita da presença de metais de transição como catalizadores, já que suas reações com oxigênio molecular não são restritas pelo spin. Paralelamente, sabe-se que a biodegradação de húmus em águas e solos através de fotoxidação utiliza o ciclo Fe(II)-Fe(III) como principal catalisador[15].

A reconhecida ação catalítica dos metais de transição na autoxidação de compostos orgânicos também tem sido observada durante a fotodegradação de polímeros[16]. Foi constatado que íons simples de metais de transição e alguns de seus complexos[17] também podem funcionar como catalisadores de fotoxidação de polímeros. Porém, em alguns casos esta ação parece estar ligada a oxidação primaria do polímero durante seu processamento, o qual introduz grupos hidroperóxidos e carbonílicos fotosensiveis[18].

Uma importante descoberta foi feita por Amim e Scott[17], onde a simples mistura de acetilacetonatos de Fe(III) e Co(III) a polietileno aceleram enormemente a oxidação deste polímero, até mesmo na ausência de luz. Além disso, a velocidade de formação de resíduos carbonílicos é diretamente proporcional à concentração destes complexos.

Copolímeros de estireno e metilvinilcetona

Nosso laboratório tem desenvolvido trabalho neta área, especificamente com copolímeros de estireno e quantidades variáveis de metilvinilcetona. Pretendemos investigar as diversas formas de degradação oxidativa destes polímeros, tentando

analisar as contribuições individuais de cada uma delas, visando, principalmente, a otimização da degradação ambiental do estireno quando exposto a condições reais.

O estireno foi escolhido como monômero base devido a facilidade de sua polimerização em solução e manuseio. O controle fino da qualidade da matéria prima é essencial para uma análise refinada dos diversos fatores envolvidos. Optamos por produzir nossas próprias resinas para facilitar esse controle de qualidade. Além disso, a literatura compreensível sobre degradação oxidativa deste tipo de polímero encontra-se centrada nos processos catalisados por luz[19]. Por último, é altamente desejável o desenvolvimento de metodologias de amplificação da degradação ambiental de poliestirenos, responsável por boa parte de lixo plástico não degradável do momento. Vale lembrar que o poliestireno é um modelo próximo de polietileno, o grande responsável pela poluição de plásticos mundial.

Uma vez que a produção de polímeros na presença de aditivos metálicos, complexados ou não, para amplificar velocidade de fotodegradação de vários tipos de polímeros tem sido estudada, e sua eficiência comprovada[17], pretendemos expandir o nosso estudo até a análise dos efeitos da aditivos metálicos, como $FeCl_3$, Co_2O_3 e Mn_2O_3 ou seus complexos, como estearatos e acetilacetonatos, na degradação oxidativa destes copolímeros.

No estágio atual, já foram produzidos e completamente caracterizados os seguintes polímeros da Tabela 1. Alguns deles como copolímeros de estireno-acroleína são praticamente desconhecidos na literatura. Sua fotólise tem sido estudada na presença ou não de aditivos de sais metálicos como o acetoacetonato férrico e o cloreto férrico. Os resultados indicam, como esperado, que o aumento da quantidade de monômero carbonílico leva a um aumento do número de cortes por tempo de irradiação, ou seja, do rendimento quântico. Também verificamos que a adição crescente de aditivo férrico causa uma aumento ainda maior, o que implica dizer que são efeitos somatórios.

Tabela 1. Percentual molar de monômero nos polímeros sintetizados.

Monômero / Polímero	Estireno	Metilvinilcetona	Acroleína
PS	100	0	0
MVK5	95	5	0
MVK10	90	10	0
MVK15	85	15	0
AC5	95	0	5
AC10	90	0	10
AC15	85	0	15

Isto tem implicações direta na qualidade de material que se queira produzir. O aumento de monômeros cetônicos a partir de um certo ponto pode implicar em mudança nas características do material, inviabilizando sua utilização. Entretanto, a utilização de ambas abordagens pode gerar materiais de boa qualidade e alta degradabilidade. Tais resultados são inéditos na literatura.

Estes resultados estão sendo atualmente publicados como parte do trabalho onde foi testado o método de monitoração em tempo real de quebra de cadeia (vide próximo ítem; **ANEXO XIV**[20]).

Espalhamento de luz como técnica de monitoramento de fotodegradação

As modificações feitas em um sistema polimérico devem ser avaliadas de acordo com sua capacidade de acelerar ou retardar a degradação. Ou seja, a avaliação mais adequada deve ser aquelas que levam em conta parâmetros cinéticos. Em se tratando de fotodegradação, um parâmetro ainda mais especifico é desejável: o rendimento quântico de quebras de cadeia. Logo, a variação de peso molecular em função do tempo de irradiação é o que deve ser monitorado. Lamentavelmente, não há descrito nenhum método de monitoração em tempo real para tal parâmentro. A maioria das

publicações da área relatam dados indiretos como velocidade de aparecimento de absorção de carbonila no infravermelho ou ultravioleta. Alguns poucos autores se dão ao trabalho de analisar amostras via cromatografia de exclusão, porém o trabalho longo e tedioso e não permite um avanço rápido.

Um grande avanço foi dado a partir dos trabalhos de Reed e colab.[21] onde descrevem um modelo cinético de depolimerização de macromoléculas quando sujeitas a cisões randômicas. O modelo é aplicado para situações onde não há considerável interação entre moléculas de tipo helicoidal randômica, ou seja, condição θ de Flory. A grande vantagem do modelo é permitir a utilização de resultados e técnicas de espalhamento de luz clássica para estudos cinéticos de cisão de moléculas. Mais importante, considerada as aproximações necessárias, não há necessidade de informações precisas de polidispersidade, raio de giração (portanto, sobre forma das moléculas) e do segundo coeficiente virial.

Inicialmente, os autores aplicaram esta teioria para a digestão de um polisacarídeo, o ácido hialurônico, pela hialuronidase[22]. Neste caso, utilizando ângulos de espalhamento relativamente altos e baixas concentrações, foi possível obter os coeficientes de Henry-Michaelis-Menten do sistema a partir de velocidades iniciais de depolimerização.

Durante algum tempo utilizamos o método de Reed para monitoração da fotodegradação de nossos polímeros em modo "batch", ou seja, através de amostragem da fotólise e análise estática. A manipulação das amostras resultou em valores obscuros de velocidade de cisão, devido principalmente a inconsistência e falta de linearidade dos dados.

Desenvolvemos, então, um sistema de fluxo baseado em poço de irradiação conectado ao goniômetro através de uma bomba de circulação. Desde então, passamos a monitorar os valores de espalhamento em tempo real. Além disso, passamos a utilizar um fotômetro de espalhamento de luz multi-ângulo, com monitoração simultânea de 18 ângulos fixos. Com o auxílio do Prof. Reed, adaptamos a teoria por ele desenvolvida para o cálculo de constantes de velocidade de fotodegradação. Com uma taxa de amostragem tipica de 5-20 segundos, e monitoração em tempo real, passamos a ter uma qualidade de resultados surpreendentemente alta.

Como um todo, esta técnica deverá revolucionar a metodologia de monitoração de depolimerização, especialmente a devida a fotodegradação. Os ensaios são

rápidos, precisos e simples. É possível obter gráficos de peso molecular versus tempo em tempo real. Também é possivel, para o caso do fotômetro multi-ângulos obter gráficos de raio de giração versus tempo, permitindo uma visialização da mudança conformacional da molécula. Além disso, para sistemas que obedecem a condição *theta*, observamos que a velocidade de depolimerização é independente do ângulo. Assim, estamos construindo um aparelho simples para monitoração de depolimerização utilizando apenas de um ângulo (90 graus). Este aparelho, que custará em torno de US$ 3000 a US$ 5000 deverá ser de de grande valia para aplicações industriais.

Bibliografia

1. Huang, J.-C., Shetty, A.S., Wang, M.-S., *Adv. Polym. Tech.,* **10**, 23 (1990).

2. Vurm, K., *Int. Polym. Sci. Tech.,* **16**, 58 (1989).

3. Schnabel, W., em *Polymer Degradation: Principles and Practical Applications*, Hanser Int., Viena, 1981.

4. Klemchuk, P.P., *Polym. Degrad. Stab.,* **27**, 183 (1990).

5. Guillet, J.E., em *Polymers and Ecological Problems*, J.E. Guillet, ed., Plenum Press, N.Y., 1973.

6. Li, S.K.L., Guillet, J.E., *J. Polym. Sci.,* **18**, 2221 (1980).

7. Kato, M., Yamazaki, M., *Makromol. Chem.*, **177**, 3455 (1976).

8. Alexandru, L.,, Guillet, J.E., *J. Polym. Sci.*, **13**, 483 (1975).

9. Kato, M., Yoneshige, M., *Makromol. Chem.*, **164**, 159, (1973).

10. Tanaka, H., Otsu, T., *J. Polym. Sci.*, **15**, 2613 (1977).

11. Hrdlovic, I., Lukac, I., Zvara, I., Kulickova, M., Berek, D., *Eur. Polym. J.*, **16**, 651 (1980).

12. Geuskens, G., Baeyens-Volant, D., Delaunois, G., Lu-Vinh, Q., Piret, W., David, D., *Eur. Polym. J.*, **14**, 291 (1978).

13. Dan, E., Guillet, J.E., *Macromolecules*, **6**, 230 (1973).

14. Miller, D.M., Buettner, G.R., Aust, S.D., *Free Rad. Biol. Med.*, **8**, 95(1990).

15. Milles, C.J., Brezonik, P.L., *Env. Sci. Tech.*, **9**, 1089 (1981).

16. Scott, G., em *Polymers and Ecological Problems*, Guillet, J.E.,ed., Plenum Press, New York, 1973, pg. 27.

17. Amin, M.U., Scott, G. *Eur. Polym. J.*, **10**, 1019 (1974).

18. Mellor, D.C., Moir, A.B., Scott, G., *Eur. Polym. J.*, **9**, 219 (1973).

19. Ranby,B.; Lucki,J., *Pure.Appl.Chem.*, **52**, 295 (1980).

20. Catalani, L.H., Rabello, A.M., Florenzano, F.H., Politi, M.F., Reed, W.F., *Int. J. Polym. Anal. Char.*, submetido.

21. Reed, C.E., Reed, W.F., *J. Chem. Phys.* **93**, 9069 (1990) e ref. citadas.

22. Reed, W.F., Reed, C.E., Byers, L.D., *Biopolymers* **30**, 1073 (1990).

ANEXOS

Anexo I: Thermolysis of 3-methyl-3-alkyl-1,2-dioxetanes: steric effecs on the activation parameters. Baumstark, A.L., Dunams, T., Catalani, L.H., Bechara, E.J.H., *J. Org. Chem.* **48**, 3713 (1983).

Anexo II: Electron transfer and chemiluminescence. Two inefficient systems: 1,4-dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide. Catalani, L.H., Wilson, T., *J. Am. Chem. Soc.* **111**, 2633 (1989).

Anexo III: The oxidation of cyclic sulfides by tetramethyldioxetane and the isobutanal/ O_2/peroxidase system: oxygen transfer versus electron transfer. Bechara, E.J.H., Catalani, L.H., *Free Rad. Biol. Med.* **18**, 731 (1995).

Anexo IV: Energy transfer of chemienergized acetone to substances that display anomalous fluorescence. Adam, W., Baader, W.J., Catalani, L.H., Cilento, G., Rychla, L. *Photochem. Photobiol.* **42**, 587 (1985).

Anexo V: Energy transfer from triplet acetophenones to 9,10-dibromoanthracene (S_1): role of its T_n state. Catalani, L.H., Wilson, T. *J. Am. Chem. Soc.*, **109**, 7458 (1987).

Anexo VI: Two water-soluble fluorescence probes for chemiexcitation studies: sodium 9,10-dibromo- and 9,10-diphenylanthracene-2-sulfonate. Synthesis, proper-ties and application to triplet acetone and tetramethyldioxetane. Catalani, L.H., Wilson, T., Bechara, E.J.H., *Photochem. Photobiol.* **45**, 273 (1987).

Anexo VII: Quenching of triplet acetone by mesitylene and durene: exciplex formation or energy transfer? Indig, G.L., Catalani, L.H., Wilson, T. , *J. Phys. Chem.*, **96**, 8967 (1992).

Anexo VIII: Quenching of chemiexcited triplet acetone by biologically important compounds in aqueos medium. Catalani, L.H., Bechara, E.J. *Photochem. Photobiol.*, **39**, 823 (1984).

Anexo IX: Synthesis of trialkylsilylated α-hydroperoxy aldehydes and ketones via ozonolysis of protected allylil hydroperoxides. Adam, W.; Catalani, L.H.; Saha-Möller, C.R.; Will, B. *Synthesis* 121 (1989).

Anexo X: Are dioxetanes chemiluminescente intermediates in lipoperoxidation ? Di Mascio, P.; Catalani, L.H.; Bechara, E.J.H. *Free Rad. Biol. Med.* **12**, 471 (1992).

Anexo XI: Esterase coupled with the H_2O_2/horseradish peroxidase system triggers chemiluminescence from 2-methyl-1-propenylbenzoate: a potential analytical tool for esterase analysis. Yavo, B.; Campa, A.; Catalani, L.H. *Anal. Biochem.* **234**, 215 (1996).

Anexo XII: Chemiluminescence triggered by hydrolase activity in an horseradish peroxidase/H_2O_2 coupled assay. Campa, A.; Andrade, A.C.; Catalani, L.H. *Photochem. Photobiol.* **63**, 742 (1996).

Anexo XIII: Photolysis of a series of α-brominated *ortho*-xylenes in apolar solvents. Rezende, D.B.; Campos, I.P.A.; Toscano, V.G.; Catalani, L.H. *J. Chem. Soc. Perkin Trans.* 1857 (1995).

Anexo XIV: Real-time determination of ultraviolet degradation kinetics of polymers in solution. Catalani, L.H., Rabello, A.M., Florenzano, F.H., Politi, M.F., Reed, W.F., Int. J. *Polym. Anal. Char.*, no prelo.

Anexo I

Thermolysis of 3-methyl-3-alkyl-1,2-dioxetanes: steric effecs on the activation parameters. Baumstark, A.L., Dunams, T., Catalani, L.H., Bechara, E.J.H., *J. Org. Chem.* **48**, 3713 (1983).

Reprinted from The Journal of Organic Chemistry, 1983, 48, 3713

Thermolysis of 3-Methyl-3-alkyl-1,2-dioxetanes: Steric Effects on the Activation Parameters

Alfons L. Baumstark,*[1] Tambra Dunams, Luiz H. Catalani, and Etelvino J. H. Bechara

Laboratory for MBS, Department of Chemistry, Georgia State University, Atlanta, Georgia 30303

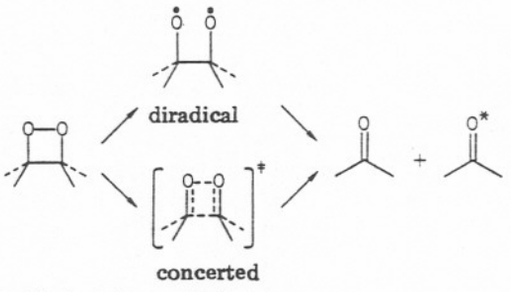
Received February 25, 1983

A series of 3-methyl-3-alkyl-1,2-dioxetanes (1–5) was synthesized by the bromo hydroperoxide method. The activation parameters for the thermal decomposition of 1–5 were determined by the chemiluminescence method [for 3-methyl-3-ethyl-1,2-dioxetane (1), E_a = 24.5 kcal/mol, log A = 13.1, $k_{60°C}$ = 1.0 × 10^{-3} s^{-1}; for 3-methyl-3-(1-propyl)-1,2-dioxetane (2), E_a = 24.6 kcal/mol, log A = 13.1, $k_{60°C}$ = 1.0 × 10^{-3} s^{-1}; for 3-methyl-3-(1-butyl)-1,2-dioxetane (3), E_a = 24.4 kcal/mol, log A = 13.0, $k_{60°C}$ = 9.6 × 10^{-4} s^{-1}; for 3-methyl-3-(2-propyl)-1,2-dioxetane (4), E_a = 25.0 kcal/mol, log A = 13.2, $k_{60°C}$ = 5.8 × 10^{-4} s^{-1}; and for 3-methyl-3-*tert*-butyl-1,2-dioxetane (5), E_a = 25.8 kcal/mol, log A = 13.3, $k_{60°C}$ = 2.6 × 10^{-4} s^{-1}]. Thermal decomposition of 1–5 produced the expected cleavage products in all cases. As expected for alkyl dioxetanes, thermolysis of 1–5 resulted in high yields (~10%) of triplet excited products and low yields (<0.01%) of singlet excited products as determined by the 9,10-dibromoanthracene/9,10-diphenylanthracene method. The data showed that increased steric interactions due to branched substituents produced increased activation energies with little or no effect observable in the $\Delta S^{\ddagger}$ terms. The results are in agreement with a diradical mechanism of dioxetane thermolysis.

The thermolysis of alkyl-1,2-dioxetanes has been shown to produce two carbonyl fragments, one of which may be produced in an excited state (high yields of excited triplets).[2] The electron-transfer-type mechanisms of chemiluminescent decomposition (high yields of excited singlets)[2e] do not occur readily with alkyl-substituted dioxetanes. Most of the experimental evidence on the thermal decomposition of simply substituted dioxetanes has been interpreted[2] in favor of a diradical (two-step) mechanism rather than a concerted mechanism (Scheme I).

The activation parameters of dioxetane thermolysis show insensitivity[2,3] to some substituent effects. Recent results have shown that substituent effects can influence the activation parameters of the thermal decomposition of alkyldioxetanes in unexpected ways.[4] 3,4-Cyclic substituents have been shown[4b–d,5] to have large effects on alkyldioxetane activation parameters. A recent study showed[4a] that the activation energy for the thermal decomposition of 3,3-diethyl-1,2-dioxetane was higher than expected. Richardson had shown[2,6] that the formal replacement of methyl groups by phenyl groups in 3,3-disubstituted dioxetanes had essentially no effect on the activation energy. Thus the E_a for 3,3-diphenyl-1,2-dioxetane and that for 3,3-dimethyl-1,2-dioxetane were found to be approximately 23 kcal/mol.[6] The E_a for 3,3-diethyl-1,2-dioxetane was found[4a] to be 1.5 kcal/mol higher than that for 3,3-dimethyl-1,2-dioxetane (little or no effect was noted on $\Delta S^{\ddagger}$). The magnitude of the steric effect for the formal replacement of 3,3-dimethyl groups with 3,3-diethyl groups was sufficient to account for the observed ΔE_a in the comparison of the E_a for tetraethyl-1,2-dioxetane[4c] with that for tetramethyl-1,2-dioxetane. An interpretation suggested[4a] that 3,3 steric interactions were of major importance when compared to 3,4 steric interactions in dioxetane thermolysis. We report here the characterization of a series of 3-methyl-3-alkyl-1,2-dioxetanes (with increasing steric bulk) to examine the effect of steric interactions on the activation parameters for dioxetane thermolysis.

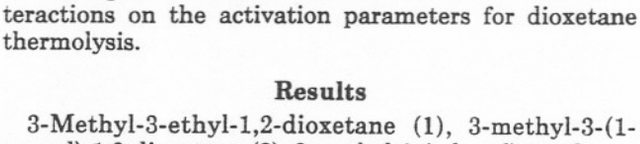
Scheme I[a]



[a] Asterisk denotes excited state.

Results

3-Methyl-3-ethyl-1,2-dioxetane (1), 3-methyl-3-(1-propyl)-1,2-dioxetane (2), 3-methyl-3-(1-butyl)-1,2-dioxetane (3), 3-methyl-3-(2-propyl)-1,2-dioxetane (4), and 3-methyl-3-*tert*-butyl-1,2-dioxetane (5) were synthesized in 5–10% yield by closure of the bromo hydroperoxides in CCl_4 with base at 0 °C. The dioxetanes, isolated by low-temperature column chromatography, were determined to be of better than 95% purity. The compounds were characterized by NMR spectroscopy and by analysis of the thermolysis products. As expected, the signals for the ring protons were observed as AB-type patterns for compounds 2–5. The signal for the ring protons was observed as a singlet for compound 1.



Thermal decomposition of 1–5 produced the expected cleavage products. The rates of thermal decomposition

(1) Fellow of the Camille and Henry Dreyfus Foundation, 1981–1986.

(2) For review, see (a) Wilson, T. *Int. Rev. Sci.: Phys. Chem. Ser. Two* **1976**, *9*, 265. (b) Adam, W. *Adv. Heterocycl. Chem.* **1977**, *21*, 437. (c) Horn, K. A.; Koo, J.; Schmidt, S. P.; Schuster, G. B. *Mol. Photochem.* **1978/1979**, *9(1)*, 1. (d) Bartlett, P. D.; Landis, M. E. In "Singlet Oxygen"; Wasserman, H. H., Murray, R. W., Ed.; Academic Press: New York, 1979; p 243. (e) Schuster, G. B.; Schmidt, S. P. *Adv. Phys. Org. Chem.* **1982**, *18*, 187. (f) Adam, W. In "Chemical and Biological Generation of Electronically Excited States"; Cilento, G., Adam, W., Ed.; Academic Press: New York, 1982; Chapter 4. (g) Baumstark, A. L. In "Singlet Oxygen" Frimer, A., Ed.; CRC Press: Boca Raton, FL, 1983, in press.

(3) (a) Koo, J.; Schuster, G. B. *J. Am. Chem. Soc.* **1977**, *99* 5403. (b) Wilson, T.; Golan, D. E.; Scott, M. S.; Baumstark, A. L. *Ibid.* **1976**, *98*, 1086. (c) Richardson, W. H.; Burns, J. H.; Price, M. E.; Crawford, R.; Foster, M.; Slusser, P.; Ardergg, J. H. *Ibid.* **1978**, *100*, 7596 and references therein.

(4) (a) Baumstark, A. L; Dunams, T. *J. Org. Chem.* **1982**, *47*, 3754. (b) Baumstark, A. L.; Wilson, C. E. *Tetrahedron Lett.* **1981**, 4363. (c) Bechara, E. J. H.; Wilson, T. *J. Org. Chem.* **1980**, *45*, 5261. (d) Lechtken, P.; Reissenweber, G.; Grubmueller, P. *Tetrahedron Lett.* **1977**, 2881.

(5) Kopecky, K. R.; Lockwood, P. A.; Gomez, R. R.; Ding, J.-Y. *Can. J. Chem.* **1981**, *59*, 851.

(6) (a) Richardson, W. H.; Yelvington, M. B.; O'Neal, H. E. *J. Am. Chem. Soc.* **1972**, *94*, 1619. (b) Richardson, W. H.; Montgomery, F. C.; Yelvington, M. B.; O'Neal, H. E. *Ibid.* **1974**, *96*, 7525.

0022-3263/83/1948-3713$01.50/0

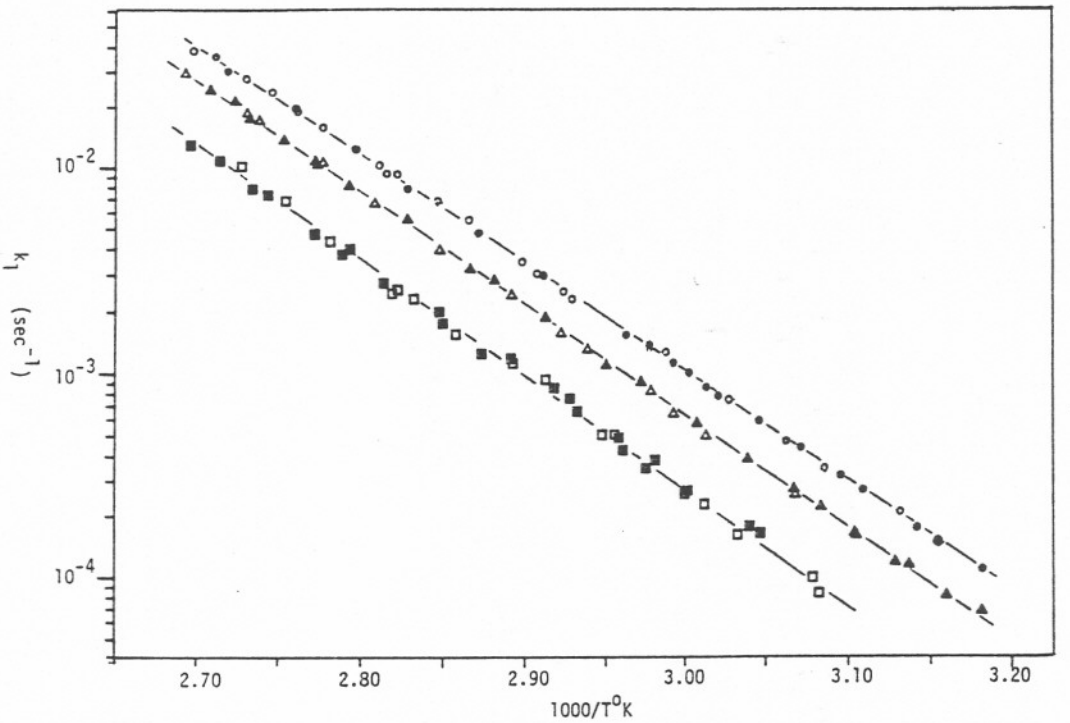


Figure 1. Arrhenius plots for the thermal decomposition of 3-methyl-3-ethyl-1,2-dioxetane [(●) DBA, (○) DPA)]; 3-methyl-3-(2-propyl)-1,2-dioxetane [(▲) DBA, (△) DPA)]; 3-methyl-3-*tert*-butyl-1,2-dioxetane [(■) DBA, (□) DPA)] in xylenes.

of **1**-**5** were determined by the chemiluminescence method. Semilog plots of relative light intensity (dioxetane concentration) vs. time were linear for at least 3 half-lives. The rates of dioxetane decomposition (initial dioxetane concentration, $\sim 10^{-4}$ M) were not effected by low concentrations of 9,10-dibromoanthracene (DBA) or 9,10-diphenylanthracene (DPA). The first-order rate constants (k_1) for compounds **1**–**3** were essentially identical over the temperature range employed (>50 °C). The k_1's for compounds **4** and **5** were readily distinguishable from one another and from those of **1**–**3**. The Arrhenius plots for the thermal decomposition of **1**, **4**, and **5** are shown in Figure 1. The activation parameter data for **1**–**5** are summarized in Table I. The activation parameters for compounds **1**–**3** are essentially identical. The activation energy for **4** is slightly larger than those of **1**–**3**, while that for **5** is clearly greater than those of the other compounds. Little or no differences are observed in log A terms for all five compounds. The observed differences in the activation energies are roughly equal in magnitude to the 95% confidence limits and must be viewed with caution. However, the results are reproducible and the relative stabilities of this series of dioxetanes are in accord with the activation energy differences.

Without added fluorescers, the thermal decompositions of **1**–**5** exhibited very weak chemiluminescence. Addition of fluorescers [9,10-dibromoanthracene (DBA) or 9,10-diphenylanthracene (DPA)] resulted in large increases in chemiluminescence intensity without increasing the rate of dioxetane decomposition. The yields of excited products directly produced during the thermolysis of **1**–**5** were determined by the DBA/DPA method. The thermal decomposition of **1**–**5** produced high yields of excited triplets (~10%) and extremely low yields of excited singlets (<0.01%).

Discussion

The present series of 3,3-dialkyl-1,2-dioxetanes has increased steric interactions compared to those of 3,3-dimethyl-1,2-dioxetane (**6**). An interpretation of the data

Table I. Activation Parameters for the Thermal Decomposition of 3-Methyl-3-alkyl-1,2-dioxetanes in Xylenes

dioxetane	3-alkyl group	E_a, kcal/mol	log A	$k_{60°C}$, s^{-1}
1	ethyl	24.5 ± 0.2[a]	13.1	1.0×10^{-3}
2	1-propyl	24.6 ± 0.4	13.1	1.0×10^{-3}
3	1-butyl	24.4 ± 0.3	13.0	9.6×10^{-4}
4	2-propyl	25.0 ± 0.3	13.2	5.8×10^{-4}
5	*tert*-butyl	25.8 ± 0.3	13.3	2.6×10^{-4}

[a] 95% confidence limits, correlation coefficient > 0.998 (for all cases).

is that an increased number of formal substitutions (of a methyl group) results in an increase in the values of the activation energies. An estimation of the magnitude of this effect can be obtained by determining the relationship of the ΔE_a of dioxetanes **1**–**6** with the number of formal substitutions. An empirical relationship for the steric interactions of 3,3-disubstituted dioxetanes of approximately +0.8 kcal/mol per formal substitution on **6** is obtained. The 95% confidence limit for this empirical relationship is ±0.6 kcal/mol. This observation may be used to predict the activation energies of new dioxetanes. For example, E_a's of 28 and 26.5 kcal/mol are predicted for 3,3-di-*tert*-butyl- and 3,3-bis(2-propyl)-1,2-dioxetane, respectively. Confirmation as to the predictive value of this empirical relationship awaits further experimentation.

The results for these compounds fit into the general framework[2] of a diradical mechanism of dioxetane thermolysis. The types and yields of electronically excited cleavage products are consistent with those reported for other disubstituted dioxetanes.[2] An interpretation of the observed increase in E_a with increased formal substitution (based on a diradical process) suggests that steric interactions are more important in the transition state (and in the diradical) than in the ground state. This explanation is consistent with 3,4 steric or 3,3 steric interactions as the origin of the steric effect. Previously, Richardson accounted[6b] for the increased stability of 3,3-dibenzyl-1,2-

dioxetane by estimation of the increased steric interactions of the diradical intermediate due to gauche-phenyl–CH_2O· interactions (a type of 3,4 steric interaction). The study on 3,3-diethyl-1,2-dioxetane suggested[4a] 3,3 steric interactions as the origin of the steric effect. The results from a study of the thermolysis of a series of *cis*/*trans*-3,4-dialkyl-1,2-dioxetanes were interpreted[7] to show that 3,4 steric interactions were of minor importance. Adam has concluded,[8] based on a study of monospiroadamantane-substituted dioxetanes, that the introduction of one adamantyl moiety was sufficient to promote stabilization of the dioxetane. It was further concluded[8] that compressional effects did not apply in that case. These studies show that 3,3-substitutions are sufficient to produce steric interactions in alkyldioxetanes that result in stabilization of the compounds. The agreement or disagreement of calculated dioxetane activation parameters by a group additivity-type method[6b,9] with the experimental values could be used, in theory, to discover the nature of the steric effect. However, at present, the thermochemical values of oxygen-containing groups required for the calculations must be estimated. Thus, unfortunately, the origin of the steric effect on dioxetane decomposition as a 3,3 steric effect or a 3,4 steric effect can not be resolved at this time.[10]

The results for dioxetanes with 3,4 cyclic substituents have been used[4b-d] to suggest a twisting mechanism[4c] of diradical formation during thermolysis. This notion has received further support in a study[5] of dicyclodioxetanes. These studies suggest that conformation can affect dioxethane activation parameters. The dioxetane, prepared by the reaction of singlet oxygen with 7-methyl-1,3,5-cycloheptatriene, was found[11] to be unusually unstable. This may represent an additional example of conformational effects on dioxetane stability. The present results could also fit into this pattern. Progress has been made in the understanding of steric interactions in the dioxetane system. However, several sets of reported activation parameters of dioxetane thermolysis do not fit the general patterns of steric effects discussed above. For example the activation energy of 3,4-di-*n*-butyl-3,4-dimethyl-1,2-dioxetane[12] and those of several tetrasubstituted dioxetanes reported by Lechtken[4d] are less than that of tetramethyl-1,2-dioxetane.[2,3b] On the basis of steric effects, these dioxetanes would have been expected to be more stable.

Experimental Section

All solvents were of reagent grade. ^1H NMR spectra were recorded on a Varian EM 360 L NMR spectrometer. Gas chromatographic studies were performed on a Varian Model 920 GC with a 6 ft × 0.25 in. SE-30 on chromosorb W column (helium flow rate of 60 mL/min). The alkenes were available commericially. 9,10-Diphenylanthracene (Aldrich) was used without further purification. 9,10-Dibromoanthracene (Aldrich) was recrystallized from xylenes (Aldrich) before use. The chemiluminescence monitoring system is essentially identical with that described previously.[13]

Dioxetane Synthesis and Purification. The following procedure[2] for the synthesis of 3-methyl-3-ethyl-1,2-dioxetane (1) was employed for the preparation of the dioxetanes. 2-Methyl-1-butene (5.16 g, 74 mmol) was converted to 1-bromo-2-hydroperoxy-2-methylbutane in 60% yield by the standard procedure developed by Kopecky.[14] The bromo hydroperoxide (an oil, **caution**!) was placed in 10 mL of carbon tetrachloride and cooled to 0 °C (ice bath). The solution was stirred rapidly (magnetically). Five grams of KOH in 20 mL of cold distilled (deionized) H_2O was added dropwise during a 15-min period to the reaction mixture in the dark. The bright yellow CCl_4 layer was separated from the reaction mixture after complete reaction of the bromo hydroperoxide (approximately 15 min) via a (cooled) separatory funnel. The CCl_4 layer was dried over anhydrous $MgSO_4$ and filtered. The dioxetane was partially purified and concentrated by low-temperature distillation. Purification and isolation were accomplished by low-temperature column chromatography. A jacketed column (15 mm i.d.) was packed with 20–25 g of silica gel/Na_2 EDTA (100/1) with pentane as the solvent. The sample of impure dioxetane in 1 mL of CCl_4 was placed on the column with the temperature at −30 °C. The sample was eluted with 50-mL portions of a step-gradient [10% (v/v)] of pentane/methylene chloride. Fractions (10 mL) were collected in vials and set on dry ice. The temperature of the column was maintained at −30 °C and the entire procedure was carried out in less than 10 min. [Pressure from a nitrogen tank was used to speed up the procedure.] The fractions were analyzed for dioxetane content in a chemiluminescence apparatus. A small aliquot from each fraction was added to a heated solution of 9,10-dibromoanthracene ($\sim 5 \times 10^{-3}$ M) in xylenes and the relative light intensity recorded. The solvent for the fractions that produced the most light intensity was removed under reduced pressure to yield the dioxetane (6%) as a light yellow oil. NMR spectroscopy and iodometric titration showed the dioxetane to be of better than 95% purity. NMR (CCl_4) for **1**: δ 0.95 (br t, J = 7 Hz, 3 H), 1.53 (s, 3 H), 1.8 (br q, J = 7 Hz, 2 H), 4.83 (s, 2 H). NMR for **2**: δ 0.95 (br t, $J \sim 6$ Hz, 3 H), 1.4 (m, 2 H), 1.53 (s, 3 H), 1.7 (m, 2 H), 4.80 (AB pattern, 2 H). NMR for **3**: δ 0.95 (br t, 3 H), 1.4 (m, 4 H), 1.53 (s, 3 H), 1.7 (m, 2 H), 4.82 (AB pattern, 2 H). NMR for **4**: δ 0.90 (d, J = 7 Hz, 3 H), 0.94 (d, J = 7 Hz, 3 H), 1.50 (s, 3 H), 2.3 (m, J = 7 Hz, 1 H), 4.81 (AB pattern, 2 H); for **5**: δ 1.01 (s, 9 H), 1.59 (s, 3 H), 4.8 (wide AB, 2 H). The dioxetanes were stored as $\sim$ 0.2 M solutions in CCl_4 at −30 °C. The dioxetane concentrations of the solutions were determined by iodometric titration by the method of Wilson and Schaap[13] and checked by NMR analysis vs. added external standard.

Product Studies. The following procedure was employed for **1–5**. A 0.2 M solution of dioxetane in CCl_4 was heated at 60 °C in a sealed NMR tube until the yellow color disappeared. The corresponding ketone and formaldehyde (trace noted) were the only products detected by NMR spectroscopy. The ketone was detected by GC analysis of the solution.

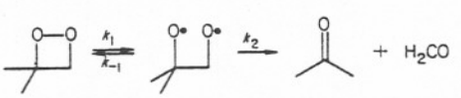
Kinetic Studies and Yields of Excited States. The temperature (±0.2 °C) of the reaction mixture in the chemiluminescence apparatus was monitored by use of a YSI Model 425C telethermometer with no. 423 probe before and after each run.

(7) Baumstark, A. L; Dunams, T.; Roskamp, P. C.; Wilson, C. E. *J. Org. Chem.* **1983**, *48*, 261.

(8) Adam, W.; Arias, L. A.; Zinner, K., 15th Congress Latinioamerican de Quimica, San Juan, PR; Oct 24–29, 1982; Abstract No. A-K-23, p 270.

(9) O'Neal, H. E.; Richardson, W. H. *J. Am. Chem. Soc.* **1970**, *92*, 6553 and correction; **1971**, *93*, 1828.

(10) A reviewer suggested the following interpretation: "For example, if thermolysis is stepwise why isn't reclosure to starting material condisered? In that case the reported rate constant would really be $k_{obsd} = (k_1/k_{-1})k_2$ or $K_{eq}\,k_2$. One might expect the substituent effects on K_{eq} and



k_2 to be in opposite directions. That is, large substitutents would depress K_{eq} (the well-known Thorpe–Ingold effect) and enhance k_2, since the product-forming step must relieve nonbinding interactions. The surprisingly small change on going from ethyl to *tert*-butyl might thus be understood as the result of partial cancellation of larger, but opposite, substituent effects". Group additivity-type calculations of dioxetane activation parameters have been based[9] (in part) on the assumptions that $k_2 \gg k_{-1}$ and that E_{-1} is constant. The activation energy is assumed to be equal to [$\Delta H_f°$(diradical) − $\Delta H_f°$(dioxetane)] + E_{-1}.[9] Bechara and Wilson have pointed out[4c] that E_{-1} may not be invarient in all cases. However, the assumption that O-O bond cleavage ($k_2 \gg k_{-1}$) is rate determining in alkyl dioxetane thermolysis seems valid.

(11) Adam, W.; Balci, M.; Cueto, O.; Pietrzak, B. *Tetrahedron Lett.* **1979**, 4137.

(12) (a) Darling, T. R.; Foote, C. S. *J. Am. Chem. Soc.* **1974**, *96*, 1625. (b) Foote, C. S.; Darling, T. R. *Pure Appl. Chem.* **1975**, *41*, 495.

(13) Wilson, T.; Schaap, A. P. *J. Am. Chem. Soc.* **1971**, *93*, 4126.

(14) Kopecky, K. R.; Filby, J. E.; Mumford, C.; Lockwood, P. A.; Ding, J.-Y. *Can. J. Chem.* **1975**, *53*, 1103.

The jacketed cell was pretreated with an aqueous Na_2EDTA solution. All runs were carried out in xylenes (Aldrich) as the solvent. The initial dioxetane concentration of a run was kept at $\sim 10^{-4}$ M to avoid induced decomposition of the dioxetane. Runs carried out without added fluorescer, with DPA, and with DBA were of the first order for at least 3 half-livers and showed no dependence on type or amount of added fluorescer. The yields of excited states produced upon dioxetane thermolysis was determined at 50.0 °C by variation of the concentration of fluorescer at constant dioxetane concentration (DBA/DPA method). The method of calculation has been discussed in detail.[2a] The value of ϕ_{ET} for energy transfer of triplet carbonyls to DBA was assumed to be 0.2 for all five cases. The apparatus was calibrated by taking the yield of triplet excited products from thermolysis of trimethyl-1,2-dioxetane determined by the DBA method, as 0.15.[15]

Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research, to NIH (Grant No. RR 09210) and to the GSU research fund. E.J.H.B. recieved grants from Fundacão de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Financiadora de Estudos e Projectos (FINEP), and Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq).

Registry No. 1, 86954-68-9; 2, 86954-69-0; 3, 86954-70-3; 4, 86954-71-4; 5, 86954-72-5; 1-bromo-2-hydroperoxy-2-methylbutane, 86954-73-6.

(15) Kopecky, K. R.; Filby, J. E. *Can. J. Chem.* **1979**, *57*, 283.

Anexo II

Electron transfer and chemiluminescence. Two inefficient systems: 1,4-dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide. Catalani, L.H., Wilson, T., *J. Am. Chem. Soc.* **111**, 2633 (1989).

Electron Transfer and Chemiluminescence. Two Inefficient Systems: 1,4-Dimethoxy-9,10-diphenylanthracene Peroxide and Diphenoyl Peroxide

Luiz H. Catalani† and Thérèse Wilson*

Contribution from The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received September 26, 1988*

Abstract: The 1,4-endoperoxide of 1,4-dimethoxy-9,10-diphenylanthracene (**1**) rearranges under mild acidic conditions to the 1,2-dioxetane **3**, which has now been characterized by NMR. The decomposition of **3** is catalyzed by fluorescers (such as **1**) in a bimolecular process which populates their singlet excited state and generates the well-known chemiluminescence. There are significant similarities between this indirect chemiluminescence and that of other peroxides which have been interpreted as examples of the CIEEL mechanism:[1] the pseudo-first-order rate of decomposition of **3** is a linear function of the activator (fluorescer) concentration, $k_{obsd} = k_1 + k_2[ACT]$, where k_2 increases as the oxidation potential of the activator decreases. A linear plot of log k_2 vs activator's E_{ox} is scattered but reasonably convincing. The quantum yield associated with the activated decomposition of **3** is only ca. 2×10^{-5} einstein per mol of **3** reacting with activator. Since this value is 4 orders of magnitude lower than that reported for the very similar reaction of diphenoyl peroxide, the latter system was reinvestigated and found, under our conditions, to be equally inefficient. The implications of these results are discussed.

The suggestion that *intermolecular* electron transfer plays a key role in some solution chemiluminescence has received a great deal of attention in recent years.[1] Fluorescers, it is proposed, can act as catalysts of the decomposition of some peroxides by a process of "chemically induced electron exchange luminescence" (CIEEL),[1a,2] which is regarded as an efficient pathway of generation of excited singlet states. We have tested the CIEEL hypothesis on one of the classic examples of chemiluminescence, that of polyacenes endoperoxides. Strong analogies between this system and the chemiluminescence of diphenoyl peroxide,[2] the prototypical example of intermolecular CIEEL, led us to reinvestigate this reaction. We found both chemiluminescences to be disappointingly inefficient.

Sixty years ago, Dufraisse and co-workers[3] reported that decomposition of polyacenes peroxides generates molecular oxygen and the polyacene. The most chemiluminescent of these compounds, the 1,4-endoperoxide **2** of 1,4-dimethoxy-9,10-diphenylanthracene (**1**), is also the most thermally labile; the formation of the peroxide is fully reversible.[3-5] This is truly a unique photochemical reaction: formation of the peroxide is a singlet oxygen reaction, while its decomposition regenerates molecular oxygen quantitatively, a large fraction, if not all of it, in the singlet excited state.[4] The sharp peak at 1.28 μm associated with the (0,0) transition of O_2 $^1\Delta_g \rightarrow {}^3\Sigma_g$ was recently recorded.[6] Moreover, the activation energy of the decomposition of **2**, ca. 19–25 kcal/mol, is roughly equal to the excitation energy of $^1O_2(^1\Delta_g)$, i.e., 22.5 kcal. Thus this process could be regarded as a spin-controlled, infrared chemiluminescent reaction with a quantum yield of ~ 1.

But the visible chemiluminescence has a different origin. The endoperoxide **2** is highly sensitive to traces of acids, which catalyze a rearrangement to aldehyde ester **4** and other minor products.[7] We previously showed[5] that it is this rearrangement, presumably via dioxetane **3**, which leads to visible chemiluminescence, not the process which regenerates **1** and $^1O_2$. In pyridine the thermolysis of **2** reforms **1** quantitatively with no visible chemiluminescence; in toluene, due to adventitious traces of acid, little **1** is reformed, while a blue-green chemiluminescence develops with a spectrum matching the fluorescence of **1** (peak at ca. 480 nm). We proposed[5] at the time that the chemiluminescence results from a compromise between the uncatalyzed path producing **1** (the emitter) and the acid-catalyzed rearrangement of **2** giving the dioxetane and a hypothetical "energy-rich precursor". This excited intermediate was thought to excite **1** via energy transfer, an assumption shown here to be incorrect.

The goals of the present study were to confirm the intermediacy of dioxetane **3** and to identify the excitation mechanism which, as that of diphenoyl peroxide, remains a topic for discussion, due to the low quantum yields of these two reactions.

Results

1. Intermediacy of a Dioxetane. Organic acids (acetic or benzoic acid) and mineral acids (HCl to H_2SO_4) as well as silica gel act as catalysts of the rearrangement of peroxide **2** to **3**; but these acids also quickly decompose the dioxetane through a nonchemiluminescent pathway, making it impossible to isolate the already thermally unstable dioxetane. Its presence was nevertheless established by low-temperature NMR spectroscopy[8] (Figure 1). The assignment of protons in position 2, 3, 5, and 6 was based on decoupling experiments and similarity with known structures.[9] The carbons at positions 1–6 were assigned on the

† Present address: Instituto de Quimica, Universidade de Sao Paulo, C.P. 20.780, Sao Paulo 01498, Brazil.

(1) Reviews: (a) Schuster, G. B.; Schmidt, S. P. *Adv. Phys. Org. Chem.* **1982**, *18*, 187. (b) Schuster, G. B. *Acc. Chem. Res.* **1979**, *12*, 366. (c) Wilson, T. In *Singlet Oxygen*; Frimer, A. A., Ed.; CRC: Boca Raton, FL, 1985; Vol. II, pp 37–57.

(2) Koo, J.-Y.; Schuster, G. B. *J. Am. Chem. Soc.* **1978**, *18*, 187.

(3) (a) Moureu, C.; Dufraisse, C.; Dean, P. M. *Compt. Rend. Acad. Sci.* **1926**, *182*, 1584. (b) Dufraisse, C.; Velluz, L. *Bull. Soc. Chim. Fr.* **1942**, *9*, 171. (c) Dufraisse, C.; Rigaudy, J.; Basselier, J. J.; Cuong, N. K. *Compt. Rend. Acad. Sci.* **1965**, *260*, 5031.

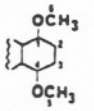
(4) Turro, N.; Chow, M.-F.; Rigaudy, J. *J. Am. Chem. Soc.* **1981**, *103*, 7218.

(5) (a) Wilson, T. *Photochem. Photobiol.* **1969**, *10*, 441. (b) Other authors reached the same conclusion independently: Lundeen, G. W.; Adelman, A. H. *J. Am. Chem. Soc.* **1970**, *92*, 3414.

(6) Wilson, T.; Khan, A. U.; Mehrotra, M. M. *Photochem. Photobiol.* **1986**, *43*, 661.

(7) (a) Rigaudy, J.; Deletang, C.; Sparfel, D.; Cuong, N. K. *Compt. Rend. Acad. Sci.* **1968**, *267*, 1714. (b) Baldwin, J. E.; Basson, H. H.; Krauss, H. *Chem. Commun.* **1968**, 984.

(8) NMR assignments (d in ppm) for protons (500 MHz, $CDCl_3$) and for carbons (125 MHz, $CDCl_3$): (**1**) ^{1}H NMR 6.61 (s, 2 H), 3.38 (s, 6 H); ^{13}C NMR (bb) 150.8, 104.2, 56.2; (**2**) ^{1}H NMR 7.09 (s, 2 H), 3.29 (s, 6 H); ^{13}C NMR (bb) 105.6, 104.1, 53.1; (**3**) ^{1}H NMR 6.31 (d, 1 H, ^2J = 6.4 Hz), 4.92 (d, 1 H, ^2J = 6.4 Hz), 3.33 (s, 3 H), 3.24 (s, 3 H); ^{13}C NMR (bb, DEPT) 160.0, 105.0, 91.5, 79.5, 55.0, 49.5; (**4**) ^{1}H NMR 9.26 (d, 1 H, ^2J = 8.1 Hz), 5.42 (d, 1 H, ^2J = 8.1 Hz), 3.61 (s, 3 H), 3.51 (s, 3 H); ^{13}C NMR (bb, DEPT) 192.5, 178.3, 168.9, 107.2, 56.8, 52.4.



0002-7863/89/1511-2633$01.50/0

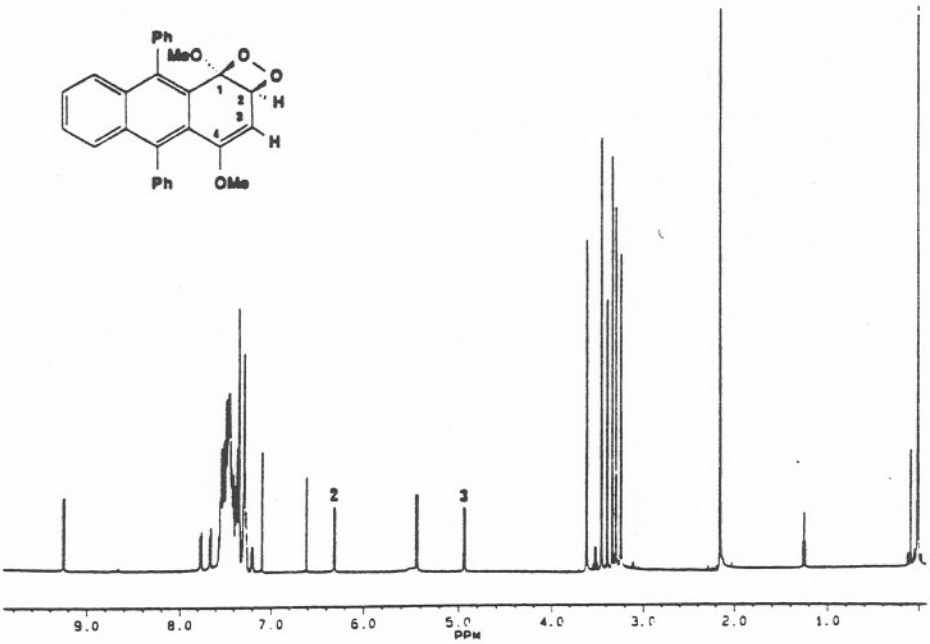
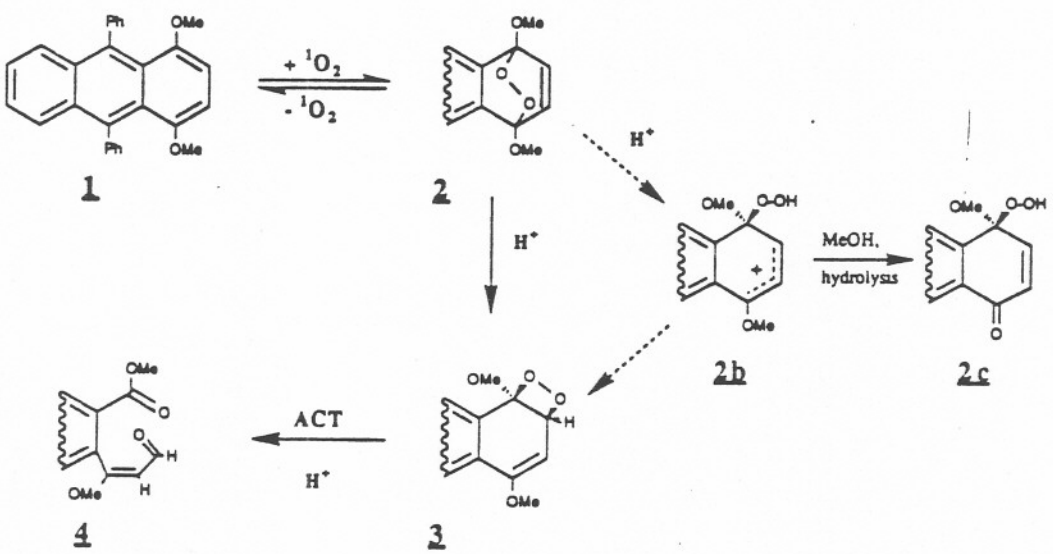


Figure 1. ^{1}H NMR spectrum of dioxetane 3 in a $CDCl_3$ solution of endoperoxide 2 and acetic acid at −35 °C.

Scheme I



basis of broad-band, DEPT, and specific-decoupling experiments against authentic material of **1**, **2**, and **4**. Upon heating in the NMR probe, the dioxetane signals decrease with concomitant increase of the peaks assigned to aldehyde ester **4**, the sole product.

The ^{1}H NMR of a solution of **2** in toluene-d_8 (no added acid) shows that **3** and **4** grow at the expense of **2**, along a time course of days at −20 °C. The relative rates of rearrangement of **2** to **3** and decomposition of **3** to **4** determine the maximum dioxetane concentration (5–10% of the initial concentration of **2** in these conditions). The addition of 1 equiv of acetic acid directly to a $CDCl_3$ solution of **2** at −35 °C resulted in up to 43% conversion of **2** to **3** but with faster decomposition of **3** to **4** (half-life of **3**, 1 h). In contrast, in pyridine-d_5 the decomposition of endoperoxide **2** led to exclusive formation of anthracene **1**, as previously reported.

The intramolecular rearrangement of endoperoxide to dioxetane is probably a double bond shift, via a hydroperoxide-like intermediate **2b** which subsequently cyclizes to the dioxetane **3**. An indication of the involvement of structure **2b** was obtained when a trapping product **2c** was detected in the reaction of **2** with acetic acid, at 20 °C in MeOH as solvent.[10]

2. Conditions for Light Emission. The time course of the chemiluminescence form a toluene solution of **2** at 50 °C is as follows. After an initial peak, attributed to the thermolysis of preaccumulated dioxetane, the light intensity increases to a maximum in ca. 1 h and then slowly decays (not first order). This behavior can be explained by the rearrangement of **2** to **3**, catalyzed by traces of acid present as impurity, and the subsequent thermolysis of **3**. If **1** is added to a solution of **2**, the intensity reaches a maximum earlier but decays faster. Addition of acetic

(9) For comparison with similar structures, see: (a) Clennan, E. L.; L'Esperance, R. P. *J. Am. Chem. Soc.* **1985**, *107*, 5178. (b) Clennan, E. L.; Lewis, K. K. *J. Am. Chem. Soc.* **1987**, *109*, 2475.

(10) Endoperoxide **2** (100 mg) was suspended in 50 mL of MeOH at 0 °C, and 50 μL of HAc was added. After 12 h 50 mL of CH_2Cl_2 was added, and the solution was washed with $NaHCO_3$ (aqueous) and brine, dried over $MgSO_4$, and rotaevaporated. The residue was purified by thin-layer chromatography (SiO_2 eluted with CH_2Cl_2/Ethylacetate, 95:5). The spot at R_f 0.35 was removed, washed, and reapplied to TLC plate (SiO_2 eluted with CH_2Cl_2/ethylacetate, 88:12). The peroxidic spot at R_f 0.47 was removed and analyzed by ^{1}H NMR. The analyses showed signals corresponding to aldehyde ester **4** and hydroperoxide **2c** in a mixture of 2:1 ratio: ^{1}H NMR for **2c** (300 MHz, $CDCl_3$) d in ppm 9.09 (b, 1 H), 7.13 (d, 1 H, ^2J = 10.5 Hz), 6.48 (d, 1 H, ^2J = 10.5 Hz), 3.12 (s, 3 H).

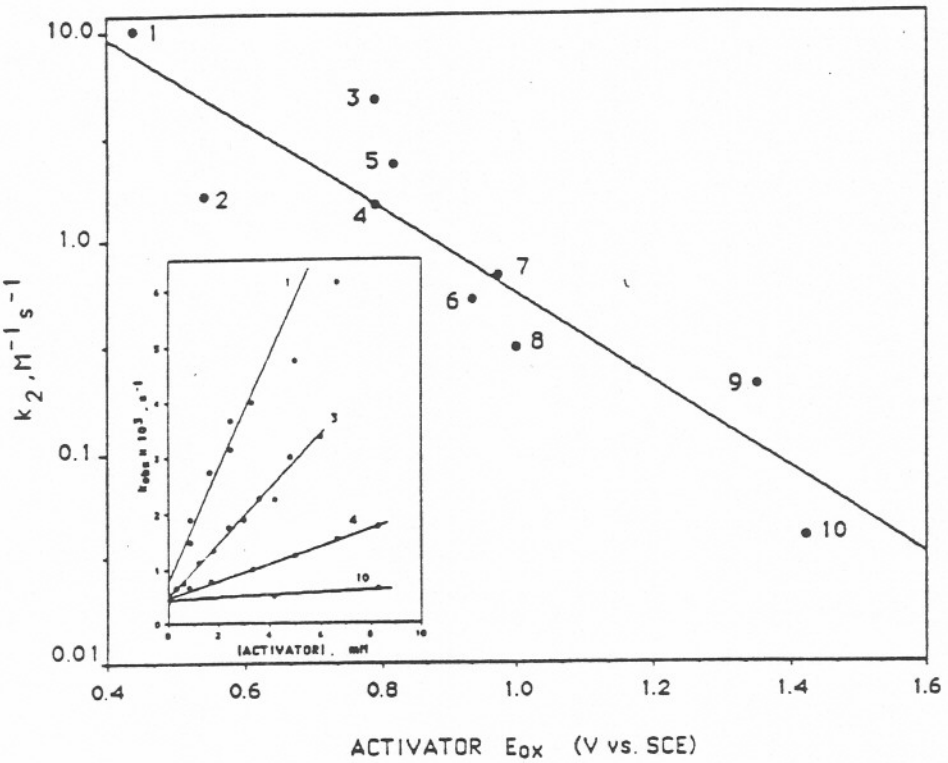


Figure 2. Dependence of catalytic rate constant k_2 on oxidation potential of activator. Inset: effect of activator concentration on rate of decay of chemiluminescence intensity (slope is k_2). All in *p*-dioxane–toluene (11/1, v/v) at 50 °C. Activators numbered as in Table I.

acid increases the peak intensity and also shortens the time needed to reach it. Pyridine or triethylamine rapidly quench the light-emitting reaction, an effect which can be reversed by addition of acid.

The effects of solvents on the course of chemiluminescence from **2** seems to be mainly a function of their ability to provide the right amount of acid as impurities. In $CHCl_3$, CH_2Cl_2, and other solvents where formation of traces of acid is difficult to prevent or control, **2** decomposes fast to **4** but with very low chemiluminescence. **2** is stable in toluene freshly filtered over basic alumina, but the concentration of **3** slowly builds up in untreated toluene (see above), where the concentration of acid-catalyzing impurities seems optimum for dioxetane accumulation.

In freshly distilled *p*-dioxane, endoperoxide **2** is fairly stable against rearrangement, and no dioxetane formation is observed. The addition of an aliquot of an "aged" solution of **2** in toluene, used as a dioxetane "stock" solution, to a solution of a fluorescer in *p*-dioxane gives a luminescence which now decays according to first-order kinetics. Thus chemiluminescence is strictly due to decomposition of dioxetane already present in the toluene solution. Because of the clean kinetics in these conditions, most experiments reported here were performed in *p*-dioxane containing a small volume of a toluene solution of dioxetane (usually *p*-dioxane–toluene, 11/1, v/v) at 50 °C. Note that such solutions are unavoidably contaminated by traces of **1**; see below.

3. Evidence for an "Activated" Chemiluminescence. In general, we found that the first-order rate constants, k_{obsd}, of chemiluminescence decay in *p*-dioxane are linearly dependent on the concentration of added fluorescer (eq 1); thus the major pathway

$$k_{obsd} = k_1 + k_2[ACT] \qquad (1)$$

to light emission is a bimolecular process between dioxetane and fluorescer (or "activator", in Schuster's terminology).[1] Figure 2 (inset) shows examples of the dependence of k_{obsd} on the concentration of added activators. (As mentioned above, the intercept k_1 comprises a small contribution from the decomposition of **3** catalyzed by traces of **1** in the toluene stock solution). With some fluorescers, notably perylene, the light intensity decay deviates from first-order: the plots of log I vs time show upward curvature, especially at high concentrations of perylene, and the plots according to eq 1 are not strictly linear either. We do not yet understand the cause of these deviations. The chemiluminescence spectrum matches the fluorescence spectrum of the activator present, and the initial chemiluminescence intensity is proportional to its concentration; plots of I_0 vs [ACT] are similar to those of Figure 2.

Table I. Dependence of Catalytic Rate Constant k_2 on Activator Oxidation Potential[a]

	activator	E_{ox}, V (vs SCE)	k_2, $M^{-1} s^{-1}$
1.	2-aminoanthracene	0.44[b]	10.1
2	1-aminonaphthalene	0.54[b]	1.64
3.	1,4-dimethoxy-9,10-diphenylanthracene (**1**)	0.79[c]	4.82
4.	1,3-diphenylisobenzofuran	0.79[d]	1.49
5	10-methylphenothiazine	0.82[e]	2.30
6.	perylene	0.93[f]	0.53
7.	rubrene	0.97[g,h]	0.69
8.	triethylamine	1.00[i]	0.31
9.	9,10-diphenylanthracene	1.35[e,j]	0.21
10.	9,10-dibromoanthracene	1.42[k]	0.04

[a] At 50 °C in *p*-dioxane–toluene (11/1, v/v). The E_{ox} values refer to measurements in acetonitrile solutions unless otherwise indicated. [b] Pysh, E. S.; Yang, N. C. *J. Am. Chem. Soc.* **1963**, *85*, 2124. [c] This work and Cruanes, M., University of Illinois, personal communication. [d] Zweig, A.; Metzler, G.; Maurer, A. H.; Roberts, B. G. *J. Am. Chem. Soc.* **1967**, *89*, 4091. [e] In dimethyl formamide: Faulkner, L. R.; Tachikawa, H.; Bard, A. J. *J. Am. Chem. Soc.* **1971**, *94*, 691. [f] This work. Range in lit: 0.12. [g] Chang, M.-M.; Saji, T.; Bard, A. J. *J. Am. Chem. Soc.* **1977**, *99*, 5399. [h] 0.82 V in CH_2Cl_2: Phelps, J.; Santhanan, K. S. V.; Bard, A. J. *J. Am. Chem. Soc.* **1967**, *89*, 1752. [i] Calcd from E_{ox} = 0.66 vs Ag/0.1 M $AgNO_3$: Mann, C. K. *Anal. Chem.* **1964**, *36*, 2424. [j] 1.20 V in CH_2Cl_2, ref h. [k] Parker, V. D. *Acta. Chem. Scand.* **1970**, *24*, 2775.

It is clear that k_2 and I_0 depend on the nature of the activator. In the case of the chemiluminescent decomposition of diphenoyl peroxide, Schuster et al.[2] showed that k_2 depends on the oxidation potential of the activator; they presented a linear free energy plot of ln k_2 vs E_{ox} for six activators with oxidation potentials in the rather narrow range 0.8–1.36 V (vs SCE).[2] Table I and Figure 2 demonstrate a similar dependence of k_2 on E_{ox}[11] over a wider

Table II. Solvent Effects on the Chemiluminescence of Diphenoyl Peroxide at 32.5 °C with Perylene as Activator[a]

solvent	$k_1 \times 10^4$, s^{-1}	k_2, M^{-1} s^{-1}	rel Φ_{CL}	ϵ[b]	η, mp[c]
CH_2Cl_2	4.4 (4.5)	1.6 (1.5)	1 (1)	8.9	3.9
benzene	6.1 (5.5)	0.6 (0.5)	2.3 (3.3)	2.3	6.0
ether	5.2 (5.6)	0.1 (0.06)	8.5 (15.4)	4.3	2.2
p-dioxane	5.4	0.5	2.1	2.2	12.0

[a] Comparison of the results of this work with those of Koo and Schuster[2] (values in parentheses). [b] Dielectric constant. [c] Viscosity.

range. A plot of log I_o vs E_{ox} has the same slope. Although the data points are scattered, in part because of uncertainties on the values of E_{ox}, the *trend* is evident; it indicates the role of either electron transfer or charge transfer.

4. Chemiluminescence Quantum Yield. Equations 2–6 where D is dioxetane **3** describe our observations without presuming the mechanism:

$$D \xrightarrow{k_1} 4 \quad (2)$$

$$D + ACT \xrightarrow{\Phi_{CL}k_2} 4 + ACT^* \quad (3)$$

$$D + ACT \xrightarrow{(1-\Phi_{CL})k_2} 4 + ACT \quad (4)$$

$$ACT^* \xrightarrow{\Phi_F k_F} ACT + h\nu \quad (5)$$

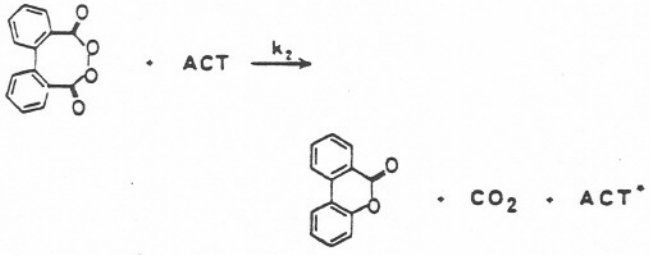
$$ACT^* \xrightarrow{(1-\Phi_F)k_F} ACT \quad (6)$$

Steady-state treatment leads to the following expression for the chemiluminescence quantum yield from the activated reaction path

$$\Phi_{CL} = I_o/\{\Phi_F k_2[ACT][D]_o\} \quad (7)$$

where I_o and $[D]_o$ are the initial intensity and concentration of dioxetane **3**, [ACT] is the concentration of the activator, and Φ_F its fluorescence efficiency in *p*-dioxane at the temperature of the experiment (Φ_F = 0.75 for **1**; 1.0 for rubrene, at 50 °C). (**Note Added in Proof**: As defined by eq 3, Φ_{CL} is an excitation yield, not an overall photon production yield. These two yields are the same with such activators as rubrene or perylene, which have fluorescence efficiencies of unity.) $[D]_o$ was calculated from the molarity of the toluene stock solution, which was determined by ^1H NMR in toluene-d_8 at −20 °C using acetone as standard 1 h prior to the experiment. The chemiluminescence quantum yield Φ_{CL} of **3** activated by **1** or by rubrene is (2 ± 1) × 10^{-5} E/mol.

5. Comparison with Diphenoyl Peroxide. Such a low value of Φ_{CL} led us to reinvestigate the chemiluminescence of diphenoyl peroxide. Table II lists the values of Φ_{CL}, k_1, and k_2 in three



different solvents with perylene as activator. All rate constants are in reasonable agreement with the literature, but our values of Φ_{CL} are *4 orders of magnitude* lower than the value (10%) reported by Koo and Schuster.[2] In our hands the perylene-activated decomposition of diphenoyl peroxide in CH_2Cl_2 at 32.5 °C has, like **3**, a quantum efficiency of (2 ± 1) × 10^{-5} E/mol.[12]

(11) In the case of 1-aminonaphthalene and triethylamine, the reaction rate was followed by monitoring the fluorescence of traces of **1** in the solution. Note that 9,10-dibromoanthracene is a poorer activator than 9,10-diphenylanthracene, indicating that excitation does not result from energy transfer from a high-energy triplet donor. The absorption spectra and the S_1 energies of these two anthracenes are very similar, so that in an energy-transfer process they should have similar efficiencies as acceptors of singlet energy.

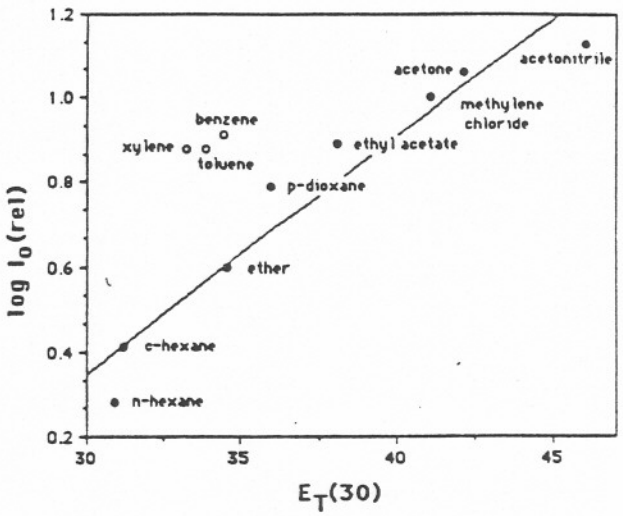


Figure 3. Effect of solvent polarity on initial intensity of chemiluminescence from diphenoyl peroxide: plot of log I_o(rel) vs solvent E_T(30), with log I_o = 1.0 for methylene chloride. Perylene (7.4 × 10^{-5}M) as activator. Diphenoyl peroxide concentration: 4.0 × 10^{-4}M. All solutions contained 3% CH_2Cl_2 per volume.

Table III. Comparison of Reaction Rates in Peroxide Chemiluminescence Reported To Follow CIEEL Mechanisms[f]

peroxide	solvent	temp, °C	k_1, s^{-1}	k_2, M^{-1} s^{-1}	k_2/k_1, M^{-1}
diphenoyl peroxide[a]	CH_2Cl_2	32.5	4.5 × 10^{-4}	1.5	3300
dimethyl-dioxetanone[b]	benzene	24.5	7.8 × 10^{-4}	5.2 × 10^{-3}	6.6
phenylethyl peroxyacetate[c]	benzene	99.5	1.1 × 10^{-4}	1.2 × 10^{-4}	1.1
malonyl peroxide[d]	CH_3CN	80.0	8.0 × 10^{-4}	1.6	2000
dioxetane **3**[e]	dioxane	50.0	4.4 × 10^{-3}	0.5	120

[a] Reference 2. [b] Reference 15. [c] Reference 16. [d] 4-Methyl-4-(1-propenyl)malonyl peroxide, ref 14. [e] This work. [f] With perylene as activator.

Table II as well as already available data in a few other solvents shows that Φ_{CL} varies little with solvent. Since with a given activator $I_o = Bk_2\Phi_{CL}$ (where *B* is a constant determined by temperature and concentrations of activator and peroxide), it is implicit in these results that I_o is quite independent of the solvent. We have verified this conclusion and extended it to a larger range of solvents. Figure 3 shows a plot of log I_o versus the polarity parameter E_T(30) based on charge-transfer transitions;[13] the initial chemiluminescence intensity increases with solvent polarity but only by a factor of 7 between nonpolar hexane and strongly polar acetonitrile. (Aromatic solvents do not fit on the line, as previously noted.)[2] The solvent viscosity appears to have very little effect on I_o judging by an experiment in dibutylphthalate, which gave an I_o value similar to that in acetonitrile. The E_T(30) value of dibutylphthalate is unfortunately not known, but since it is both aromatic and more polar than benzene, the high viscosity of this solvent (ca. 20× that of benzene) has clearly no major effect on I_o.

Discussion

The role of long-suspected dioxetane **3** was confirmed. We have shown that fluorescers, including anthracene **1**, are active participants in its chemiluminescent thermolysis, not mere acceptors of energy, and that the oxidation potential of these fluorescers influences both reaction rate and chemiluminescence intensity.

(12) At our request, Dr. Schuster kindly sent us copies of J.-Y. Koo's primary data (luminescence intensity vs concentration of perylene in the case of diphenoyl peroxide and intensity vs [DBA] in the case of tetramethyldioxetane which was used for calibration). From these data we calculate Φ_{CL} ~ 10^{-4} E/mol. We were pleased to learn, after submission of this paper, that Gary Schuster agrees with this re-evaluation and thus with the very low efficiency of the diphenoyl peroxide chemiluminescence.

(13) Reichert, C. *Solvent Effects in Organic Chemistry*; Verlag-Chemie: Weinheim, 1979.

Thus in all major aspects, the reaction of dioxetane **3** follows a course similar to that of diphenoyl peroxide. Apart from the important difference in quantum yield, we reproduced here the key points (eq 1 and 7 and Figure 2) which led Schuster and co-workers to formulate the influential CIEEL mechanism. These authors have described three peroxide reactions as prime examples of intermolecular CIEEL:[14] diphenoyl peroxide,[2] dimethyldioxetanone,[15] and peroxyesters.[16] Table III compares the k_1 and k_2 values in these reactions with the corresponding values in the reaction of **3**; it shows that in the cases of **3**, the ratio k_2/k_1, although not as high as in the case of diphenoyl peroxide, is still quite favorable to the study of the activated pathway of peroxide decomposition.

The CIEEL hypothesis is compatible with our results. It provides a framework for discussion by substituting concrete steps 8–10 for the formal reactions 3 and 4 above, as follows:

$$3 + \mathrm{ACT} \rightleftharpoons [3^{\bullet-}\ \mathrm{ACT}^{\bullet+}] \tag{8}$$

$$[3^{\bullet-}\ \mathrm{ACT}^{\bullet+}] \rightarrow [4^{\bullet-}\ \mathrm{ACT}^{\bullet+}] \tag{9}$$

$$[4^{\bullet-}\ \mathrm{ACT}^{\bullet+}] \rightarrow 4 + \mathrm{ACT}^{*} \tag{10}$$

The rate-controlling step, eq 8, is viewed as a dissociative electron transfer. The electron affinity of the peroxide is expected to increase as the O–O bond stretches, in agreement with MO calculations,[17] so that bond cleavage and electron transfer may occur simultaneously. While still in the original solvent cage, $3^{\bullet-}$ rearranges fast to $4^{\bullet-}$, a stronger reductant than $3^{\bullet-}$; the annihilation of $4^{\bullet-}$ and $\mathrm{ACT}^{\bullet+}$ then results in electronic excitation of the activator, eq 10.

The main argument for a CIEEL mechanism is the LFER plots of log k_2 (or log I_0) vs activator E_{ox} (Figure 2). Here the slope of the plot (which is equal to $\alpha F/2.3RT$)[18] is only 2.0 V^{-1}, which at 50 °C corresponds to $\alpha = 0.13$. This value of α is ca. 2 times smaller than the values reported by Schuster in the cases of diphenoyl peroxide, dimethyldioxetanone, and peroxyesters. It is comparable, however, to the α values obtained in the quenching of triplet ketones by amines[19] or by enol ethers[20] or of naphthalene fluorescence by strained saturated hydrocarbons,[21] for example. Although it has been argued that such small α is not incompatible with "full electron transfer" from the activator, it implies that the dioxetane must already have undergone, thermally, considerable stretching of the O–O bond.[22]

Within the framework of the CIEEL model, a high quantum yield of chemiluminescence requires that several conditions be satisfied, besides the trivial one of a high efficiency of activator fluorescence: (a) the bond rearrangement (eq 9) must be fast to compete with exothermic back electron transfer (note that our expression for Φ_{CL} is independent of back transfer) and with diffusion of the radical ions out of the solvent cage; (b) this rearrangement and the subsequent charge annihilation must also be faster than intersystem crossing in the caged pair of radicals, originally singlet; and (c) the enthalpy $-\Delta H$ released in the critical electron transfer between $3^{\bullet-}$ and $\mathrm{ACT}^{\bullet+}$ must be sufficient for promotion of the activator to its singlet excited state. We will now examine how these requirements apply to the cases of **3** and of diphenoyl peroxide. If a CIEEL type of mechanism obtains, what are the factor(s) responsible for the very low quantum yields (ca. 10^{-5})? By definition (eq 7), these yields refer to the number of excited activator molecules generated per molecule of peroxide decomposed via electron transfer (second order rate constant k_2) in the bimolecular reaction with activator.

Following Weller and Zachariasse,[23]

$$-\Delta H = E_{ox} - E_{red} + \Delta\Delta H - e_0^2/\epsilon a \tag{11}$$

where $\Delta\Delta H$ is a correction term necessary when the reaction solvent (here *p*-dioxane) is not the solvent in which the redox potentials were measured (acetonitrile). E_{red} of **4** and E_{ox} of **1** are −1.55 and 0.78 V, respectively, from cyclic voltammetry measurements.[24] In *p*-dioxane, $\Delta\Delta H$ is ca. 2 eV, and the coulombic energy is ca. 1 eV. Thus $-\Delta H \simeq 3.3$ eV, which is sufficient for excitation of **1** ($E_S \simeq 63$ kcal). Singlet excitation of rubrene, 2,5-diphenylisobenzofurane, perylene, naphthacene, and 9,10-diphenylanthracene should also be exothermic. It seems therefore unlikely that the energetics of the charge annihilation are responsible for the low quantum yield with these fluorescers. Excitation of 2-aminoanthracene and of 10-methylphenothiazine may just be energetically possible also, whereas excitation of 1-aminonaphthalene should be endothermic by ca 0.5 eV.[25] No chemiluminescence was observed with this activator, even though it is a strong catalyst of the decomposition of **3**.

Escape of either of the radical anions $3^{\bullet-}$ or $4^{\bullet-}$ from the proximity of the counter ion would evidently reduce the chemiluminescence efficiency. But if most radicals escaped and reacted with solvent, one would expect to find major products other than **4**, contrary to data. In the case of diphenoyl peroxide, formation of small amounts (5%) of diphenic acid was interpreted as resulting from the reaction of some radical anions (prior to CO_2 loss) with solvent;[2] but clearly this minor side reaction does not account for the low Φ_{CL}. Schuster has argued that ion escape from the primary solvent cage should be facilitated by solvents of high polarity and low viscosity and has presented some evidence for such solvent effects.[2,26]

Another possible cause of low quantum yield is a competition between two parallel bimolecular processes involving activator: one "dark" process and one leading to excited-state generation, both a function of the oxidation potential of the activator. If this were the case, one would expect two different values of activation energies, one for the catalytic rate constant k_2 based on rates of peroxide decomposition, the other based on chemiluminescence intensities. Koo and Schuster found no differences between these two activation energies with either perylene, rubrene, or DPA. Therefore it is most likely that the efficiency of chemiluminescence is primarily governed by losses *after* a common transition state.[30]

(14) The case of malonyl peroxides is more complex, see below: Porter, J. E.; Schuster, G. B. *J. Org. Chem.* **1985**, *50*, 4068.
(15) Schmidt, S. P.; Schuster, G. B. *J. Am. Chem. Soc.* **1980**, *102*, 306.
(16) Dixon, B. G.; Schuster, G. B. *J. Am. Chem. Soc.* **1981**, *103*, 3068.
(17) (a) Schmidt, S. P.; Vincent, M. A.; Dykstra, C. E.; Schuster, G. B. *J. Am. Chem. Soc.* **1981**, *103*, 1292. (b) Yamaguchi, K. In *Singlet Oxygen*; Frimer, A. A., Ed.; CRC Press: Boca Raton, FL, 1985; Vol. III, p 119.
(18) α is considered as analogous to the transfer coefficient in electrode reactions: the activation energy of reaction 8 is only a fraction of the total free-energy change in this redox process,[18,19] i.e., $k_2 = Ae(-\alpha E_{redox}/RT)$. If only E_{ox} varies with activator, then the plot of log k_2 vs E_{ox} should be linear with slope $\alpha F/2.3RT$.
(19) Guttenplan, J. B.; Cohen, S. G. *J. Am. Chem. Soc.* **1972**, *94*, 4040.
(20) Schore, N. E.; Turro, N. J. *J. Am. Chem. Soc.* **1975**, *97*, 2482.
(21) Gassman, P. G.; Olson, K. D.; Walter, L.; Yamaguchi, R. *J. Am. Chem. Soc.* **1981**, *103*, 4977.
(22) Scandola, F.; Balzani, V.; Schuster, G. B. *J. Am. Chem. Soc.* **1981**, *103*, 2519.
(23) Weller, A.; Zachariasse, K. In *Molecular Luminescence*; Lim, E. C., Ed.; Benjamin: New York, 1969; p 895.
(24) In acetonitrile vs SCE; we thank Prof. R. Holm for permission to use his equipment. The E_{ox} of **1** was also kindly determined in the same solvent by Maria Cruanes at University of Illinois; the two determinations were in excellent agreement.
(25) The energy requirement for singlet excitation is $\Delta H > E_S$, with $\Delta H = E_{ox} + 2.55$ eV. For 2-aminoanthracene, $E_{ox} = 0.44$ V, $\Delta H = 3.0$ eV, $E_S = 2.9$ eV. For 1-aminonaphthalene, $E_{ox} = 0.54$ V, $\Delta H = 3.1$ eV, $E_S = 3.6$ eV. For 10-methylphenothiazine, $E_{ox} = 0.82$ V, $\Delta H = 3.4$ eV, $E_S = 3.4$ eV.
(26) However, tempting,[27] it seems premature to try to apply Marcus' theory of electron transfer to these chemiluminescence reactions, where the transition states can only be reached after considerable bond reorganization. The effect of solvent polarity is necessarily very complex, since not one but three electron-transfer steps are involved (eq 8 and the back reaction and eq 10). In spite of having the lowest viscosity, the solvent in which the chemiluminescence yield from diphenoyl peroxide is the highest is diethyl ether, where k_2 is the smallest. In the case of dioxetane **3** we found *p*-dioxane to be the best solvent. Both ether and *p*-dioxane are good n-donors in CT complexes,[29] which may be significant.
(27) Eberson, L. *Chem. Scr.* **1982**, *20*, 29.
(28) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265 and references therein.
(29) See: Foster, R. *Molecular Complexes*; Academic Press: New York, 1975.
(30) In the case of the thermolysis of 4-methyl-4-(1-propenyl)malonyl peroxide, the activation barrier for chemiluminescence is reported to be a few kcal higher than the barrier to ground-state products. This may explain the apparently very low quantum yield of this fluorescer-catalyzed reaction.[14]

Intersystem crossing can occur at different times during the reaction course.[31] Once the spins of two radical ions are randomly oriented, electron transfer between these radicals will produce 75% triplets and 25% singlets. This "interpair" process[31] could thus reduce the yield of singlet excited products by a factor of 4 but not by 4 orders of magnitude. Prior to that point, however, hyperfine interactions can bring about fast "intrapair" intersystem crossing in the original singlet contact ion pair presumably present at the transition state. If intrapair intersystem crossing is important, then the modest solvent polarity effects observed on I_o (and Φ_{CL}) could be rationalized by increased ion solvation and faster formation of the solvent-separated ion pair. Although Koo and Schuster[2] have been unsuccessful at detecting triplet products, the possibly important role of intersystem crossing in reducing Φ_{CL} deserves to be further addressed. The low triplet energies of the activators, which are not phosphorescent in solution, render this search difficult. The effect of an external magnetic field may be worth exploring; by reducing intrapair intersystem crossing, it may result in modest enhancement of Φ_{CL}.

To summarize the discussion so far, it is difficult to single out one specific step in the CIEEL mechanism (eq 8–10) as the cause of the reaction inefficiency, although it would appear that intersystem crossing could be significant. Turning the question around, one might ask whether an intermolecular CIEEL mechanism is indeed capable of generating high yields of excited products. Our results with 3 and with diphenoyl peroxide reopen this question. Clearly neither diphenoyl peroxide nor 3 (with $\Phi_{CL} \lesssim 10^{-4}$) can be regarded as a realistic model of efficient chemiluminescence. The question therefore is the following: what specific properties of the peroxide would render an intermolecular CIEEL more efficient?

The groups of Schuster[15] and Adam[32] reported high quantum yields also from dimethyldioxetanone in the presence of easily oxidized activators: 10% according to Schuster, coincidentally the same yield as he reported for diphenoyl peroxide. We have not checked this value. Even if it was overestimated by an order of magnitude, a Φ_{CL} of 0.01, i.e., 100 times higher than from 3 or from diphenoyl peroxide, would still be a very significant result, since it may lead to an understanding of why the same mechanism, with the same activators, could be orders of magnitude more efficient in one system than in another. Why, for example, would one pair of radical ions spend less time than another as a contact ion pair, susceptible to intrapair intersystem crossing? CO_2 is a product of the decomposition of diphenoyl peroxide as well as of dioxetanone. The reduction potential of CO_2 is −2.2 V[33] compared to −2.3 V (all vs SCE) for acetone[34] and −1.92 V for benzocoumarin,[2] the other main product from DPP. Therefore dissociative electron transfer is expected to generate the radical anion of CO_2 in the dioxetanone case but the anion of benzocoumarin in the case of diphenoyl peroxide (Table IV). Is $CO_2^{\bullet-}$ likely to be an especially suitable partner in the final electron-transfer step which generates the excited state? The electrochemical work of Bard et al.[33] does not suggest it, partly because the small size of $CO_2^{\bullet-}$ should lead to a large difference in solvation energy between it and uncharged CO_2 and thus to a slower electron-transfer rate. On the other hand, strong solvation could favor the quicker separation of this anion from the proximity of the cation and consequently reduce intersystem crossing in the contact ion pair.

The discussion of $CO^{\bullet-}$ brings to mind the very efficient chemiluminescences from oxalate esters/hydrogen peroxide,[35] where electron transfer from fluorescers has long been proposed to play a determining role.[36] The main reaction products are

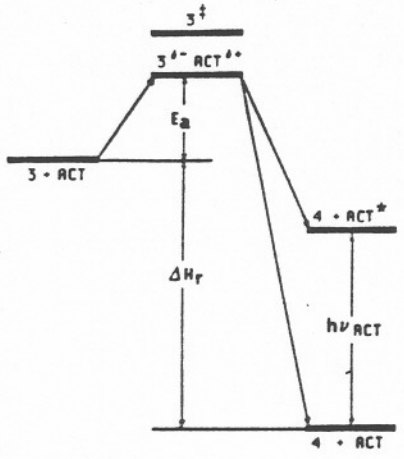


Figure 4. Representation of energy levels in a hypothetical charge-transfer mechanism of activated chemiluminescence.

phenols which do not participate in the photoexcitation step and again CO_2. Conflicting evidence exists for the involvement of dioxetanedione, the hypothetical four-membered ring dimer of CO_2, as one of possibly several energy-rich intermediates,[35,37] which again suggests a possible connection between product CO_2 and high quantum yield. To be sure, the generation of CO_2 from dioxetanone or dioxetanedione are very exothermic processes. Thermochemical estimates of the energy released in the reactions discussed here are listed in Table IV; chemiluminescence yields and reaction enthalpy run suggestively parallel. Whatever the reaction pathway, more energy is definitely available in the oxalate system than in the other examples in Table IV, and the annihilation step would also be more exothermic. Provided enough energy is available for excitation in reaction 10, the CIEEL hypothesis does not offer an immediate rationale for an increase in Φ_{CL} with ΔH. In fact, if the generation of excited activator by reaction 10 was too exothermic, even this electron transfer could fall in Marcus' "inverted region" and be slower. The quantum yield may depend on the size and rigidity of the radical anion involved in the final charge annihilation and/or the degree of charge delocalization. Although too little is known to speculate further on the factors reponsible for the high efficiency of the oxalate–ester reaction, it is important to keep in mind that an intermolecular activated chemiluminescence *can* be a remarkably efficient process.

Conclusions

In our opinion, the mechanism of peroxide chemiluminescence activated by fluorescers of low oxidation potentials, of which the reaction of 3 is unquestionably an example, is still open to question. Although our results are consonant with a CIEEL scheme, is it *necessary* to postulate the successive and distinct steps of a CIEEL mechanism? Or could one account for the observations by a simpler hypothesis? One such mechanism might assume as the rate-determining step the formation of a charge-transfer complex between vibrationally excited dioxetane and fluorescer, resulting in peroxide breakdown with simultaneous generation of some singlet-excited fluorescer, directly from the transition state (Figure 4). The small slope of the LFER plot of k_2 vs E_{ox} is compatible

$$3 + \mathrm{ACT} \rightarrow [3^{\bullet-}\mathrm{ACT}^{\bullet+}] \rightarrow 4 + \mathrm{ACT}^* \rightarrow 4 + \mathrm{ACT} \qquad (12)$$

with this interpretation. Similarly, the correlation between I_o and solvent polarity is more understandable, since I_o now reflects the rate of a single process. The energy available for excitation would be $-(\Delta H_r + E_a)$, where E_a is the activation energy for peroxide decomposition catalyzed by fluorescer, i.e., the energy required

(31) Weller, A. *Z. Phys. Chem. Neue Folge* **1982**, *130*, 129.
(32) (a) Adam, W.; Simpson, G. A.; Yany, F. *J. Phys. Chem.* **1974**, *78*, 2559. (b) Adam, W.; Cueto, O.; Yany, F. *J. Am. Chem. Soc.* **1978**, *100*, 2587.
(33) Chang, M.-M.; Saji, T.; Bard, A. J. *J. Am. Chem. Soc.* **1977**, *99*, 5399.
(34) Loufty, R. O.; Loufty, R. O. *J. Phys. Chem.* **1973**, *77*, 336.
(35) Reviews: (a) Rauhut, M. M. *Acc. Chem. Res.* **1969**, *2*, 80. (b) Mohan, A. G. In *Chemi- and Bioluminescence*; Burr, J., Ed.; Marcel Dekker: New York, 1985; pp 245–258.
(36) McCapra, F. *Prog. Org. Chem.* **1973**, *8*, 273.
(37) (a) Alvarez, F. J.; Parekh, N. J.; Mauszewski, B.; Givens, R. S.; Higuchi, T.; Schowen, R. L. *J. Am. Chem. Soc.* **1986**, *108*, 6435. (b) Catheral, C. L. R.; Palmer, T. F.; Cundall, R. B. *J. Chem. Soc., Faraday Trans.* **1984**, *80*, 823, 837. (c) White, E. H.; Wildes, P. D.; Wiecko, J.; Doshan, H.; Wei, C. C. *J. Am. Chem. Soc.* **1973**, *95*, 7050.

Table IV. Chemiluminescence Quantum Yields, Energies Released in Redox Process (with Rubrene Radical Cation)[a] and Heats of Reaction from Peroxides to Products[b]

peroxide	products	E_{red}[c]	E_{redox}[a]	ΔH_r[b]	Φ_{CL}[d]
3	4	−1.55	2.52		$<10^{-4}$ [e]
diphenoyl peroxide	benzocoumarin	−1.92*	2.89*	3.04	$<10^{-4}$ [e]
	CO_2	−2.2	3.17		
dimethyl dioxetanone	acetone	−2.3	3.27	3.5	0.1[f]
	CO_2	−2.2*	3.17*		
dioxetane dione?	CO_2	−2.2	3.17	4.8	0.3[g]

[a] For comparison between peroxide reactions, calculated as $E_{red} - E_{ox}$ with $E_{ox} = 0.97$ V; in eV. The asterisk indicates the most likely redox couple. [b] Literature values from thermochemical calculations; in eV. Not calculated for **3** because of unavailability of some group values. [c] In V vs SCE; the asterisk indicates the radical anion most likely to be generated. [d] In einstein per mol of peroxide reacting via the activated route. [e] This work. [f] Reference 15. [g] Reference 35.

to elongate the O–O bond and form the charge-transfer complex. The trend toward higher Φ_{CL} as ΔH_r increases, so clear in Table IV, cannot be rationalized without a knowledge of the potential surfaces involved; but there is no longer a need to explain how two identical annihilation processes between two identical ion pairs could have different efficiencies. A mechanism which does not call for the transfer of a "full electron", back and forth between donor and acceptor, may be a more realistic picture of intramolecularly activated chemiluminescence, as in the series of efficient dioxetanes synthesized by Schaap[38] and by McCapra.[39] Alternatively, the nonvertical electron-transfer process of CIEEL and the admittedly more vague charge-transfer mechanism suggested above could represent two extremes in a spectrum of activated reactions, within which each example of Table IV occupies a different position. In any case, one must remember that unless a sharp, mechanistic distinction is drawn between promotion to a singlet or to a triplet excited state, the classic dioxetanes such as tetramethyldioxetane are still remarkably efficient ($\Phi \sim 0.3$) converters of chemical energy to electronic energy,[40] apparently without the development of charge.

Irrespective of the precise mechanism of chemiexcitation, the thermolysis of anthracene endoperoxide **2** is an interesting example of peroxide chemiluminescence. Subtle changes in medium have a profound effect on the course of the reaction, leading to either the near infrared emission of singlet oxygen or, via an unstable dioxetane, to visible light of any color, depending on the fluorescer present. Features of the reaction path, with its many branching points, make it an interesting model for some bioluminescences.

Experimental Section

General Methods. The solvents used in chemiluminescence experiments were of spectral grade and distilled from calcium hydride. Perylene, rubrene, 9,10-diphenylanthracene, 9,10-dibromoanthracene, naphthacene, 2-aminoanthracene, and 1-aminonaphthalene were purchased from Aldrich and vacuum sublimed prior to use. 10-Methylphenothiazine from Eastman was recrystallized twice from benzene, 1,3-diphenylisobenzofuran was purchased from Columbia Org. Chem. and recrystallized twice from ethanol.

1,4-Dimethoxy-9,10-diphenylanthracene (**1**) was synthesized according to Dufraisse and Velluz.[3b] Its photooxygenation to peroxide **2** was performed at −20 °C by irradiation of a 10 mM ethyl ether solution containing a few crystals of tetraphenylporphyrine and a few drops of pyridine, to avoid acid-catalyzed decomposition of the product. After 1 h of irradiation the product **2** (insoluble in ether) was filtered and washed several times with ether. This crude peroxide was then resuspended in ether and washed with ether prior to use in any experiments.

Diphenoyl peroxide was synthesized by the method of Ramirez et al.[41] and freshly recrystallized from $MeOH/CH_2Cl_2$ at −20 °C prior to use.

NMR Spectra of Dioxetane 3. Peroxide **2** (8 mg, 19 μmol) was dissolved in 0.5 mL of $CDCl_3$ and frozen in dry ice. HAc (11 μL, 19 μmol) was placed on top of the frozen solution. This sample was then placed in the spectrometer at −35 °C, and spectra recorded after the solution thawed.

Chemiluminescence Measurements. Chemiluminescence intensity was followed in either photon counting mode (cooled PMT Hamamatsu R943-02) or in analog mode (cooled PMT EMI 9558-B and Keithley electrometer). Quantum yields were determined on the basis of the Hastings–Weber light standard[42] and confirmed by comparison with the tetramethyldioxetane/DBA chemiluminescent system.[40]

Acknowledgment. This work was supported by the National Science Foundation (Grant CHE-8209863 and Grant DMB-8616522 to J. W. Hastings). We have enjoyed discussions with Professor Hastings and his group and with Professor Y. Kishi and Dr. Hideshi Nakamura, who gave us many helpful suggestions and major help with the NMR spectra. L.H.C. is grateful for a fellowship from the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) of Brazil.

Registry No. 1, 6274-79-9; 2, 118949-89-6; 3, 118949-90-9; 4, 21768-85-4; diphenoyl peroxide, 6109-04-2; 2-aminoanthracene, 613-13-8; 1-aminonaphthalene, 134-32-7; 1,3-diphenylsiobenzofuran, 5471-63-6; 10-methylphenothiazine, 1207-72-3; perylene, 198-55-0; rubrene, 517-51-1; triethylamine, 121-44-8; 9,10-diphenylanthracene, 1499-10-1; 9,10-dibromoanthracene, 523-27-3.

(38) Schaap, A. P.; Chen, T.-S.; Handley, R. S.; DeSilva, R.; Giri, B. P. *Tetrahedron Lett.* **1987**, *28*, 1155 and references therein.

(39) McCapra, F.; Perring, K. D. In *Chemi- and Bioluminescence*; Burr, J., Ed.; Marcel Dekker: New York, 1985; pp 115–152, and references therein. Dr. McCapra arrived at similar conclusions by a different route, see: Gundermann, K.-D.; McCapra, F. *Chemiluminescence in Organic Chemistry*; Springer-Verlag: Berlin, 1987; pp 60–65.

(40) Reviews: (a) Adam, W. In *Chemical and Biological Generation of Excited States*; Adam, W., Cilento, G., Eds.; Academic Press: New York, 1982; pp 115–152. (b) Wilson, T. *Int. Rev. Sci.; Ser. Two* **1976**, *9*, 265.

(41) Ramirez, F.; Desai, M. B.; Mitra, R. B. *J. Am. Chem. Soc.* **1961**, *83*, 492.

(42) Hastings, J. W.; Weber, G. *J. Opt. Soc. Am.* **1963**, *53*, 1410. This calibration probably overestimates the photon flux by a factor of ca. 2.5; see, for example: Michael, P. R.; Faulkner, L. R. *Anal. Chem.* **1976**, *48*, 1188 and references therein.

Anexo III

The oxidation of cyclic sulfides by tetramethyldioxetane and the isobutanal/ O_2/peroxidase system: oxygen transfer versus electron transfer. Bechara, E.J.H., Catalani, L.H., *Free Rad. Biol. Med.* **18**, 731 (1995).

Pergamon

Free Radical Biology & Medicine, Vol. 18, No. 4, pp. 731–738, 1995

0891-5849/95 $9.50 + .00

0891-5849(94)00194-4

Original Contribution

THE OXIDATION OF CYCLIC SULFIDES BY TETRAMETHYLDIOXETANE AND THE ISOBUTANAL/O_2/PEROXIDASE SYSTEM: OXYGEN TRANSFER VERSUS ELECTRON TRANSFER

ETELVINO J. H. BECHARA* and LUIZ H. CATALANI†

*Departamento de Bioquímica and †Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil

(*Received* 4 *April* 1994; *Revised* 10 *August* 1994; *Accepted* 9 *September* 1994)

Abstract—The oxidation of chlorpromazine (CPZ) by tetramethyldioxetane (TMD) and isobutanal (IBAL)/O_2/horseradish peroxidase (HRP) system was investigated. The reaction with TMD proved to be of the oxygen transfer type, generating chlorpromazine-5-oxide (CPZO) and tetramethylethylene-oxide, and not by single-electron transfer, as previously reported. In contrast, the reaction of CPZ with IBAL/O_2/HRP leads to formation of chlorpromazine cation radical, through reaction with active intermediates Compound I and II, following its dismutation and hydrolysis to CPZO. For comparison, 10-methylphenothiazine was also tested. Despite the fact that both systems are known to generate oxidizing triplet acetone, this species does not participate in the oxidation path in either case.

Keywords—Chlorpromazine, Chlorpromazine oxidation, Triplet acetone, Isobutyraldehyde oxidation, Tetramethyldioxetane, Single electron transfer, Horseradish peroxidase, Oxygen transfer, Free radicals

INTRODUCTION

The recent demonstration of genotoxicity of 1,2-dioxetanes[1,2] led to new questions about the interaction of these molecules with others of biological interest, especially in view of dioxetanes' oxidative properties and ability to generate electronically excited species through their thermolysis.[3] Moreover, the role of dioxetanes in photobiological processes is well documented, specially as intermediates in oxidative reactions catalyzed by peroxidases and luciferases.[4]

Within the range of low oxidation potential biomolecules, these peroxides are known to act upon sulfide and thiols, as demonstrated by Adam and coworkers.[5] They concluded that the genotoxicity of dioxetanes is severely reduced by biological thiols like glutathion and cystein-rich proteins, preventing "oxidative stress" upon genomic material of the tested cells.

At the same time, a well established path of decomposition of these cyclic peroxides via electron transfer has been reinvestigated, and the efficiency in creating excited states by the so-called CIEEL mechanism (Chemically Initiated Electron Exchange Luminescence), a proposed mechanism for bioluminescence reactions, has been questioned.[6] The quantum yield of the bimolecular reaction most often suggested as a model of CIEEL (perylene-activated decompositon of diphenoyl peroxide) proved to be surprisingly low, even though the reaction does involve a peroxide decomposition catalyzed by electron transfer. Recently, it has been shown that catalyzed decomposition of peroxides and peroxide reduction via single electron transfer (SET) can take place in the reaction between 1,2-dioxetanes and a series of biological reductants.[7,8] This led to the search of a new general mechanism where, most probably, luminescence will stand as a by-product.

Taking into consideration that: (i) peroxides are known to promote oxidation of organic sulfides to the corresponding sulfoxides; (ii) the oxidation of isobutanal (IBAL) by horseradish peroxidase (HRP) proceeds via highly oxidizing intermediates such as HRP compounds I and II, α-hydroperoxy-isobutanal, and probably 3,3-dimethyl-4-hydroxy-1,2-dioxetane[4]; and (iii) the human metabolism of chlorpromazine (CPZ), a well-known tranquilizer, antiemetic and sedative,[9] produces chlorpromazine-sulfoxide (CPZO) accompanied

Address correspondence to: Luiz H. Catalani, Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, Caixa Postal 20780, 01498-970 - São Paulo, Brazil. E-mail: LHCATALA@QUIM.IQ.USP.BR

by phototoxic effects,[10] we have decided to carry out a detailed investigation of CPZ oxidation to CPZO by tetramethyldioxetane (TMD) and IBAL/O_2/HRP system; 10-Methylphenothiazine (MPZ) was taken as a model.

The aim of this study is to test the existence of a general catalytic mechanism for 1,2-dioxetane decomposition based on SET and to compare it with its enzymatic counterpart.

MATERIALS AND METHODS

Horseradish peroxidase Type VI, 2,4-hexadienoic acid (sorbic acid) and *tert*-butylhydroperoxide (Sigma), tetramethylethylene (TME), spectroscopic grade acetone and *m*-chloroperbenzoic acid (Aldrich), CPZ (Rhodia), MPZ (Kodak) indole (Baker), H_2O_2 30% (Carlo Erba), and Na_2EDTA (Merck) were used without further purification. IBAL (Fluka) was freshly distilled under N_2. Hydrogen peroxide stock solutions were determined by Cotton and Dunford's procedure.[11] Sodium 9,10-dibromoanthracene-2-sulfonate (DBAS), recrystallyzed (twice) from water, was prepared according to Catalani et al.[12]

Tetramethyl-1,2-dioxetane was prepared according to Kopecky's method.[13] The dioxetane gives a single absorption peak in ^{1}H-NMR spectrum at 1.50 ppm in $CDCl_3$. Tetramethylethylene-oxide (TMEO) and 2,3-dimethyl-2,3-butanediol were prepared according to known procedures.[14] The epoxide was assigned in the ^{1}H-NMR spectrum as a sharp singlet at 1.32 ppm ($-CH_3$) in $CDCl_3$, whereas the diol peaks at 1.19 ppm ($-CH_3$) and 4.00 ppm ($-OH$) in acetone-d_6.

Absorption spectra were recorded and kinetic studies performed on a Zeiss DMR 10 spectrophotometer. ^{1}H-NMR spectra were obtained on a Brucker AC-200 spectrometer (200MHz) using tetramethylsilane as internal reference. Oxygen consumption was followed on a Yellow Spring Instruments Model 53F oxygen monitor. Chemiluminescence was measured, depending upon its intensity, on either a Hamamatsu TV Photon counter C-767, or on a Mitchell–Hastings type photometer of in-house construction with a Hamamatsu 1P28 photomultiplier.

The standard enzymatic reaction mixtures for oxygen uptake and spectrophotometric studies, unless otherwise stated, contained 2 mM HRP and 80 mM IBAL in 0.25 M phosphate buffer pH 7.4. The concentration of HRP was determined spectrophotometrically at 403 nm ($\epsilon = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$; ref. 15), and the reaction was initiated by addition of either TMD, *tert*-butylhydroperoxide, or H_2O_2. The reaction was followed at 341 nm where CPZO has an absorption maximum,[16] using an automatic device that permits absorbance readings every 30 s without irradiating CPZ in the intervals.

Table 1. Relative Yield of Reaction Products

Exp	Reagents (solvent)	Acetone	Epoxide	Diol
1	TMD + CPZ ($CDCl_3$)	48%	52%	0%
2	TMD + CPZ (D_2O/Ac-d_6, 1:1)	30%	19%	51%
3	TMD + MPZ ($CDCl_3$)	88%	8%	4%
4	TMD + MPZ (D_2O/Ac-d_6, 1:1)	54%	8%	38%
5	TME + MCPBA ($CDCl_3$)	0%	100%	0%
6	TME + MCPBA (D_2O/Ac-d_6, 1:1)	0%	50%	50%

Data calculated from integrated ^{1}H-NMR signals of methyl groups after 1 h reaction at 60°C (δ in $CDCl_3$ (ppm): acetone 2.16; tetramethylethylene-oxide (III; Fig. 1) 1.32; tetramethylethylene-glycol (II; Fig. 1) 1.19; the signal of an unknown structure at 1.14 ppm was observed in experiments 1–4, possibly due to the metastable adduct I shown in Figure 1. Considering this signal as being from a singlet of four equivalent methyl groups, the adduct would be formed in 4–12% yield. Initial equimolar quantities of reagents = 0.10 M.

RESULTS

Reaction of TMD with CPZ and MPZ

^{1}H-NMR studies. The reaction of equimolar amounts of TMD and CPZ (0.10 M) in $CDCl_3$ at 60°C showed the disappearance of the TMD signal concomitant with the rising absorptions of tetramethylethylene-oxide (TMEO) absorption at 1.32 ppm, and of acetone, which is formed in the well known thermal cleavage of TMD[3]. When the same reaction was run in a mixture of acetone-d_6/D_2O (1:1) the ^{1}H-NMR spectrum showed a third product, the 2,3-dimethyl-2,3-butanediol together with acetone and epoxide. The reaction of TMD with MPZ resulted in a higher dioxetane conversion to acetone than with CPZ. The relative yields of these three products are shown in Table 1.

The finding that the diol is formed in D_2O/acetone-d_6 mixture does not mean that it is a direct product of the oxygen insertion reaction between the promazines and the dioxetane (Experiments 2 and 4; Table 1), but instead it could be the hydrolysis product of the primary epoxide, as seen in $CDCl_3$ (Experiments 1 and 3; Table 1). Indeed, when tetramethylethylene (TME) reacted with equimolar amounts of *m*-chloroperbenzoic acid (MCPBA) in $CDCl_3$, the epoxide was observed as a sole product (Experiment 5; Table 1), whereas the same reaction run in D_2O/acetone-d_6 produced a mixture of epoxide/diol with a ratio around 1:1 (Experiment 6; Table 1).

Another possibility is that the TMD-CPZ adduct (I), of the type shown in Figure 1 could be formed as a primary product. In fact, a fourth compound of unknown structure was observed in the reactions described here (see footnote of Table 1). The ^{1}H-NMR

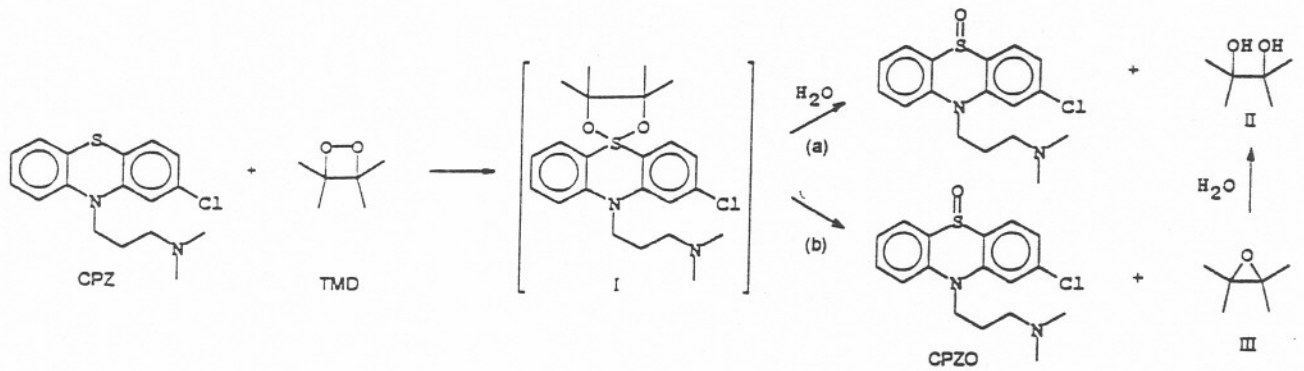


Fig. 1. A proposed reaction mechanism to account for epoxide and diol formation in the reaction of CPZ and TMD.

showed a fourth singlet at ≈ 1.14 ppm, right above the diol at 1.19 ppm in $CDCl_3$. This compound is very unstable and disappears upon prolonged heating.

Note that the unimolecular decomposition of the proposed insertion product I can be responsible for epoxide formation (path b), whereas its direct hydrolysis (path a) would render the diol, as shown in Figure 1. This fact would explain why the ratio diol/epoxide formed in CPZ + TMD and MPZ + TMD reactions in D_2O/acetone-d_6 is not the same as that observed in the TME + MCPBA reaction in the same solvent mixture.

Spectrophotometric studies. Formation of CPZO was revealed by recording the UV spectra of the spent reaction of TMD with CPZ. Figure 2 shows the absorption spectra of normally aerated aqueous solutions containing 20 μM CPZ and increasing concentrations of TMD (60–600 μM), after 90 min reaction at 60°C. Formation of CPZO as a main product is indicated by its characteristic absorption peaks at 239, 273, 299, and 341 nm and the isosbestic points at *ca* 265 and 315 nm.[16] The reaction of MPZ with TMD promoted identical spectral changes when water was the solvent, indicating formation of its sulfoxide as well.

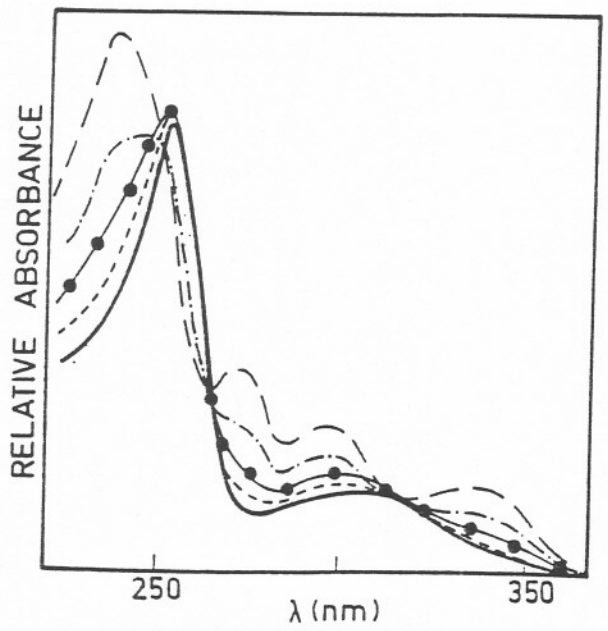


Fig. 2. Spectral modification of CPZ by reaction with TMD in water. The samples were heated for 90 min at 60°C. (——) control −20 μM CPZ; (----) idem + 60 μM TMD; (— ● —) idem + 120 μM TMD; (—·—) idem + 300 μM TMD and (— —) idem + 600 mM TMD.

Duran and coworkers[17] proposed that CPZ could be oxidized by energy transfer from triplet acetone generated by the IBAL/HRP/O_2 system. To test this possibility and because TMD is a known source of triplet acetone, we have added indole to the CPZ/TMD reaction mixture in an amount sufficient to quench 50% of triplet acetone formed.[18] The yields of CPZO formed at different initial TMD/CPZ ratios were reduced in less than 20%, as shown in Table 2.

The intermediacy of chlorpromazine radical cation ($CPZ^{+\cdot}$) was ruled out by two different experiments:

1. With N_2 purging the absorption spectra were the same as those shown in Figure 2. If the oxidation of CPZ by TMD follows a single electron transfer path, $CPZ^{+\cdot}$ should be the first intermediate formed, and its reaction with triplet oxygen should be an important path for CPZO formation. However, the same yield of CPZO was observed in the presence and absence of O_2. This result also supports the

Table 2. Chemical Yields of CPZO Formation

Ratio TMD/CPZ[a]	No Indole	With Indole[b]
6	0.28	0.23
15	0.60	0.49
30	1.00	0.81

Yields calculated after 90 min reaction at 60°C in water.

[a] Initial [CPZ] = 20 μM.

[b] [indole] = 200 μM.

Table 3. Second Order Rate Constants for CPZ Oxidation

Oxidant	k_2 (M^{-1} s^{-1})[a]
TMD	$(5.4 \pm 0.5) \times 10^{-1}$
H_2O_2	$(1.1 \pm 0.1) \times 10^{-2}$
t-BuOOH	$(1.8 \pm 0.2) \times 10^{-3}$

[a] Rate constants were determined spectrophotometrically by monitoring CPZO formation at 341 nm[16] (in water at 62°C) using pseudo-first order conditions on CPZ.

preceding observation (see Table 2) that triplet acetone is not responsible for CPZ oxidation, because O_2 is as good a triplet acetone quencher as indole, and the concentration of O_2 in water is the same as that of indole in the experiments of Table 2.

2. CPZ was irradiated in normally aerated water using a low pressure mercury lamp (main emission at 254 nm) in the presence of TMD at 20°C. UV irradiation is known to generate $CPZ^{+\cdot}$, which reacts with O_2 to form the sulfoxide.[18] One could also argue that TMD itself could act upon the cation radical primarily formed, promoting its oxidation to CPZO. We have observed, however, that the yield of CPZO formed during irradiation of a CPZ solution in water at 20°C did not change in the presence of TMD. One should note that at this temperature and in the time required for total photooxidation ($\approx$ 5 min), the amount of CPZ oxidation by the bimolecular reaction with TMD was insignificant.

To compare the oxidizing activity of TMD with that of other classes of peroxides, we measured the second order rate constants for the reaction of CPZ with TMD and, for comparison, those observed with H_2O_2 and *tert*-butylhydroperoxide. The results are listed in Table 3. At least for CPZ, TMD was found to be a better oxygen transfer species.

Quenching studies. Stern–Volmer plots for the quenching of TMD generated triplet acetone by CPZ were obtained using DBAS as emission sensitizer. These studies were carried out in 50 mM phosphate buffer pH 7.4, at 50°C (200 μM TMD and 40 to 250 μM CPZ). The chemiluminescence measurements were done in a time where no appreciable reaction between TMD and CPZ had occurred. The results were corrected for the direct quenching of DBAS fluorescence by CPZ.[18] The $k_q\tau^\circ$ values (k_q is the quenching rate constant; τ° is the lifetime of triplet acetone in the absence of quencher) obtained were 5×10^3 M^{-1} in normally aerated solution and 5×10^4 M^{-1} in N_2-purged solutions.

The 10 fold difference between these values is in satisfactory agreement with prior findings of 1.2 μs and 7 μs lifetime for triplet acetone,[12] in air equilibrated and N_2 purged aqueous solutions at 45°C, respectively; therefore, $k_q = 4 - 7 \times 10^9$ $M^{-1}s^{-1}$. This value is 10 orders of magnitude larger than the rate constant for the oxygen transfer reaction (Table 3) and indicates a diffusion controlled quenching process.

CPZ oxidation promoted by enzymatic systems

System IBAL/O_2/HRP. That CPZ is oxidized to CPZO when exposed to enzymatic system IBAL/HRP/O_2 in buffered solutions is revealed by the absorption spectrum of the spent reaction mixture (10 min), characteristic of CPZO as shown in Figure 3.

Figure 4 shows the effect of CPZ on the kinetics of oxygen uptake by IBAL/HRP. The oxygen uptake curves exhibit two phases: Initially, the reaction rate is slower the higher the concentration of CPZ added to the reaction mixture, but as CPZ is consumed, the kinetics of oxygen uptake in its absence is restored.

In the normal catalytic cycle of IBAL oxidation, HRP-native form (Fe^{3+}) is first oxidized to HRP-compound I (formally FeO^{3+}) by perisobutyric acid, followed by two consecutive reductions steps to HRP-Compound II (formally FeO^{2+}) and back to HRP-native.[20] Figure 5 shows the time course of the enzymatic reaction both in the absence and presence of CPZ, followed spectrophotometrically at 420 nm (λ_{max} HRP Compound II), 411 nm (isosbestic point for HRP Compound II and native HRP), and 400 nm (λ_{max} for both native HRP and HRP Compound I). The shapes of the

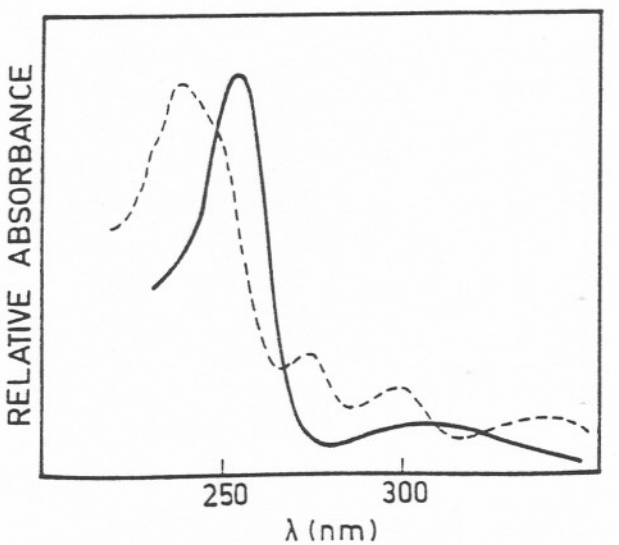


Fig. 3. Spectral modification of CPZ by reaction with IBAL/O_2/HRP system. 20 μM CPZ was incubated with the standard enzymatic mixture for 10 min at 40°C. CPZ absorption spectrum before (——) and after (----) incubation.

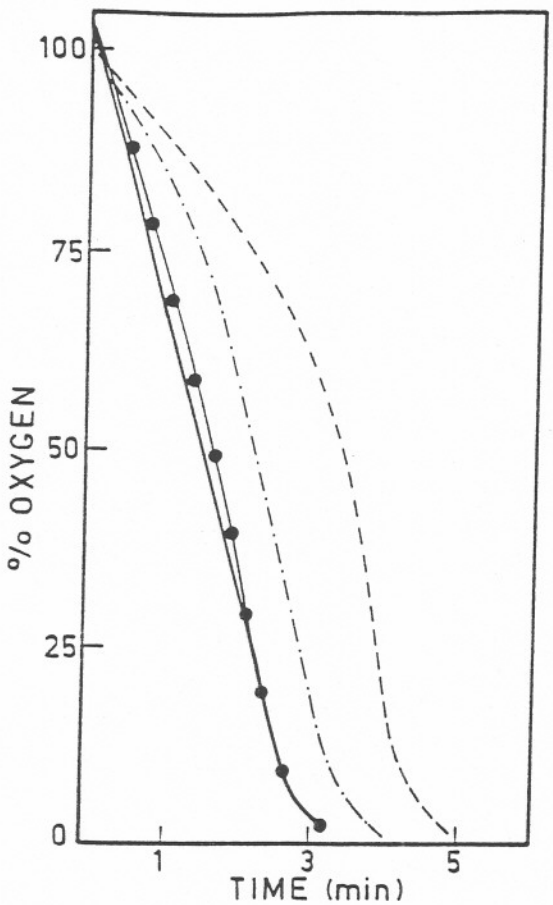


Fig. 4. Uptake of O_2 by IBAL (80 mM)/HRP(2 μM) in phosphate buffer 0.1 M pH 6.4 at 40° containing 33 μM EDTA. (——) control—no CPZ; (— ● —) with 17 μM CPZ; (—·—) with 25 μM CPZ and (— —) with 33 μM CPZ.

curves show clearly that CPZ quenches the enzymatic oxidation of IBAL and suggest that once CPZ has been completely oxidized, the normal enzymatic cycle for IBAL oxidation is recovered. Moreover, this effect is proportional to the CPZ initial concentration.

System H_2O_2/HRP. HRP-compound I was prepared by preincubation of native HRP and H_2O_2. We observed a direct reaction of CPZ with this intermediate, as shown in Figure 6. Increasing concentrations of CPZ enhances the rate of its decomposition to HRP-native form.

Applying steady state conditions to the system, which implies that

$$[H_2O_2] \gg [CPZ],$$

one can demonstrate that:

$$-\frac{\partial[CPZ]}{\partial t} = K_{obs}\,[CPZ][E_0]$$

where E_0 is the total concentration of enzyme (HRP native + Compound I + Compound II). The values of K_{obs}, expressed in terms of initial rates, were estimated by following the decay of CPZ absorbance at 255 nm, where $\epsilon_{CPZ} = 3.40 \times 10^4\ M^{-1}cm^{-1}$ and $\epsilon_{CPZO} = 1.44 \times 10^4\ M^{-1}cm^{-1}$,[16] and were found to be independent of CPZ and enzyme concentrations, provided the steady state conditions are met. A K_{obs} value of $7 \pm 1 \times 10^4\ M^{-1}s^{-1}$ was found for the range 10–50 mM CPZ and 20–60 nM HRP, using 1 mM H_2O_2 (0.25 M phosphate buffer, pH 7.4). Direct reaction between CPZ and H_2O_2 in the same experimental conditions was found to occur at a much slower rate.

DISCUSSION

Formation of CPZO as the main product of the reaction of CPZ with TMD is clearly demonstrated in Figure 2. Moreover, the ^{1}H-NMR data showed that in absence of water, formation of tetramethylethylene oxide is the only pathway competitive with the unimolecular cleavage of the dioxetane. The presence of triplet quenchers, such as indole, affected neither the kinetics of CPZO formation nor its chemical yields (see Table 2), leading to the conclusion that triplet acetone is not involved in the CPZ oxidation. Figure 7 shows the possible reactions of CPZ with TMD.

The basic difference in the behavior of this reaction in chloroform and in aqueous medium can be accounted for by the hydrolysis of the epoxide formed. The diol formed when D_2O was the solvent was also observed as a product of tetramethylethylene oxidation by MCPBA. Adam and coworkers[7,8] observed the formation of glycol II (Fig. 1) during reaction of TMD and a series of phenothiazines. The authors proposed that the reduction of the dioxetane was promoted by the promazines through a single electron transfer (SET) mechanism. Although this mechanism cannot be fully disregarded, we believe that in those cases an oxygen transfer reaction followed by hydrolysis better explains the results.

Reactions of diphenylsulfide with trimethyl-1,2-dioxetane yielding the corresponding sulfoxide and epoxide is described in the literature.[21] The authors described this bimolecular process as being a sulfur atom insertion in the peroxidic bond, producing a sulfurane or a zwitterion intermediate, followed by its decomposition. They also report the detection of a sulfurane-like adduct in the reaction between dioxetanes and dialkoxy sulfides. The same type of adduct was observed when TMD reacted with trivalent phosphorus compounds like triphenylphosphine, trimethylphosphite, and triethylphosphite.[22,23] The authors observed the formation of a stable phosphorane, which decomposed to epoxide and the oxygenated phosphorus de-

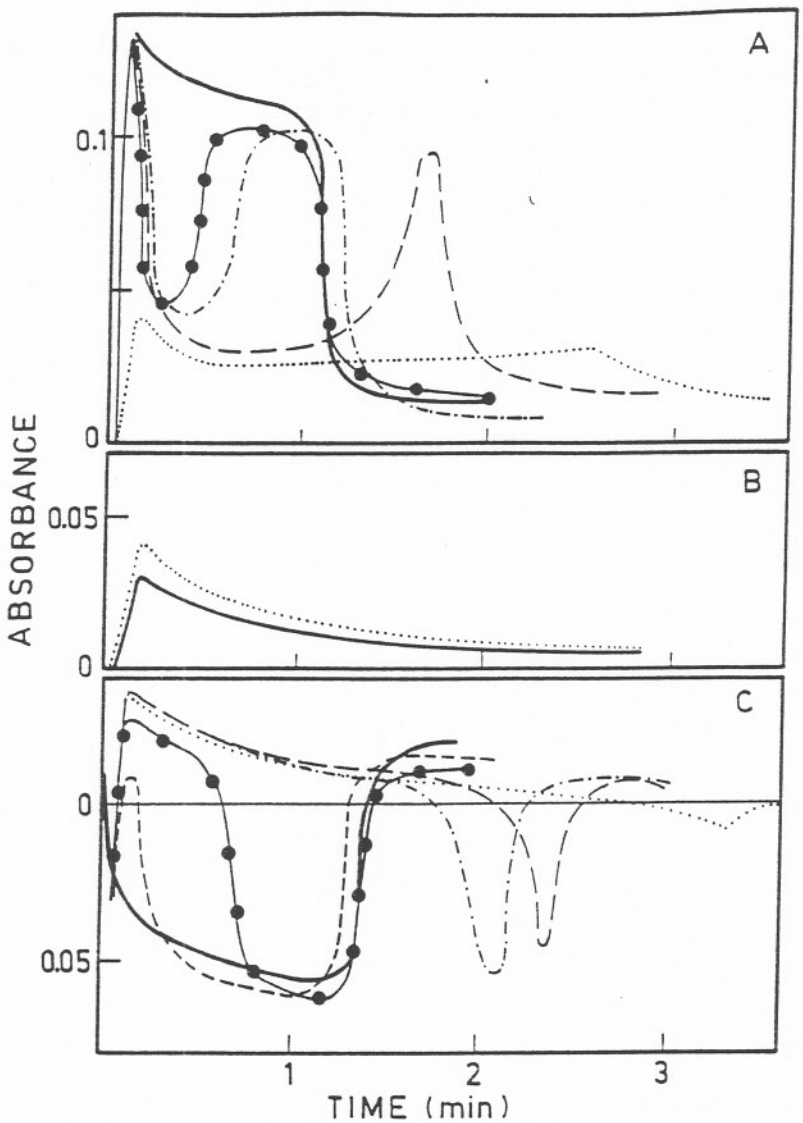


Fig. 5. Peroxidase cycle kinetics followed spectrophotometrically as a function of time. (A) The differential absorbance centered at 420 nm (λ_{max} of HRP native form); (B) the differential absorbance centered at 411 nm (isosbestic point between HRP native form and Compound I); and (C) the differential absorbance centered at 400 nm (λmax of HRP-Compound I). The assays were done using IBAL 80 mM and HRP 2 μM, in phosphate buffer 0.25 M pH 7.4 at 40°C. (——) control—CPZ absent; (-----) 8 μM CPZ; (— ● —) 16 μM CPZ; (—·—) 24 μM CPZ; (— —) 28 μM CPZ; and (·····) 32 μM CPZ.

rivative upon heating. The characterization of CPZO and TMEO as products of the reaction indicates that this mechanism is also operative here through pathway a (Fig. 7). This confirms the oxygen transfer ability of dioxetanes to some classes of compounds, in keeping with general peroxide reactivity.

The quenching of the triplet acetone by CPZ (pathway b; Fig. 7) certainly occurs, but it does not exclude or interfere with the oxygen transfer reaction (pathway a; Fig. 7). The $k_q\tau^\circ$ obtained in deaerated solution compares well with the value previously reported of 2.9×10^4 M^{-1} for the quenching of CPZ upon IBAL/O_2/HRP system.[17] Considering that the lifetime of triplet acetone in the enzymatic system is very close to that measured in a deaerated solution (due a special "enzyme protection"[18]), one can infer quenching of triplet acetone by CPZ and O_2 follow the same mechanism. Taking the lifetime of triplet acetone in deaerated water[12] at 50°C as 7 μs, the rate constant for quenching by CPZ (7×10^9 $M^{-1}s^{-1}$) falls within the diffusion-controlled limit.

Duran and coworkers[17] showed that system IBAL/O_2/HRP promotes the oxidation of CPZ to CPZO, by using paper chromatography and spectrofluorimetry as detection techniques. We comfirm this result by spectrophotometry, as is shown in Figure 3. The resulting spectrum is the same as that obtained when TMD was used as oxidizing system (Fig. 2), and it is the CPZO

spectrum.[16] Indole in concentration sufficient to quench 50% of the triplet acetone formed, only showed a small effect upon the yield of CPZO formation. This small effect, as well as the effect observed by Duran using DBAS as quencher, can be explained as resulting of reaction of the quencher either with $CPZ^{+\cdot}$, or with the radicals formed in the enzymatic cycle, but not triplet acetone quenching.

Figures 4 and 5 show that CPZ interacts directly with the HRP enzymatic cycle probably by consuming one or both intermediates (compounds I and II). CPZO formation can be explained by an electron transfer step from CPZ to compounds I and II yielding $CPZ^{+\cdot}$, as shown by Duran et al.,[17] followed by dismutative formation of the phenathionium ion and its hydrolysis to CPZO.[4,25] In fact, the same behavior had been also observed by Cavanaugh[26] and Piette et al.[25] when CPZ was reacted with H_2O_2/HRP system. Our data show that HRP-Compound I (prepared by reaction of HRP with H_2O_2) reacts very fast ($\approx$ 30 s) with CPZ when the stoichiometry is 1:2.5 (Fig. 6). The rate constant for this reaction was found to be two orders of magnitude slower than the value of 4.5×10^6 $M^{-1}s^{-1}$ reported by Piette et al.,[25] who used electron spin ressonance at milimolar concentration of CPZ. Again, these facts

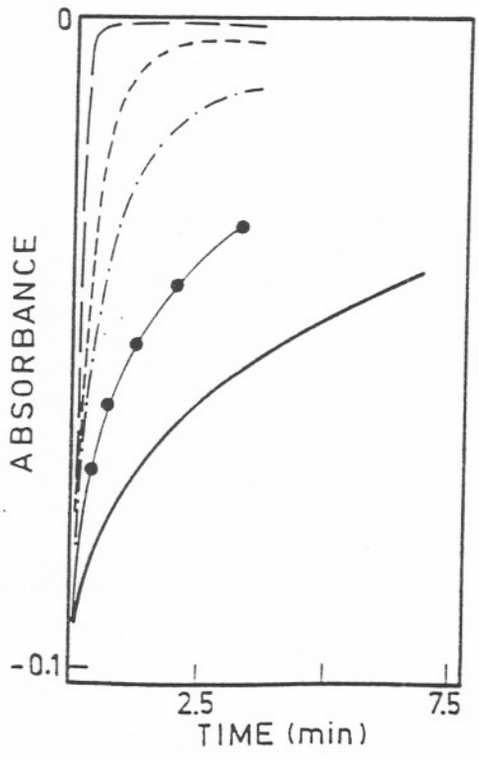


Fig. 6. Kinetics of HRP-Compound I conversion to the native form by reaction with CPZ. Compound I was prepared by reaction of 2 μM H_2O_2 with 2 μM HRP in acetate buffer 0.1 M pH 3.8 at 40°C, and its differential absorbance at 400 nm was followed with the time. (——) control—no CPZ; (— ● —) 0.8 μM CPZ; (—·—) 1.7 μM CPZ; (----) 2.5 μM CPZ; and (— —) 5.0 mM CPZ.

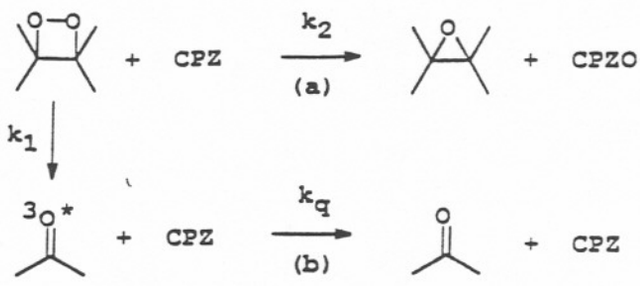


Fig. 7. Possible interactions between TMD and CPZ.

attest that triplet acetone is not responsible for CPZ oxidation, even when IBAL/O_2/HRP is the oxidizing system.

SUMMARY CONCLUSIONS

The phototoxic effects of CPZ in human skin and eyes has been well documented and attributed to light-induced binding with membrane proteins.[27,28] The fact that CPZO is the main metabolite excreted by dogs and humans remained unexplained.[29] Our data show that CPZ oxidation promoted by endogenous excited states is unlikely. At the same time, enzymatic oxidative systems[26,30] (e.g., myoglobin, lactoperoxidase, myeloperoxidase, catalase, etc.) turn out to be the best candidates to promote the in vivo CPZ oxidation. According to previous findings, dioxetanes proved to be a general class of peroxides, promoting oxygen transfer reactions with certain types of molecules, like sulfides.

Acknowledgements — The authors thank Dr. Thérèse Wilson (Harvard University) and Dr. Alfons L. Baumstark (Georgia State University) for critically reviewing this article. This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), the Financiadora de Estudos e Projetos (FINEP), the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Volkswagenwerk Stiftung.

REFERENCES

1. Nassi, L.; Schiffmann, D.; Favre, A.; Adam, W.; Fuchs, R. Induction of the SOS function sfiA in *E. coli* by systems which generate triplet ketones. *Mutation Res.* **198**:53–60; 1988.
2. Nassi, L.; Epe, B.; Schiffmann, D.; Adam, W.; Beinhauer, A.; Griesbeck, A. Induction of morphological transformation and micronuclei in Syrian hamster embryo fibroblasts by 1,2-dioxetanes: Correlation with single-strand breaks in HL-60 cells. *Carcinogenesis*, **8**:947–953; 1988.
3. Wilson, T.; Golan, D. E.; Harris, M. S.; Baumstark, A. L. Thermolysis of tetramethoxy- and p-dioxenedioxetanes: Kinetic parameters, chemiluminescence, and yields of excited products. *J. Am. Chem. Soc.* **98**:1086–1091; 1976.
4. Cilento, G.; Adam, W. Photochemistry and photobiology without light. *Photochem. Photobiol.* **48**:361–368; 1988.
5. Adam, W.; Epe, B.; Schiffmann, D.; Vargas, F.; Wild, D. Facile reduction of 1,2-dioxetanes by thiols as potential protective measure against photochemical damage of cellular DNA. *Angew. Chem. Int. Ed. Engl.* **27**:429–431; 1988.

6. Catalani, L. H.; Wilson, T. Electron transfer and chemiluminescence. Two inefficient systems: 1,4-dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide. *J. Am. Chem. Soc.* **111**:2633–2639; 1989.
7. Adam, W.; Hückmann, S.; Vargas, F. Direct observation of electron transfer between phenothiazines and 1,2-dioxetanes. *Tetrahedron Lett.* **30**:6315–6318; 1989.
8. Adam, W.; Vargas, F.; Epe, B.; Schiffmann, D.; Wild, D. Single-electron-transfer in the reduction of 1,2-dioxetanes by biologically active substrates. *Free Rad. Res. Comms.* **5**:253–258; 1989.
9. Freeman, H. The therapeutic value of combinations of psychotropic drugs: A review. *Psychopharm. Bull.* **4**:1–27; 1967.
10. Huang, C. L.; Sands, F. L. Effect of ultraviolet irradiation on chlorpromazine: II. Anaerobic conditions. *J. Pharm. Sci.* **56**:259–264; 1967.
11. Cotton, M. L.; Dunford, H. B. Studies on horseradish peroxidase: XI. On the nature of compounds I and II as determined from the kinetics of the oxidation of ferrocyanide. *Can. J. Chem.* **51**:582–587; 1973.
12. Catalani, L. H.; Wilson, T.; Bechara, E. J. H. Two water-soluble fluorescence probes for chemiexcitation studies: Sodium 9,10-dibromo- and 9,10-diphenylanthracene-2-sulfonate. Synthesis, properties and application to triplet acetone and tetramethyldioxetane. *Photochem. Photobiol.* **45**:273; 1987.
13. Kopecky, K. R.; Filby, J. E.; Munford, C.; Lockwood, P. A.; Ding, J.-Y. Preparation and thermolysis of some 1,2-dioxetanes. *Can. J. Chem.* **53**:1103–1122; 1975.
14. Vogel, A., ed. *Textbook of practical organic chemistry (4th ed.).* London: Longman Inc.; 1978.
15. Ohlsson, P.-I.; Paul, K.-G. The molar absorptivity of horseradish peroxidase. *Acta. Chem. Scand. B* **30**:373; 1976.
16. Warren, R. J.; Eisdorfen, I. B.; Thompson, W. E.; Zarembo, J. E. Spectra-structure correlation of phenothiazines by infrared, ultraviolet, and nuclear magnetic resonance spectroscopy. *J. Pharm. Sci.* **55**:144–150; 1966.
17. Duran, N.; Haun, M.; Faljoni, A.; Cilento, G. Photochemical oxidation of chlorpromazine in the dark induced by enzymically generated triplet carbonyl compounds. *Biochem. Biophys. Res. Commun.* **81**:785–790; 1978.
18. Catalani, L. H.; Bechara, E. J. H. Quenching of chemiexcited triplet acetone by biologically important compounds in aqueous medium. *Photochem. Photobiol.* **39**:823–830; 1984.
19. Iwaoka, T.; Kondo, M. Mechanistic studies of the photooxidation of chlorpromazine in water and ethanol. *Bull. Chem. Soc. Jpn.* **47**:980–986; 1974.
20. Baader, W. J.; Bohne, C.; Cilento, G.; Dunford, H. B. Peroxidase-catalyzed formation of triplet acetone and chemiluminescence from isobutyraldehyde and molecular oxygen. *J. Biol. Chem.* **260**:10217–10225; 1985.
21. Campbell, B. S.; Denney, D. B.; Denney, D. Z.; Shih, L. S. Reactions of dioxetanes with sulfoxylates and sulfides: Preparation of novel tetraalkoxysulfuranes. *J. Am. Chem. Soc.* **97**:3850–3851; 1975.
22. Bartlett, P. D.; Baumstark, A. L.; Landis, M. E. An insertion reaction of triphenylphosphine with tetramethyl-1,2-dioxetane. Deoxygenation of a dioxetane to an epoxide. *J. Am. Chem. Soc.* **95**:6486–6487; 1973.
23. Bartlett, P. D.; Baumstark, A. L.; Landis, M. E.; Lerman, C. L. Phosphorane formation from the reaction of trivalent phosphorus compounds with tetramethyl-1,2-dioxetane. *J. Am. Chem. Soc.* **96**:5267–5268; 1974.
24. Merkle, F. H.; Discher, C. A.; Felmeister, A. Separation and investigation of a stable solid free radical of chlorpromazine. *J. Pharm. Sci.* **53**:965–966; 1964.
25. Piette, L. H.; Bulow, G.; Yamazaki, I. Electron-paramagnetic-resonance studies of the chlorpromazine free radical formed during enzymic oxidation by peroxidase-hydrogen peroxide. *Biochim. Biophys. Acta* **88**:120–129; 1964.
26. Cavanaugh, D. J. Oxidation of chlorpromazine by peroxidase and catalase. *Science* **125**:1040–1041; 1957.
27. Rosenthal, I.; Ben-Hur, E.; Prager, A.; Riklis, E. Photochemical reactions of chlorpromazine: Chemical and biochemical implications. *Photochem. Photobiol.* **28**:591–594; 1978.
28. Testylier, G.; Daveloose, D.; Leterrier, F.; Buchmann, O.; Shimoni, M. Photochemical binding of phenothiazines on biological membrane protein. *Photochem. Photobiol.* **39**:273–276; 1984.
29. Salzman, N. P.; Moran, N. C.; Brodie, B. B. Identification and pharmacological properties of a major metabolite of chlorpromazine. *Nature* **176**:1122–123; 1955.
30. Cilento, G.; Nascimento, A. L. T. O. Generation of electronically excited triplet species at the cellular level: A potential source of genotoxicity. *Toxicol. Lett.* **67**:17–28; 1993.

ABBREVIATIONS

CIEEL—Chemically Initiated Electron Exchange Mechanism
CPZ—chlorpromazine
CPZO—chlorpromazine-5-oxide
DBAS—sodium 9,10-dibromoanthracene-2-sulfonate
HRP—horseradish peroxidase
IBAL—isobutyraldehyde
MCPBA—meta-chloroperbenzoic acid
MPZ—10-methylphenothiazine
SET—Single Electron Transfer
TMD—tetramethyl-1,2-dioxetane
TME—tetramethylethylene
TMEO—tetramethylethylene-oxide

Anexo IV

Energy transfer of chemienergized acetone to substances that display anomalous fluorescence. Adam, W., Baader, W.J., Catalani, L.H., Cilento, G., Rychla, L. *Photochem. Photobiol.* **42**, 587 (1985).

Photochemistry and Photobiology Vol. 42, No. 5, pp. 587 – 589, 1985
Printed in Great Britain.

0031-8655/85 $03.00+0.00

ENERGY TRANSFER FROM CHEMIEXCITED ACETONE TO SUBSTANCES THAT DISPLAY ANOMALOUS FLUORESCENCE

WALDEMAR ADAM[1]*, WILHELM J. BAADER[1,2]‡, LUIZ H. CATALANI[1,2§], GIUSEPPE CILENTO[2] and LYDA RYCHLÁ[1,‖]

[1]Institute of Organic Chemistry, University of Würzburg, D-8700 Würzburg, W. Germany and [2]Department of Biochemistry, Instituto de Química, Universidade de São Paulo, C.P. 20.780, São Paulo, Brazil

(*Received* 15 *April* 1985; *accepted* 14 *June* 1985)

Abstract—Excited acetone generated in the thermolysis of tetramethyldioxetane elicits the anomalous $S_2 \rightarrow S_0$ fluorescence from azulene and from xanthione. In the case of azulene it could be demonstrated that (i) only the acetone singlets transfer energy to the S_2 state and (ii) the acetone triplets are quenched. These energy transfer processes are diffusion-controlled.

INTRODUCTION

Triplet acetone, generated from the thermolysis of tetramethyldioxetane, TMD† (Adam, 1982) or enzymically (Cilento, 1984), transfers energy to an upper triplet (T_n) of anthracene, DBA, xanthene dyes and most likely also of chlorophyll (Nassi and Cilento, 1985). Provided appropriate conditions exist, for instance heavy atom perturbation, ISC ($T_n \rightarrow S_1$) occurs to the fluorescent state in competition with internal conversion ($T_n \rightsquigarrow T_1$). It became, therefore, of interest to verify whether triplet acetone could excite molecules that emit directly from upper states, i.e. those which violate Kasha's rule (Turro *et al.*, 1978). Since TMD does also generate—albeit in small yield—S_1 acetone, the opportunity presented itself to investigate energy transfer to upper excited singlet states (S_1–S_n) of a suitable acceptor.

Since azulene (Beer and Longuet-Higgins, 1955; Viswanath and Kasha, 1956) and xanthione (Birks, 1972; Mahaney and Huber, 1975; Huber and Mahaney, 1975) are known to display $S_2 \rightarrow S_0$ fluorescence, these were, therefore, selected for this investigation. The S_1 energy of acetone (E_S = 356 mJ/mol or 85 kcal/mol; Calvert and Pitts, 1966) is sufficiently higher than the S_2 energy of azulene (E_S = 80.9 kcal/mol; Turro *et al.*, 1978), to make a S_1–S_2 energy transfer process feasible.

*To whom correspondence should be addressed.
‡Postdoctoral Fellow jointly funded by the Stiftung Volkswagenwerk and the 400-Jahre-Jubiläumsstiftung der Universität Würzburg.
§Postdoctoral Fellow of the Alexander von Humboldt Foundation.
‖Visiting scientist sponsored by the DAAD program.
†*Abbreviations*: TMD, tetramethyldioxetane; ISC, intersystem crossing; DPA, 9,10-diphenylanthracene; DBA, 9,10-dibromoanthracene; AZ, azulene (in the figures); XT, xanthione (in the figures).

MATERIALS AND METHODS

Materials. Azulene was purchased from Aldrich. Xanthione was prepared according to Anderson *et al.* (1976). TMD was synthesized according to Kopecky *et al.* (1975). Toluene was stirred with ethylenediaminetetraacetic acid and distilled. The solvent was transparent in the spectral range of the first and second absorption bands of azulene.

Excited acetone generated by thermolysis of TMD. Unless otherwise stated, the solvent was normally aerated toluene. When singlet and triplet acetone, generated by thermolysis of TMD, were monitored respectively by DPA and DBA sensitized emission in the presence of azulene, the quenching by azulene was plotted according to Eq. 1 (Rivas-Suárez *et al.*, 1983), in which I_0 and I are the

$$\frac{I_0/I}{1 + k_q^* \tau_0^* [AZ]} = 1 + k_q \tau_0 [AZ] \qquad (1)$$

observed intensities, respectively in the absence and presence of azulene, [AZ] is the azulene concentration, $k_q \tau_0$ is the Stern–Volmer constant for the quenching of acetone singlets or triplets and $k_q^* \tau_0^*$ refers to quenching of excited singlet DPA or DBA. The latter term was determined by measuring the effect of the azulene upon the fluorescence of photoexcited DPA or DBA, using a Perkin–Elmer Model MPF-44B spectrofluorimeter, which was provided with differential spectral correction. The $k_q \tau_0$ (corrected for azulene quenching by DPA or DBA) was obtained as the slope of the plot of the left-hand term of Eq. 1 against [AZ].

The quenching experiments of the DPA and DBA enhanced chemiluminescence promoted by TMD were carried out in a Mitchell-Hastings photometer (in-house construction), containing a 1P28 Hamamatsu photomultiplier. The sample compartments of the photometer and the spectrofluorimeter were connected to thermostated circulating baths.

RESULTS

Excitation of azulene by singlet acetone

Figure 1 shows the spectrum of the emission originating from the thermolysis of TMD, both in the absence and presence of azulene. The emission from

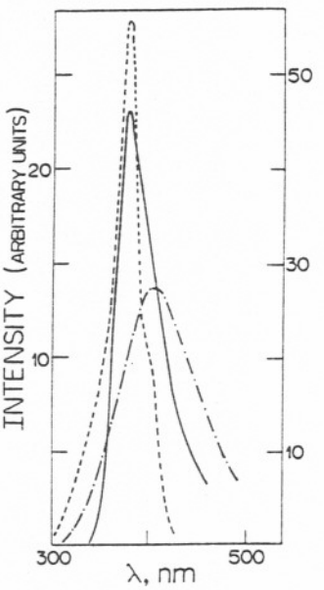


Figure 1. Fluorescence spectrum of azulene promoted by the thermolysis of 0.01 *M* TMD at 61.3°C (——) and by 355-nm light excitation (----). The lower curve (–·–·–) is the chemiluminescence spectrum (identical to acetone fluorescence) of the TMD thermolysis. [AZ] = 1.0 m*M* in the TMD experiment and [AZ] = 5.0 m*M* in the optical experiment (ordinates at right).

TMD alone was identical to acetone fluorescence; in the presence of azulene it matched that of azulene $S_2 \rightarrow S_0$ fluorescence. In order to obtain energy transfer parameters, the effect of increasing azulene concentration upon the emission spectrum was first investigated (Fig. 2). Unfortunately, the overlap of the two emission bands precluded the measurement of Stern–Volmer kinetics. The effect of temperature on the azulene-promoted chemiluminescence indicated an activation energy (E_a) of 103.8 kJ/mol (24.8 kcal/mol). Since this value is sufficiently close to that of the direct chemiluminescence of TMD (E_a = 105 ~ 113 kJ/mol or 25–27 kcal/mol; Adam and Zinner, 1982), azulene appears to be solely involved in electronic energy transfer. Since in the presence of 5×10^{-2} *M* biphenyl, a quencher of triplet acetone (Leigh and Scaiano, 1983; Cilento, 1980), the TMD promoted fluorescence of azulene was not quenched, S_1 acetone appears to be the donor in this energy transfer process.

An estimate of $k_q\tau$ could be obtained by monitoring the acetone singlets with the highly fluorescent DPA in the presence of azulene, investigating the quenching effect by the latter. This procedure is valid because azulene (ϕ_F = 0.031; Turro *et al.*, 1978) is much less fluorescent than DPA (ϕ_F ~ 0.90; Hamai and Hirayama, 1983). For this purpose the quenching effect of azulene upon the optically excited DPA was first ascertained. The Stern–Volmer plot was linear with $k_q{}^*\tau_0{}^* = 98\ M^{-1}$. From the slope of the corrected Stern–Volmer plot (*cf.* Eq. 1) $k_q\tau_0 = 60\ M^{-1}$ was obtained for the azulene quenching of S_1 acetone (derived from the thermolysis of TMD) monitored by DPA fluorescence. Taking τ_S = 2 ns for the lifetime of S_1 acetone (Halpern and Ware, 1980), the rate constant for energy transfer was estimated to be $k_q = 3 \times 10^{10}\ M^{-1}\,s^{-1}$. This k_q value indicates that the energy transfer process is diffusion controlled.

It is of interest to mention that for micellar solubilized azulene, using sodium dodecyl sulfate (SDS), the $S_2 \rightarrow S_0$ azulene fluorescence was observed during optical excitation and also during TMD thermolysis. Thus, upper excited singlet states can also be energized in aqueous systems.

It is not surprising that triplet acetone fails to excite azulene to its S_2 state. On one hand, spectral overlap for a T-S transfer between T_1 acetone and S_2 azulene is less favorable, on the other hand, T_1 acetone (80 kcal/mol; Calvert and Pitts, 1966) is somewhat lower in energy than S_2 azulene (80.9 kcal/mol). Still less likely would be transfer to populate an azulene triplet level above S_2 followed by ISC to the latter state.

Anomalous fluorescence of xanthione elicited by TMD thermolysis

When TMD was heated at 70°C in toluene in the presence of xanthione, the acetone fluorescence was quenched. Concomitantly a new emission band arose at 480 nm (Fig. 3). The latter was identical to the $S_2 \rightarrow S_0$ fluorescence of xanthione in toluene, indicating that energy transfer occurred from S_1 acetone to generate S_2 xanthione. A control experiment revealed that biphenyl (5×10^{-2} *M* in toluene) did

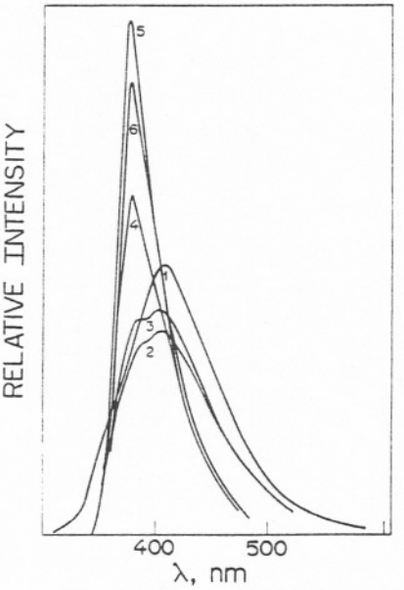


Figure 2. The effect of azulene concentration upon the chemiluminescence spectrum of 0.01 *M* TMD at 60°C. Curve 1: [AZ] = 0.0; curve 2, 5.3×10^{-4} *M*; curve 3, 1.1×10^{-3} *M*; curve 4, 3.0×10^{-3} *M*; curve 5, 6.0×10^{-3} *M*; curve 6, 1.1×10^{-2} *M*.

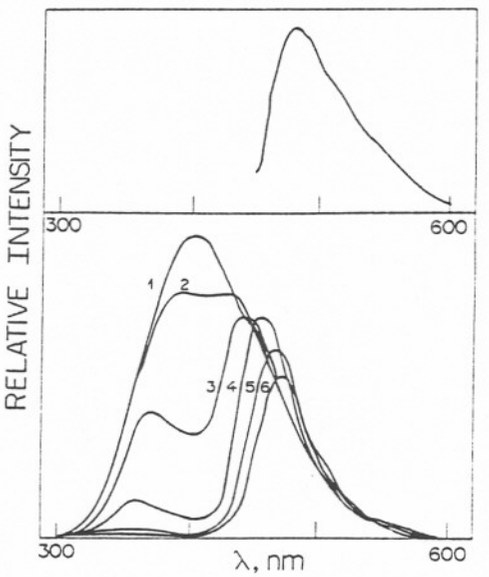


Figure 3. The effect of xanthione concentration upon the chemiluminescence spectrum of 0.01 *M* TMD at 70°C. Curve 1: [XT] = 0.0; curve 2, 3.3×10^{-5} *M*; curve 3, 2.0×10^{-4} *M*; curve 4, 1.0×10^{-3} *M*; curve 5, 5.0×10^{-3} *M*; curve 6, 1.0×10^{-2} *M*. Emission slit = 20 nm. The upper curve is the optically excited spectrum of 1.0×10^{-3} *M* XT (λ_{exc} = 400 nm; *T* = 70°C; both slits = 10 nm). This spectrum has been corrected for solvent fluorescence.

not quench the xanthione emission. Unfortunately, the overlap of the two bands in Fig. 3 precluded a quantitative Stern–Volmer analysis. Nor was it possible to carry out studies employing DPA and DBA as monitors because their absorption spectra completely overlap with that of xanthione. Also Rose Bengal was not suitable because of its low solubility and small fluorescence efficiency.

We attempted energy transfer with enzyme-generated triplet acetone to micelle-solubilized azulene and xanthione. Unfortunately, both substances undergo extensive chemical transformation, precluding a quantitative investigation.

CONCLUDING REMARKS

This is the first report of sensitized anomalous fluorescence promoted in the thermolysis of dioxetanes. Thus, dioxetanes provide interesting opportunities to study the behavior of upper excited states without the use of radiation sources (White *et al.*, 1974).

Acknowledgements—Generous funding by the Stiftung Volkswagenwerk is gratefully appreciated. Additionally the authors of the Universidade de São Paulo wish to express their gratitude to FINEP (Rio de Janeiro), FAPESP (São Paulo) and CNPq (Brasilia) and the authors of the University of Würzburg to the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie for financial support. They also thank Dr. Etelvino J. H. Bechara for a critical reading of the manuscript.

REFERENCES

Adam, W. (1982) In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 115–152. Academic Press, New York.

Adam, W. and K. Zinner (1982) In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 153–189. Academic Press, New York.

Anderson, Jr., R. W., R. M. Hochstrasser and H. J. Pownall (1976) *Chem. Phys. Lett.* **43**, 224–227.

Beer, M. and H. C. Longuet-Higgins (1955) *J. Chem. Phys.* **23**, 1390–1391.

Birks, J. B. (1972) *Chem. Phys. Lett.* **17**, 370–372.

Calvert, J. G. and J. W. Pitts Jr. (1966) *Photochemistry*. Wiley, New York.

Cilento, G. (1980) *Acc. Chem. Res.* **13**, 225–230.

Cilento, G. (1984) *Pure Appl. Chem.* **56**, 1179–1190.

Halpern, A. M. and W. R. Ware (1971) *J. Chem. Phys.* **54**, 1271–1276.

Hamai, S. and F. Hirayama (1983) *J. Phys. Chem.* **87**, 83.

Huber, J. R. and M. Mahaney (1975) *Chem. Phys. Lett.* **30**, 410–412.

Klemp, D. and B. Nickel (1983) *8th Meeting of the Photochemistry Section*, German Chemical Society, Abstracts, pp. 33–36. Tübingen, West Germany.

Kopecky, K. R., J. E. Filby, C. Mumford, P. A. Lockwood and J.-Y. Ding (1975) *Can. J. Chem.* **53**, 1103–1122.

Leigh, W. J. and J. C. Scaiano (1983) *J. Am. Chem. Soc.* **105**, 5652–5657.

Mahaney, M. and J. R. Huber (1975) *Chem. Phys.* **9**, 371–378.

Nassi, L. and G. Cilento (1985) *Photochem. Photobiol.* **41**, 195–201.

Rivas-Suárez, E., L. H. Catalani, E. J. H. Bechara and G. Cilento (1983) *Photochem. Photobiol.* **37**, 93–97.

Turro, N. J., V. Ramamurthy, W. Cherry and W. Farneth (1978) *Chem. Revs.* **78**, 125–145.

Viswanath, H. and M. Kasha (1956) *J. Chem. Phys.* **24**, 574–577.

White, E. H., J. D. Miano, C. J. Watkins and E. J. Breaux (1974) *Angew. Chem. Int. Ed.* **13**, 229–243.

Wilson, T. and A. M. Halpern (1980) *J. Am. Chem. Soc.* **102**, 7279–7283.

Wilson, T. and A. P. Schaap (1971) *J. Am. Chem. Soc.* **93**, 4126–4136.

Anexo V

Energy transfer from triplet acetophenones to 9,10-dibromoanthracene (S_1): role of its T_n state. Catalani, L.H., Wilson, T. *J. Am. Chem. Soc.*, **109**, 7458 (1987).

Energy Transfer from Triplet Acetophenones to 9,10-Dibromoanthracene (S_1): Role of Its T_n State

Luiz H. Catalani and Thérèse Wilson*

Contribution from The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received May 6, 1987*

Abstract: The efficiency of energy transfer to 9,10-dibromoanthracene (S_1) (DBA, 0.5–5 × 10^{-4} M) from triplet acetophenone, methyl-substituted acetophenones, and indanone (**1**) (0.02 M, E_T = 71.5–75.8 kcal) was measured as a function of temperature (1–57 °C) in acetonitrile. The decay of DBA fluorescence follows double-exponential functions, with a ca. 2-ns component (direct DBA excitation) and a ca. 0.1–3-μs component (sensitization by triplet ketone). The rate parameters are the same for all ketones, but the amplitude of the slow component depends on the triplet energy of the ketone. With Φ_{TS} defined as k_{TS}/k_{ET}, Φ_{TS} = 0.3 for **1** (E_T = 75.8 kcal) and 0.004 for 3,5-dimethylacetophenone (E_T = 71.5 kcal) at 20 °C; DBA deactivates all triplet ketones with k_{ET} = 1.15 × 10^{10} M^{-1} s^{-1}. The temperature dependence of Φ_{TS} shows the involvement of DBA (T_n), ca. 4 kcal above S_1, in a two-step exchange process: TT transfer to T_n (k_{TT_n}) and then isc $T_n \rightarrow S_1$. The E^a of k_{TT_n} matches the gap between E_T and DBA (T_n). Φ_{TS} of **1** is close to the limit set by the isc efficiency in DBA (ca. 0.3–0.4); thus, the 35-kcal exothermic triplet transfer to DBA (T_1) is unimportant compared with the near-isothermic transfer to T_n.

Many studies have addressed the role of higher excited states of anthracenes in intersystem-crossing (isc) and energy-transfer processes.[1,2] For example, the effect of temperature and of meso substituents on the fluorescence quantum yield can be readily understood on the basis of the position of higher triplet states relative to S_1 in these compounds.[3-7] A case in point is 9,10-dibromoanthracene (DBA). Its fluorescence efficiency Φ_F decreases from unity at 77 K[4,7] to ca. 0.1 at room temperature in ethanol,[6] because fluorescence competes with intersystem crossing to a higher triplet manifold; this mediated isc process[2] depends on temperature, according to an Arrhenius equation (eq 1), where

$$k_{isc} = A_{isc} \exp(-E_{isc}/RT) \quad (1)$$

$E_{isc} \approx 4 \pm 1$ kcal.[7] Thus, in DBA at least one (and possibly several) higher triplet state(s) is estimated to be located ca. 4 ± 1 kcal above S_1.[8,9] Solvents shift S_1 and T_n ($n \geq 2$) to different extents and therefore E_{isc} and Φ_F are solvent dependent.[6] Recent experiments of Amirav and Jortner with jet-cooled isolated DBA, prepared in the S_1 state with excess vibrational energy, have refined the understanding of mediated isc.[2]

This T_n state may also be important in exothermic energy transfer from triplet donors to DBA, a process known to result in part in DBA (S_1).[10] With some triplet donors, such as acetophenone, this spin-forbidden process has a remarkably high efficiency, $\Phi_{TS} \approx 0.1$,[11] defined here by eq 2, where k_{TS} and k_{ET} are the rate constants of reactions 3 and 4. Equation 3 is the

$$\Phi_{TS} = k_{TS}/k_{ET} \quad (2)$$

$$^3K^* + DBA \xrightarrow{k_{TS}} K + DBA\ (S_1) \quad (3)$$

$$^3K^* + DBA \xrightarrow{k_{ET}} K + DBA \quad (4)$$

overall process leading to the formation of DBA (S_1), while eq 4 represents all processes of deactivation of the triplet donor $^3K^*$ by DBA, which result mostly in the formation of DBA (T_1). Such high values of Φ_{TS} as 0.1 allow DBA (or a water-soluble sulfonated derivative[12b]) to serve as a fluorescence probe for the generation of triplet excited molecules in chemical or biochemical reactions.[12a] This property, which has been widely exploited, adds a practical interest to the study of energy transfer to DBA. Yet the exact mechanism of this process has not been definitely established, and significant differences in values of Φ_{TS} between triplet carbonyl donors, for example, remain unexplained. The present paper addresses, and answers, some of these questions.

Both long-range (Forster) and collisional (exchange) interactions could in principle be at play in eq 3; arguments in support of both have indeed been offered. A major contribution from Forster energy transfer can, however, be safely ruled out, since the efficiency of such a process should not be affected by the presence of heavy atoms in the acceptor.[13] Yet chemiluminescence and time-resolved fluorescence studies have established, for example, that Φ_{TS} is 1 order of magnitude higher with DBA than with 9,10-dichloroanthracene, although these two anthracenes have almost identical absorption spectra.[15,16]

If, on the other hand, the energy-transfer process is collisional and requires prior formation of an encounter complex (eq 5), two

$$^3K^* + DBA \underset{k_{-diff}}{\overset{k_{diff}}{\rightleftharpoons}} (^3K^*DBA) \quad (5)$$

distinct mechanisms need be considered. In the first, eq 3 may be viewed as representing two consecutive processes, shown in Figure 1, a triplet–triplet energy transfer resulting in excitation of DBA (T_n) (eq 6) followed by isc from this higher triplet to DBA (S_1) (eq 7). The rate of the latter process is enhanced by the

(1) Birks, J. B. *Photophysics of Aromatic Molecules*; Wiley-Interscience: London, 1970; pp 142–192.
(2) Amirav, A.; Jortner, J. *Chem. Phys. Lett.* **1986**, *132*, 335.
(3) Bennett, R. G.; McCartin, P. J. *J. Chem. Phys.* **1966**, *44*, 1969.
(4) Lim, E. C.; Laposa, J. D.; Yu, J. M. H. *J. Mol. Spectrosc.* **1966**, *19*, 412.
(5) Dreescamp, H.; Pabst, J. *Chem. Phys. Lett.* **1979**, *61*, 262.
(6) Wu, K.-C.; Ware, W. R. *J. Am. Chem. Soc.* **1979**, *101*, 5906.
(7) Kearvell, A.; Wilkinson, F. *Transitions Non Radiatives dans les Molecules*; Paris, 1969; *J. Chim. Phys.* **1970**, *20*, 125.
(8) Gillispie, G. D.; Lim, E. C. *J. Chem. Phys.* **1976**, *65*, 2022; *Chem. Phys. Lett.* **1979**, *63*, 355.
(9) Tanaka, M.; Tanaka, I.; Tai, S.; Hamanoue, K.; Sumitani, K.; Yoshihara, K. *J. Phys. Chem.* **1983**, *87*, 813.
(10) Vasil'ev, R. F. *Nature (London)* **1963**, *200*, 773. Belyakov, V. A.; Vassil'ev, R. F. *Photochem. Photobiol.* **1970**, *11*, 179.
(11) Wilson, T.; Halpern, A. M. *J. Am. Chem. Soc.* **1980**, *102*, 7272.
(12) (a) Wilson, T. *Int. Rev. Sci.: Phys. Chem., Ser. Two* **1976**, *9*, 265. Turro, N. J.; Lechtken, P.; Schore, N. E.; Schuster, G.; Steinmetzer, H.-C.; Yekta, A. *Acc. Chem. Res.* **1974**, *7*, 97. Cilento, G. *Pure Appl. Chem.* **1984**, *56*, 1179. (b) Catalani, L. H.; Wilson, T.; Bechara, E. J. H. *Photochem. Photobiol.* **1987**, *45*, 273.
(13) The experiments of Turro et al.[14] with chemically generated triplet acetone (from tetramethyldioxetane) and DBA in polystyrene matrix were interpreted as suggesting an important contribution from long-range TS transfer. But the possibility of energy migration along the phenyl groups of the polymer and also of some SS transfer in these fluorescence intensity measurements complicates the analysis, as does the possible role of exciplexes of triplet acetone with the phenyl groups[15] of the polymer.
(14) Turro, N. J.; Steinmetzer, H.-C. *J. Am. Chem. Soc.* **1974**, *96*, 4677, 4679. Turro, N. J.; Kochevar, I. E.; Nogochi, Y.; Chow, M.-F. *Ibid.* **1978**, *100*, 3170.
(15) Wilson, T.; Halpern, A. M. *J. Am. Chem. Soc.* **1980**, *102*, 7279.
(16) Schmidt, R.; Kelm, H.; Brauer, H.-D. *Ber. Bunsen-Ges. Phys. Chem.* **1977**, *81*, 402. *J. Photochem.* **1979**, *11*, 145.

0002-7863/87/1509-7458$01.50/0

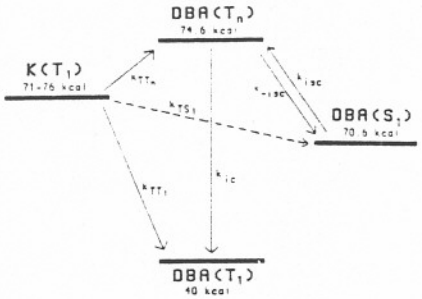


Figure 1. Diagram of excited states of an acetophenone (K) and 9,10-dibromoanthracene (DBA) illustrating the energy-transfer processes considered here.

bromine substituents and therefore able to compete with internal conversion (eq 8). This two-step mechanism was first proposed

$$(^3\text{K*DBA}) \xrightarrow{k_{TT_n}} \text{K} + \text{DBA}\ (T_n) \qquad (6)$$

$$\text{DBA}\ (T_n) \xrightarrow{k_{-isc}} \text{DBA}\ (S_1) \qquad (7)$$

$$\text{DBA}\ (T_n) \xrightarrow{k_{ic}} \text{DBA}\ (T_1) \qquad (8)$$

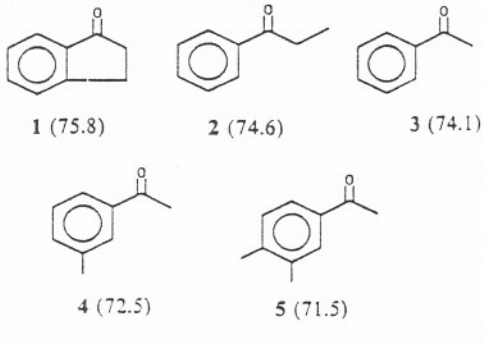
$$(^3\text{K*DBA}) \xrightarrow{k_{TT_1}} \text{K} + \text{DBA}\ (T_1) \qquad (9)$$

by Schmidt et al.[16] on the basis of high-pressure experiments with chemically generated triplet acetone.[17] The conclusions of this study, designed primarily to evaluate the role of a long-range mechanism, were questioned by Wu, who proposed an alternative pathway, still within the framework of an exchange energy-transfer mechanism.[18] He argued that the bromine atoms introduce considerable triplet character in the singlet wave functions of DBA, and vice versa. Because of this mixing of states, reaction 3 is not truly "spin-forbidden". Therefore, within an encounter complex of ^{3}K* and DBA, direct energy transfer to form DBA*(S_1) takes place in competition with triplet–triplet transfer to form T_1 and T_n. Thus, Φ_{TS} now reflects the efficiency of formation of the encounter complex (eq 5) and the partition within the complex between reaction 10 and reactions 6 and 9.

$$(^3\text{K*DBA}) \xrightarrow{k_{TS_1}} \text{K} + \text{DBA}\ (S_1) \qquad (10)$$

As Wu pointed out, these alternative mechanisms (eq 5–7 or 5 and 10) lead to the same kinetic expression for Φ_{TS}. Neither can be dismissed a priori on the basis of unreasonable demand on the magnitude of the rate constants involved. Thus, whether or not T_n is involved cannot be decided on the basis of the available experimental data.

We have designed an experiment that provides a clear-cut answer to this question. Consider a series of energy donors of similar structures: indanone, acetophenone, and three methyl-substituted acetophenones (listed with their corresponding triplet energies, in kilocalories).[19a] Their triplet states are all above DBA



1 (75.8) **2** (74.6) **3** (74.1)

4 (72.5) **5** (71.5)

(17) The solvent was toluene, which like benzene and methyl derivatives is known to form triplet exciplexes with acetone; these exciplexes are efficient energy donors to DBA.[15] This adds a complication to an already complex system.

(18) (a) Wu, K.-C. *J. Photochem.* **1980**, *12*, 363. (b) See, however: Schmidt, R.; Kelm, H.; Brauer, H.-D.; *Ibid.* **1980**, *14*, 261.

(S_1) at ca. 70.6 kcal; therefore, TS energy transfer is exothermic in all cases. But some of these donors have their triplet above and some below DBA (T_n),[20] so that TT_n energy transfer is either slightly exothermic, isothermic, or endothermic by up to 3 kcal. If T_n is not involved, then eq 10 predicts that the relative Φ_{TS} values of these donors will be determined by the spectral overlap between the phosphorescence spectra of the donors and the absorption spectrum of DBA (eq 11).[21]

$$k_{TS} \propto \int_0^\infty P_D(\bar{\nu})\ \epsilon_{DBA}(\bar{\nu})\ d\bar{\nu} \qquad (11)$$

On the other hand, if eq 6 and 7 represents the dominant mechanism, then one expects the energy-transfer process to be temperature dependent when the donor's triplet energy is below DBA (T_n), with a corresponding activation energy predictable on the basis of the energy gap between the donor triplet and DBA (T_n). This, in fact, is what we found.

Note that our analysis is simplified by the very short lifetime (ca. 2 ns) of the singlet excited state of DBA, the energy acceptor. The observed rates of forward TS energy transfer do not require correction for the rate of back-transfer, as is normally the case in triplet–triplet transfer studies when the process is near isothermicity and both triplets have long lifetimes.[21b]

The values of Φ_{TS}, k_{ET}, and therefore k_{TS} (eq 2) were determined by the time-correlated single-photon counting method, which has previously been shown to be ideally suited to the determination of these parameters in the case of acetophenone and two alkanones as triplet donors.[11,15] Pulsed UV excitation of solutions of ketones and DBA generates both direct and sensitized fluorescence of DBA. Consequently, the fluorescence of DBA decays as the sum of two exponentials of very different rate parameters (eq 12). DBA (S_1) is the emitter of the prompt as

$$I_t \propto \alpha \exp(-k_E t) + \exp(-k_{obsd} t) \qquad (12)$$

well as the delayed fluorescence; thus, factors such as temperature that affect its rate of deactivation cancel out here. All necessary information on rates and yields can be extracted from eq 12, notably k_{ET} and Φ_{TS}.

The same method was also applied to the quenching of triplet ketones **1**, **3**–**5** by another brominated anthracene, 1,5-dibromo-9,10-bis(phenylethynyl)anthracene (DBPEA), also an efficient TS fluorescence probe.[22] Here Φ_{TS} was found to be the same for these four ketones, the differences in their triplet energies notwithstanding.

The implications of our results will be discussed in the context of the photophysics of DBA and DBPEA and of their use as triplet probes in chemiluminescence studies.

Experimental Section

Indanone (**1**), propiophenone (**2**), acetophenone (**3**), 3-methylacetophenone (**4**), and 3,4-dimethylacetophenone (**5**) were obtained from Aldrich and distilled before use. 9,10-Dibromoanthracene (DBA, Aldrich) was vacuum sublimed. 1,5-Dibromo-9,10-bis(phenylethynyl)-anthracene (DBPEA) was a gift from Dr. A. P. Schaap, Wayne State

(19) (a) E_T values from the O–O band of phosphorescence emission in a polar solvent at 77 K determined by: S. L. Murov Ph.D. Thesis, University of Chicago, 1966. Listed in: Murov, S. L. *Handbook of Photochemistry*; Marcel Dekker: New York, 1973. (b) In parentheses, net energies of the triplet states, i.e., the energy differences between relaxed T_1 and S_0, not the energies associated with vertical transitions. For justification, see: Kiri, A.; Thomas, J. K. *J. Phys. Chem.* **1974**, *78*, 196. Gessner, F.; Scaiano, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7206. These net triplet energies were obtained from the E_T values of Murov[19a] by adding half of the Stokes shifts (1.1 ± 0.3 kcal for **1**–**3** with n,π* lowest triplet; 0.7 ± 0.3 kcal for **4** and **5** with lowest triplet of π,π* configuration; see ref 26). We also corrected for the relaxation of n,π* (but not ^{3}π,π*) ketone triplets in fluid solutions, by substracting 2 kcal from the rigid solution values as recommended by Wagner.[26] Note that our conclusions are the same whether we adopt the E_T values from Murov or the revised net triplet energies, because the energy of the S_1 of DBA is always below the E_T of **1**–**5**.

(20) Estimated to be 74.5 kcal above S_0.[7]

(21) (a) Dexter, D. L. *J. Chem. Phys.* **1953**, *21*, 836. (b) Reference 1, pp 537–544.

(22) Schaap, A. P., private communication. Lampert, R. A.; Meech, S. R.; Metcalfe, J.; Phillips, D.; Schaap, A. P. *Chem. Phys. Lett.* **1983**, *94*, 137.

Table I. Efficiency and Temperature Dependence of Energy Transfer from Triplet Acetophenones to 9,10-Dibromoanthracene[a]

ketones	E_T[b]	Φ_{TS}[c]	spectral overlap[d] (rel)	E_{TT_n}[a,e]	$E_T + E_{TT_n}$[a,f] (rel)	Φ_{TS}[c] (rel)	$\exp(-E_{TT_n}/RT)$[c]
1	75.8 (74.9)	0.30		(0)[g]	(75.8)[g] (74.9)[g]	2.3	4.6
2, 3	74.3_5 (73.4)	0.13	1.00	0.91 (0.34)	75.2 (74.3)	1.00	1.00
4	72.5 (73.2)	0.025	0.9	1.27 (0.41)	73.7 (74.5)	0.2	0.5
5	71.5 (72.2)	0.004	0.7	2.83 (0.47)	74.3 (75.0)	0.03	0.04
					av = 74.7 (74.7)		

[a] All energies in kilocalories/mole. [b] From Murov.[19a] In parentheses, net triplet energies estimated as in footnote 19b. All E_T ±0.3 kcal/mol. [c] At 20 °C. [d] See Figure 4 and the Results. [e] Standard errors calculated with the following numbers of data points: 31 for **2** and **3**, 13 for **4**, and 16 for **5**. [f] In parentheses, values based on net E_T. [g] Since Φ_{TS} is temperature independent, this is a guess; see Discussion.

University. Acetonitrile, benzene, and cyclohexane ("distilled in glass", Burdick and Jackson) were used as received. Absorption spectra and extinction coefficients were obtained with a Uvikon 820 (Kontron) spectrophotometer.

For the fluorescence decay measurements, the samples were deaerated by nitrogen purging following a standardized procedure. The experimental techniques and instrumentation for time-resolved single-photon counting have been described earlier.[23] In the experiments with DBA, fluorescence was monitored through a wide-band interference filter centered at 433 nm (MicroCoating, 50-nm fwhm). With DBPEA the filter was centered at 550 nm (Baird Associates, 100-nm fwhm).

Results

1. Energy Transfer to DBA. Effect of Temperature. Acetonitrile solutions of ketone (ca. 0.02 M) and DBA (ca. (0.5–5) × 10^{-4} M) were excited at 320 nm; the ratios of absorbances of ketone to DBA were >4 in all cases. All the decay curves of DBA fluorescence were strictly single exponentials in the absence of ketone: $\tau_F = 1/k_F = 2 \pm 0.1$ ns at 20 °C, $E_{isc} = 4.0$ kcal (±0.1 SD), eq 1. In acetonitrile, S_1 was determined to be 70.6 kcal above S_0, from the absorption and fluorescence spectra, which puts T_π at 74.6 kcal. In the presence of acetophenone, the decay curves were all strictly double exponentials (eq 12), as expected from the simplified reaction scheme presented in eq 13–17. Here eq

$$K + h\nu \rightarrow {}^1K^* \rightarrow {}^3K^* \quad (13)$$

$$DBA + h\nu \rightarrow DBA\ (S_1) \quad (14)$$

$$^3K^* + DBA \xrightarrow{k_{TS}} K + DBA\ (S_1) \quad (3)$$

$$^3K^* + DBA \xrightarrow{k_{ET}} K + DBA \quad (4)$$

$$^3K^* \xrightarrow{k_d} K \quad (15)$$

$$DBA\ (S_1) \xrightarrow{k_r} DBA + h\nu \quad (16)$$

$$DBA\ (S_1) \rightarrow DBA \quad (17)$$

3 represents either eq 6, 7, or 10, as discussed in the introduction. The rate parameter of the slow-decay component depends linearly on the concentration of DBA,[11] according to eq 18. The plots

$$k_{obsd} = k_d + k_{ET}[DBA] \quad (18)$$

of k_{obsd} vs [DBA] with acetophenone at three temperatures (7, 20, and 40 °C) are linear. At 20 °C $k_{ET} = 1.15 \times 10^{10}$ M^{-1} s^{-1}, consistent with diffusion-controlled quenching of $^3K^*$ by DBA. The temperature dependence of k_{ET} indicates an activation energy of 1.8 kcal/mol. All ketones have the same k_{ET}.

In contrast, there are striking differences between the ketones regarding the amplitudes α of the slow component of fluorescence decay (eq 12). This is illustrated in Figure 2, which compares the results obtained with three ketones in similar conditions of concentrations, temperature, and time scale; the curves are normalized at I_{max}. Triplet dimethylacetophenone (**5**), especially, is evidently a much poorer sensitizer of DBA fluorescence than acetophenone (**3**), for example. An expression for this efficiency, Φ_{TS} (eq 2), was previously derived[23] in terms of the experimentally

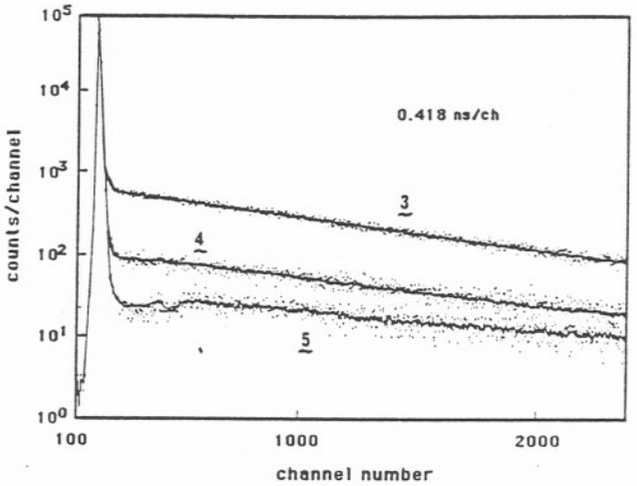


Figure 2. Fluorescence decay curves of acetonitrile solutions of 0.02 M ketones **3**–**5** and DBA (ca. 2 × 10^{-4} M) at 20 °C. λ_{ex} = 320 nm; fluorescence monitored in the 420–460-nm range.

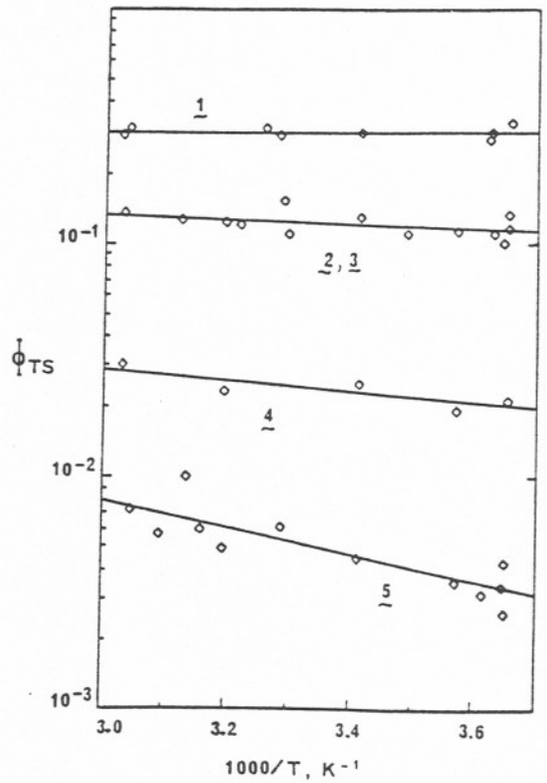


Figure 3. Temperature dependence of Φ_{TS} for ketones **1**–**5**. Some symbols represent the average of several data points.

available rate constants and the ratio R of absorbances of DBA and the ketone ($R = \epsilon_{DBA}[DBA]/\epsilon_K[K]$):

$$\Phi_{TS}^{DBA} = R(k_F - k_{obsd})/(1 + \alpha)k_{ET}[DBA] \quad (19)$$

Once k_{ET} has been determined at a given temperature, then every decay curve provides the information for a value of Φ_{TS}. We used

(23) Wilson, T.; Frye, S. L.; Halpern, A. M. *J. Am. Chem. Soc.* **1984**, *106*, 3600.

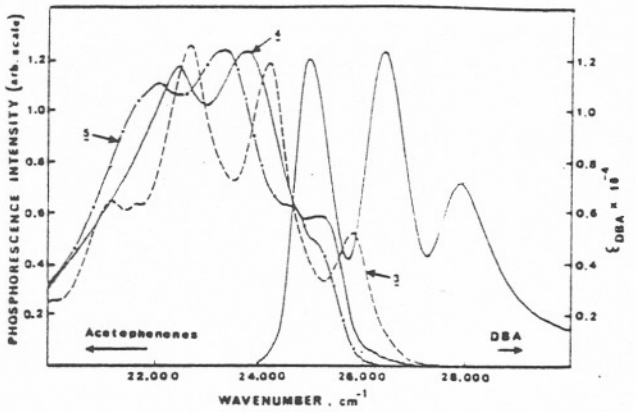


Figure 4. Phosphorescence spectra of acetophenones **3–5** (as labeled; data from ref 28) and absorption spectrum of DBA in acetonitrile.

the values of k_{ET} determined for acetophenone in the calculation of the Φ_{TS} of all ketones. The results at 20 °C are listed in Table I. Φ_{TS} was determined at several temperatures between 0 and 57 °C (Figure 3). It is independent of temperature in the case of **1** but increases with temperatures for the other ketones. The effect is largest with **5**; its Φ_{TS} doubles in this temperature range.

For reasons to be discussed later, one experiment with **5** was carried out in benzene and one in cyclohexane instead of acetonitrile. This ketone remains a very poor energy donor in these other solvents ($\Phi_{TS} \leq 0.004$).

2. Energy Transfer to DBPEA at 20 °C. DBPEA was synthesized by Schaap for use as a fluorescence probe in the study of dioxetanes when the cleavage products have triplet energies too low (<70 kcal) to be monitored by DBA. DBPEA is intensely fluorescent ($E_s \approx 57$ kcal, $\Phi_F \approx 1$, $\lambda_{max_F} \approx 512$ nm in acetonitrile). Its fluorescence efficiency, in contrast to that of DBA, is practically temperature independent.[22]

Excitation at 330 nm of acetonitrile solutions of DBPEA (3.3×10^{-5} M) and ketones **1** and **3–5** (0.013–0.017 M) at 20 °C resulted in the emission of DBPEA fluorescence that exhibited double-exponential decay. The short-decay component indicates a fluorescence lifetime of ca. 6 ns, while the rate parameters of the long component were in keeping with the values of k_{obsd} calculated from eq 18 (substituting the concentration of DBPEA for [DBA]). But in sharp contrast to the decay curves obtained with these ketones and DBA, here the amplitudes α of the slow component were all roughly the same. This is reflected in the corresponding values of Φ_{TS}^{DBPEA}, which are identical for the four ketones, 0.08 ± 0.004.

Discussion

The experiments described here were designed to identify which of two possible pathways of collisional energy transfer is primarily responsible for the efficient sensitization of DBA fluorescence by triplet ketones. The two alternatives considered were (a) direct excitation of singlet DBA within an "encounter complex" (or possibly an exciplex) of DBA and the triplet donor (eq 10) or (b) excitation of a higher triplet of DBA followed by intersystem crossing to S_1 (eq 6 and 7). These two mechanisms are, of course, not mutually exclusive. Nevertheless, we believe that the evidence presented here demonstrates the major role of a T_n state of DBA.

If mechanism (a) was the dominant one, then the relative values of Φ_{TS} should be proportional to the extent of spectral overlap between the phosphorescence emission of the ketones and the absorption spectrum of DBA (eq 11). Figure 4 shows the relevant spectra,[24] from which the relative values of spectral overlaps, listed in Tables I for ketones **3–5**, were estimated. This comparison gives a clear answer: whereas the values of Φ_{TS} for **3** and **5**, for example, differ by nearly 2 orders of magnitude, the extent of spectral overlap accounts for a difference of less than a factor of 2. (The order of the relative values of Φ_{TS} and spectral overlaps is the same, since it reflects the triplet energies of the respective ketones.)

If this is a strong negative argument against direct population of DBA (S_1) (eq 10) as an important pathway, the effect of temperature on Φ_{TS} is a compelling argument in support of the intermediacy of T_n via mechanism (b) above. A look at Table I and Figure 3 immediately suggests this interpretation. The efficiency Φ_{TS} has the highest value and is temperature independent in the case of **1**, the ketone with the highest triplet energy. Φ_{TS} becomes increasingly temperature dependent as the triplet energy E_T^K of the ketone decreases in the order **1–5**, even though E_T^K is always above DBA (S_1). Table I shows that the uncertainties attached to the proper values of the ketones E_T do not alter this conclusion. These results are easily interpreted on the basis of reactions 6–8 and the quenching process (eq 9), as shown in Figure 1.

If deactivation of triplet donors proceeds exclusively via TT transfer to generate either the higher or the lower triplet of DBA, then the experimentally determined efficiency Φ_{TS} of TS transfer is given by eq 20, where $\Phi_{-isc} = k_{-isc}/(k_{-isc} + k_{ic})$. Φ_{-isc}, the

$$\Phi_{TS} = \Phi_{-isc} k_{TT_n}/(k_{TT_n} + k_{TT_1}) \qquad (20)$$

efficiency of intersystem crossing in DBA, is assumed to be independent of the ketone. A lower limit for this value is 0.3, since Φ_{TS} of indanone (**1**) is 0.3. (The Φ_{TS} of the triplet exciplex of acetone and benzene is also ca. 0.3.)[15] Using a double-excitation method to populate the T_n state of anthracenes in ethanol, Kokubun et al. found $\Phi_{-isc} = 0.27$ for DBA, later revising this value to 0.19.[25a] Importantly, they found Φ_{-isc} to be temperature independent, as expected since the two processes involved, ic and −isc, are exothermic. In this discussion we will *assume* $\Phi_{-isc} = 0.35 \pm 0.05$.[25b]

Rearrangement of eq 20 gives eq 21. Since k_{TT_1} can also be assumed to be independent of temperature, plots of $\ln [\Phi_{TS}/(\Phi_{-isc} - \Phi_{TS})]$ vs $1/T$ should be linear, with slopes $E_{TT_n}^a/R$, where $E_{TT_n}^a$ is the activation energy of k_{TT_n} for each ketone. The values of $E_{TT_n}^a$ are listed in Table I.

$$\Phi_{TS}/(\Phi_{-isc} - \Phi_{TS}) = k_{TT_n}/k_{TT_1} \qquad (21)$$

In the case of **4** and **5**, since Φ_{TS} is small compared with Φ_{-isc}, the exact value of Φ_{-isc} has little effect on $E_{TT_n}^a$. With ketones **2** and **3**, the uncertainty attached to Φ_{-isc} introduces an uncertainty of about 10% on the values of $E_{TT_n}^a$.

Table I shows that for ketones **2–5**, the sum of the ketone triplet energy E_T^K and $E_{TT_n}^a$ corresponds to an average energy of 74.7 ± 1 kcal. This is the total energy available for excitation of DBA; it matches well the level of the T_n state, which we located at 74.6 kcal (see Results). Given the uncertainties attached to the values of the triplet energies of the ketones,[19] our results are fully consistent with excitation of DBA (T_n) prior to DBA (S_1). Thus the "TS" transfer of energy from the triplet ketones is, in truth, a

(24) Dusenbery, R. Ph.D. Thesis, University of Chicago, 1970. We thank Prof. N. C. Yang for providing us with this information.

(25) (a) Kikuchi, K.; Fukumura, H.; Kokubun, H. *Chem. Phys. Lett.* **1986**, *123*, 226. Kobayashi, S.; Kikuchi, K.; Kokubun, H. *Chem. Phys.* **1978**, *27*, 399. (b) A direct and reliable photophysical determination of Φ_{-isc} in acetonitrile would be highly desirable. If, as assumed here, only one higher triplet state T_n is involved in isc to and from S_1, then[6] $1/\tau_F = k_r + k_{isc}k_{ic}/(k_{ic} + k_{-isc})$, where τ_F is the measured lifetime of DBA (S_1) and k_r its radiative rate of decay ($k_r = \Phi_F/\tau_F \approx 8 \times 10^7$ s^{-1} at 20 °C). With these values, we have $k_{isc}(1 - \Phi_{-isc}) \approx 4.2 \times 10^8$ s^{-1}. It can reasonably be assumed[1,7,25a] that $k_{isc} = k_{-isc} \exp(E_{isc}/RT) = 1.3 \times 10^{-3}\, k_{-isc}$. Thus one can calculate k_{isc}, k_{-isc}, and k_{ic} for different values of Φ_{-isc}. For example, if $\Phi_{-isc} = 0.35$ as assumed here, then $k_{isc} \approx 7 \times 10^8$ s^{-1}, $k_{-isc} \approx 5 \times 10^{11}$ s^{-1}, and $k_{ic} \approx 10^{12}$ s^{-1}, which indicates a very short lifetime for T_n ($\tau_{T_n} = 1/[k_{ic} + k_{isc}k_r/(k_r + k_{-isc})] \approx 1$ ps), in contrast with the 200 ps estimated from chemical sensitization experiments. See: Liu, R. S. H.; Edman, J. R. *J. Am. Chem. Soc.* **1969**, *91*, 1492. Other values of Φ_{-isc} lead to similarly short T_n lifetimes. This difficulty, which has been perceived and discussed in different contexts by others,[8,9,25a] can be circumvented only by new assumptions. For example, there may be another triplet state slightly below T_n that gets populated very fast by internal conversion from T_n and has a longer lifetime, in keeping with the large energy gap between it and T_1. This triplet state, which we call T_2, would be responsible for the energy-transfer experiments of Liu and Edman and perhaps be the origin of the TT fluorescence emission at 840 nm; see footnote 27. Further speculation along these lines is outside the scope of this paper.

(26) Wagner, P. J.; Thomas, M. J.; Harris, E. *J. Am. Chem. Soc.* **1976**, *98*, 7675.

spin-allowed but temperature-activated TT process, followed by isc to give DBA (S_1).[27,28]

Our results lead to another interesting conclusion concerning the relative rates of energy transfer from the triplet ketones to the upper or to the lowest triplet of DBA. If $\Phi_{-isc} \approx 0.35$, then $\Phi_{TS} \approx \Phi_{-isc}$ in the case of **1**, and k_{TT_1} must be small compared with k_{TT_n} (eq 21). Like k_{TT_1}, k_{TT_n} is likely to have no or a very small activation energy. The triplet energy of **1** may thus be entirely channeled into the upper triplet of DBA, because reaction 6, which is nearly isothermic, is faster than reaction 9, exothermic by ca. 35 kcal. Thus a large energy gap actually slows down an exothermic energy-transfer process.

With no preassumption regarding the relative Φ_{TS} from either n,π^* or π,π^* triplets, we ascertained that the inefficiency of ketones **4** and **5** as donors was not a consequence of the electronic configuration of their lowest triplet states, which are π,π^*, whereas the lowest triplets of **1**–**3** are n,π^* in acetonitrile. **4** and **5** have T_2 states of n,π^* configuration slightly above T_1. Going to a nonpolar solvent is expected to lower these T_2 (n,π^*) states without affecting T_1 (π,π^*), which remains the lowest triplet. This shift increases the mixing of these states and confers more n,π^* character to the lowest triplet.[28] Two experiments with **4**, one in cyclohexane and one in benzene, resulted in values of Φ_{TS} only minimally different from that in acetonitrile reported in Table I.

Our experiments with the second brominated anthracene, DBPEA, had the same broad objective of ruling out other possible photophysical or chemical interpretations of the low Φ_{TS} values observed with **4** and **5**, not solely resting on the level of their T_1 state. We find the results with DBPEA compelling in that regard, since energy transfer from ketones **1** and **3**–**5** all generated DBPEA (S_1) with the same efficiency, $\Phi_{TS} \approx 0.08$. This is an interesting result in itself. Since the fluorescence quantum yield of this anthracene is reported to be near unity and temperature independent,[22] it is likely that its T_2 state is located relatively high above S_1, as in 9,10-diphenylanthracene for example,[3] but most probably well below even the triplet state of **5**. Thus one would not anticipate energy transfer to DBPEA from any of these ketones to require an activation energy.

The second concern of this paper was to elucidate the causes of the differences in Φ_{TS} between different triplet donors. Ketones **1**–**5** were purposefully selected for their structural similarity, all being substituted acetophenones, and for having T_1 levels critically located around DBA (T_n). Clearly, between these ketones the differences in Φ_{TS} can be entirely rationalized on the basis of their respective triplet energies. This is demonstrated in the last two columns of Table I, where the relative values of Φ_{TS}, normalized for acetophenone, are compared with the relative values of $\exp(-E_{TT_n}/RT)$ at room temperature. The agreement is good, considering the experimental errors. But this rationalization can probably not be extended to triplet donors unrelated to acetophenone, other than to predict the obvious: molecules with triplet energies much below DBA (T_n) will be poor donors. What are the factors that determine the preexponential factor in k_{TT_n}? Are ketones with T_1 much above DBA (T_n) as good donors as indanone, with $E_T^K = 75.8$ kcal? The answer is no, or not necessarily: ^{3}n,π^* acetone ($E_T^K = 78$ kcal) has a lower Φ_{TS} than acetophenone ($E_T^K = 74.6$ kcal). The fact that triplet exciplexes of acetone with benzene derivatives have higher Φ_{TS} than uncomplexed triplet acetone,[15,29] although their triplet energies are surely lower, is also significant. Further discussion would be too speculative at this point.

In view of the results presented here, a note of caution must be reiterated regarding the use of DBA for the determination of yields of chemically generated triplet carbonyl products, in the thermolysis of dioxetanes for example.[30] If the specific values of Φ_{TS} have not been predetermined by a reliable method, it is evidently unwarranted to assume that different carbonyl compounds, even closely related, have the same Φ_{TS}. The use of DBPEA in these systems would be a valuable check.

Conclusions

The well-known and relatively efficient sensitization of DBA fluorescence by triplet ketones, such as acetophenones, has been shown to involve the intermediacy of a higher triplet state of DBA. Thus the overall TS energy transfer is, in fact, a temperature-activated TT transfer followed by intersystem crossing to DBA (S_1), a process facilitated by the presence of the bromine substituents. It is noteworthy that the efficiency Φ_{TS} of indanone, with a T_1 state probably slightly above but close to DBA (T_n), must be equal or close to the limit set by the efficiency of intersystem crossing in DBA, assumed here to be ca. 0.35; a reliable value of Φ_{-isc} in acetonitrile would evidently be very valuable. Of interest also is the implication of this result regarding the relative rates of energy transfer from triplet indanone to DBA (T_n) and to DBA (T_1), 35 kcal below. The latter rate is at least 6 times smaller than the first. Thus energy transfer becomes less efficient at large exothermicity (as does electron transfer in the "inverted region").[31] This result could be obtained only because the rate limitations imposed by diffusion are circumvented here, where we measure the relative rates of energy transfer either to a higher or to a lower triplet state of an acceptor already in an encounter complex with the donor. Since $k_{TT_1} \geq 1.2 \times 10^{10}$ M^{-1} s^{-1}, $k_{TT_n} \geq 7 \times 10^{10}$ M^{-1} s^{-1}, in keeping with previous estimates of energy transfer in an encounter complex.[32]

Within the substituted acetophenone family, the T_1 energy seems to be the sole determinant of Φ_{TS}. The factors other than triplet energy that determine the Φ_{TS} of unrelated triplet donors have not yet been identified.

Acknowledgment. This work was supported by the National Science Foundation (Grant CHE-8209863). L.H.C. is grateful for a fellowship from the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) of Brazil. We thank Paul Schaap for a sample of DBPEA and information on its properties.

(27) We tried to establish the intermediacy of DBA (T_n) by a more incisive experiment. Gillispie and Lim[8] reported the observation of the fluorescence transition T_n–T_1 at ca. 840 nm, following excitation of DBA (S_1) in solution. Having confirmed this result, we had hoped to observe the sensitization of this extremely weak emission in conditions where acetophenone absorbs most of the exciting light (313 nm). Unfortunately, even with solutions only partially deaerated, a requirement for the detection of TT energy transfer to DBA, we only saw the well-known intense phosphorescence ($T_1 \rightarrow S_0$) of DBA, which unfortunately obscures the entire 700–900-nm region of interest. We thank Michel Bermane for performing these experiments.

(28) Yang, N. C.; McClure, S. D.; Murov, S. L.; Houser, J. J.; Dusenbery, R. *J. Am. Chem. Soc.* **1967**, *89*, 5466. Yang, N. C.; Dusenbery, R. L. *Ibid.* **1968**, *90*, 5899.

(29) Johnston, L. J.; Scaiano, J. C.; Wilson, T. *J. Am. Chem. Soc.* **1987**, *109*, 1291.

(30) Wilson, T. In *Singlet O_2*; Frimer, A. A., Ed.; CRC: Boca Raton, FL, 1985; Vol. II, pp 37–57.

(31) Marcus, R. A. *J. Chem. Phys.* **1956**, *24*, 966. Miller, J. R.; Beitz, J. V.; Huddleston, R. K. *J. Am. Chem. Soc.* **1984**, *106*, 5057.

(32) Saltiel, J.; Marchand, G. R.; Kirkor-Kaminska, E.; Smothers, W. K.; Mueller, W. B.; Charlton, J. L. *J. Am. Chem. Soc.* **1984**, *106*, 3144. Anderson, R. W.; Hochstrasser, R. M.; Lutz, H.; Scott, G. W. *J. Chem. Phys.* **1974**, *61*, 2500. Wagner, P. J.; Kochevar, I. *J. Am. Chem. Soc.* **1968**, *90*, 2232.

Anexo VI

Two water-soluble fluorescence probes for chemiexcitation studies: sodium 9,10-dibromo- and 9,10-diphenylanthracene-2-sulfonate. Synthesis, proper-ties and application to triplet acetone and tetramethyldioxetane. Catalani, L.H., Wilson, T., Bechara, E.J.H., *Photochem. Photobiol.* **45**, 273 (1987).

Photochemistry and Photobiology Vol. 45, No. 2, pp. 273–281, 1987
Printed in Great Britain.

0031-8655/87 $03.00+0.00

TWO WATER-SOLUBLE FLUORESCENCE PROBES FOR CHEMIEXCITATION STUDIES: SODIUM 9,10-DIBROMO- AND 9,10-DIPHENYLANTHRACENE-2-SULFONATE. SYNTHESIS, PROPERTIES AND APPLICATION TO TRIPLET ACETONE AND TETRAMETHYLDIOXETANE

LUIZ H. CATALANI[1], THÉRÈSE WILSON[2] and ETELVINO J. H. BECHARA[1*]
[1]Instituto de Quimica da Universidade de São Paulo, Caixa Postal 20.780, São Paulo, Brazil and
[2]The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA

(*Received* 29 *April* 1986; *accepted* 15 *July* 1986)

Abstract—The syntheses of sodium 9,10-dibromo- and 9,10-diphenylanthracene-2-sulfonate (DBAS and DPAS, respectively) are described and their photophysical properties determined. These two probes were used in aqueous solution studies of the kinetic parameters of tetramethyldioxetane thermolysis, which were found to be the same as in organic solvents. The yields of triplet and singlet acetone generated by the decomposition of this dioxetane in water are also comparable to the literature values in organic medium. The lifetime of triplet acetone in water was determined to be 13 ± 2 μs by a method based on the measurement of the fluorescence decay of DBAS excited *via* energy transfer from triplet acetone, by the time-correlated single-photon counting technique. Sorbate ion quenches triplet acetone from tetramethyldioxetane with a rate constant smaller but close to the diffusion-controlled limit.

INTRODUCTION

Since Vassil'ev discovered (1963) that anthracenes enhance the weak chemiluminescence emitted by autoxidation reactions, 9,10-dibromo- and 9,10-diphenylanthracene (DBA and DPA†) have been widely used to monitor the presence of chemi-or photoexcited triplet and singlet ketones in organic solvents. The excitation energy of the carbonyl compounds, usually poor emitters, is transferred to these fluorescers which then emit efficiently their own characteristic fluorescence. In the case of DPA, the energy transfer is predominantly of the singlet–singlet type (Eq. 1, where A is the ketone). When this spin-allowed energy transfer is exothermic, the transfer efficiency Φ_{SS} is assumed to be unity; i.e. at infinite concentration of DPA all singlet excited ketone is quenched by DPA with concomitant excitation of its S_1 state. The fluorescence of DBA may result also from collisional energy transfer from a triplet donor (Eq. 2). The efficiency Φ_{TS} (Eq. 4) of this triplet–singlet energy transfer can in fact be quite high.

$$^1A^* + DPA \xrightarrow{k_{SS}} {}^1DPA^* + A \qquad (1)$$

$$^3A^* + DBA \xrightarrow{k_{TS}} {}^1DBA^* + A \qquad (2)$$

$$^3A^* + DBA \xrightarrow{k_{TT}} {}^3DBA^* + A \qquad (3)$$

$$\Phi_{TS} = \frac{k_{TS}}{k_{TS} + k_{TT}} = \frac{k_{TS}}{k_{ET}} \qquad (4)$$

It is possible that it proceeds through the intermediacy of an upper triplet state of DBA located a few kcal above S_1 (Lim *et al.*, 1966; Kearvell and Wilkinson, 1970), followed by intersystem crossing to S_1, a radiationless transition facilitated by the two bromine substituents (Schmidt *et al.*, 1978; however, see also Wu, 1980, and Schmidt *et al.*, 1980). The transfer efficiency Φ_{TS} depends on the donor, the acceptor and the solvent. For example, it is 0.10 for triplet acetophenone–DBA–benzene, ~0.30 for triplet acetone–DBA–benzene, ~0.05 for triplet acetone–DBA–cyclohexane (Wilson and Halpern, 1980a,b).

The DBA–DPA method, which has been extensively used in mechanistic studies of autoxidation and peroxide decomposition, especially dioxetanes and dioxetanones, is unfortunately limited to organic solvents (for reviews, see Wilson, 1976; Adam, 1982; Adam and Zinner, 1982). Thus very little is known about the behavior of dioxetanes and dioxetanones in water, even though such peroxides are considered to be the high-energy intermediates in many bioluminescences (McCapra, 1976) as well as in reactions responsible for "photobiochemistry in the dark" (Cilento, 1984). To circumvent the solubility problem, Cilento and coworkers (1978) introduced the use of water-soluble sodium salts of sulfonated DBA and DPA, DBAS and DPAS, respectively. Recently, Catalani and Bechara (1984) used DBAS to study the quenching of triplet acetone, generated from tetramethyldioxetane in aqueous solution, by biologically important com-

*To whom correspondence should be addressed.

Abbreviations: DBA, 9,10-dibromoanthracene; DBAS, 9,10-dibromoanthracene-2-sulfonate ion; DPA, 9,10-diphenylanthracene; DPAS, 9,10-diphenylanthracene-2-sulfonate ion; TMD, tetramethyldioxetane.

pounds such as indoles, tyrosines and quinones. Yet the syntheses of DBAS and DPAS remain inadequately described in the literature and their photophysical properties have not been fully reported. They will be the main object of this paper. In addition, the usefulness of the DBAS–DPAS method will be illustrated by studies of the lifetime of photoexcited triplet acetone and of the thermal decomposition of tetramethyldioxetane, both in aqueous solutions.

MATERIALS AND METHODS

Reagents

All reagents for synthetic work were used as purchased from Eastman Kodak Co. (Rochester, NY) (9,10-dibromo- and 9,10-diphenylanthracene), Merck ($H2SO_4$.60% SO_3), Aldrich Chemical Co., Inc. (Milwaukee, WI) (2,3-dimethyl-2-butene and 1,2-dibromo-5,5′-dimethylhydantoin), Baker (silver acetate), Sigma Chemical Co. (St. Louis, MO) (2,4-hexadienoic acid, i.e. sorbic acid) and Merck (Na_2EDTA). 9,10-Dibromoanthracene and 9,10-diphenylanthracene were recrystallized from benzene. The organic solvents were of spectroscopic grade (Aldrich and Baker Instra-Analyzed). Water was purified either by double distillation from glass or deionization by Chelex. Nitrobenzene (Carlo Erba) was distilled from P_2O_5.

Syntheses

Tetramethyldioxetane (*TMD*) was prepared according to Kopecky *et al.* (1975). Pure TMD (yellow crystals) was obtained with 9% yield. Stock solutions of TMD in acetonitrile were quantified by GC (Bechara and Wilson, 1980).

Sodium 9,10-dibromoanthracene-2-sulfonate (*DBAS*). One mℓ of H_2SO_4.60% SO_3 (23 mmol SO_3) was added dropwise to a vigorously stirred solution of 4.5 g of DBA (13.4 mmol) in 30 mℓ nitrobenzene at 10°C. The resulting dark green slurry was stirred first for *ca.* 4 h at 50°C and then overnight at room temperature. After neutralization with conc. aqueous NaOH in an ice bath, the nitrobenzene was removed by steam distillation. The residue was extracted 5 times with *ca.* 1 ℓ of boiling water and the combined filtrates let to stand overnight in the refrigerator. Two recrystallizations of the precipitate from boiling water gave 4.0 g of pale yellow crystals (68% yield; calculated for DBAS). TLC runs of these crystals in silica gel–n-butanol revealed traces of a blue-fluorescent contaminant with a much lower R_f (disulfonated DBA?). Further purification by ion exchange column chromatography (Dowex 1 × 1, 50–100 meshes, 10 × 0.8 cm), using a gradient of HCl (0–0.5 *M*) in methanol (200 mℓ total volume) as eluent, removed the contaminant. Twenty-mℓ fractions were collected from the column. Sixty mℓ of water were added to each of the fractions containing DBAS (as indicated by TLC), followed by filtration of the precipitate (yellow crystals) after letting it stand overnight in the refrigerator. Elemental analysis: C, 37.33%; H, 1.81%; Br, 36.91%; S, 7.31% (calculated for $C_{14}H_7O_3Br_2NaS$: C, 38.39%; H, 1.61%; Br, 36.48%; S, 7.32%). ^{1}H-NMR (400 MHz; CD_3OD/d_6-DMSO, 2:1) δ: 7.80, t, 1H; 7.82, t, 1H; 8.03, d, 1H; 8.56, d, 1H; 8.58, d, 1H; 8.60, d, 1H; 8.96, s, 1H. ^{13}C-NMR (100 MHz; CD_3OD/d_6-DMSO, 2:1) δ: 123.20, s; 124.23, s; 124.86, d; 126.33, d; 128.63, d; 128.90, d; 129.02, d; 130.42, s; 130.88, s; 131.45, s; 135.51, s; 146.89, s.

Sodium 9,10-diphenylanthracene-2-sulfonate (*DPAS*). DPA treated with H_2SO_4.60% SO_3 in nitrobenzene according to the same synthetic procedure described above for DBA led to a mixture of mono- and polysubstituted anthracene sulfonic derivatives. The same purification procedure used for DBAS afforded light yellow-green crystals of DPAS.$5H_2O$ in 43% yield. Elemental analysis: C, 60.20%; H, 5.22%; S, 6.06% (calculated for $C_{26}H_{27}O_8SNa$: C, 59.76%; H, 5.21%; S, 6.14%). ^{1}H-NMR (400 MHz; CD_3OD/d_6-DMSO, 2:1) δ: 7.45–7.51, m, 6H; 7.64–7.72, m, 10H; 8.11, s, 1H. ^{13}C-NMR (100 MHz; CD_3OD/d_6-DMSO, 2:1) δ: 123.84, d; 126.12, d; 126.27, d; 127.14, d; 128.41, d; 128.44, d; 129.24, d; 129.81, d; 130.64, s; 130.69, s; 137.46, s; 138.67, s; 138.85, s; 138.97, d; 143.95, s.

Methods

Absorption spectra and extinction coefficients were obtained with a Zeiss DMR 10 or a Cary 219 spectrophotometer and fluorescence spectra with a Perkin–Elmer MPF-4 spectrofluorimeter. Chemiluminescence from solutions containing TMD was measured on either a Hamamatsu C-767 photon counter equipped with Toshiba filters or on a Mitchell–Hastings (1971) type photometer of in-house construction with a 1P28 photomultiplier. GC analyses were performed on a Hewlett–Packard Model 5750 flame ionization chromatograph.

The fluorescence quantum yields Φ_F of both DBAS and DPAS in aqueous medium were determined according to Demas and Crosby (1971), using DPA in benzene as reference (Φ_F = 0.90, at 20°C; Hamai and Hirayama, 1983). Aqueous solutions of these fluorescers (5 μ*M*) were excited at 310 nm (broad valley in the absorption spectrum) and the intensity of the resulting fluorescence, integrated over the entire fluorescence spectrum, compared to that of a DPA/benzene solution. Corrections were made for the differences in the absorbance at 310 nm and the refractive index of the solvent. These Φ_F values were confirmed by a second set of experiments carried out with fluorescer concentrations of 0.5 μ*M*. The fluorescence intensity of DBAS and DPAS decreases with increasing temperature; the corresponding negative activation energies were calculated from the slopes of the Arrhenius plots of the log of the fluorescence quantum yields *vs* $1/T$ (K^{-1}).

Triplet acetone, generated by the thermolysis of TMD, is quenched by sorbate ion. This quenching was studied indirectly by a Stern–Volmer analysis of the effect of sorbate on the fluorescence of 10 μ*M* added DBAS, used to monitor the concentration of triplet ketone. The experiments were carried out in air-equilibrated as well as in nitrogen-purged aqueous solutions of 200 μ*M* TMD. The concentration range of sorbate ion was 30–330 μ*M*. Since singlet excited DBAS is itself weakly quenched by sorbate ion, a correction factor was determined from a Stern–Volmer plot of the fluorescence of *photo*excited DBAS *vs* sorbate concentration. Equations 2–10, where Q is the sorbate ion, describe the reaction system (with DBAS replacing DBA in Eqs. 2 and 3).

$$\text{TMD} \xrightarrow{k} {}^3\text{A}^* + \text{A} \qquad (5)$$

$${}^3\text{A}^* \xrightarrow{k_a} \text{A} \qquad (6)$$

$${}^3\text{A}^* + \text{Q} \xrightarrow{k_q} \text{A} + \text{Q} \qquad (7)$$

$${}^1\text{DBAS}^* + \text{Q} \xrightarrow{k_q} \text{DBAS} + \text{Q} \qquad (8)$$

$${}^1\text{DBAS}^* \xrightarrow{k_F} \text{DBAS} + h\nu \qquad (9)$$

$${}^1\text{DBAS}^* \xrightarrow{k_{III}} \text{DBAS} \qquad (10)$$

Application of the steady-state assumption to the concentrations of triplet acetone $^3A^*$ and of $^1DBAS^*$ gives Eq. 11

$$I_0/I = (1 + k_R\tau_F[Q])(1 + k_q\tau_A[Q]) \quad (11)$$

where I_0 and I are the luminescence intensity of DBAS in the absence and in the presence of sorbate ion, respectively; $\tau_F = 1/(k_F + k_{ic})$ is the lifetime of $^1DBAS^*$ and $\tau_A = 1/(k_A + k_{ET}[DBAS])$ is the lifetime of triplet acetone in the absence of quencher (with $k_{ET} = k_{TS} + k_{TT}$). The rate constant for quenching of $^1DBAS^*$ by Q is k_R; k_A is the rate constant for the decay of triplet ketone by all paths not involving DBAS and Q and k_{ic} is the rate constant of radiationless decay of $^1DBAS^*$.

The intensity of chemiluminescence from aqueous solutions of TMD (80 μM, with 10 μM EDTA) containing either DBAS or DPAS was measured as a function of the concentration of sensitizer. From double-reciprocal plots of the chemiluminescence intensity *vs* concentration of DBAS or DPAS, one can calculate (Eq. 12) the ratio $^3\Phi/^1\Phi$, where $^3\Phi$ is the quantum yield of chemiexcited triplet acetone and $^1\Phi$ that of singlet excited acetone generated by reaction 13 (Wilson, 1976).

$$\frac{^3\Phi}{^1\Phi} = \frac{\gamma^{DPAS}\Phi_{SS}\Phi_F^{DPAS}}{\gamma^{DBAS}\Phi_{TS}\Phi_F^{DBAS}} \quad (12)$$

$$TMD \rightarrow {}^1A^* + A \quad (13)$$

In Eq. 12, γ^{DPAS} and γ^{DBAS} are the reciprocals of the intensities extrapolated to infinite concentration of either DPAS or DBAS, Φ_{SS} and Φ_{TS} are the efficiencies of energy transfer from chemically generated singlet and triplet acetone to DPAS and DBAS, respectively, yielding the first excited singlet state of these fluorescers. The ratio of intercept to slope of each of these double reciprocal plots yields the product $k_{ET}\tau$, where k_{ET} is the rate constant for quenching of either singlet or triplet excited acetone by DPAS or DBAS and τ is the lifetime of the excited acetone donor, either singlet or triplet, in the absence of sensitizer, Eq. 14 (Wilson, 1976).

$$k_{ET}\tau = \frac{\gamma^{DBAS}\,(\text{or } \gamma^{DPAS})}{\text{slope}} \quad (14)$$

Equations 12 and 14 are derived from Eq. 13 and Eqs. 1–10 by making the steady-state assumptions on the concentration of the excited molecules. These experiments were performed in normally aerated solutions at 42°C. In the measurement of intensities *vs* fluorescer concentrations, appropriate cut-off filters (Toshiba *Y*-45 for DPAS and *Y*-43 for DPAS), which selected the long-wavelength tail of the fluorescences, were used in order to minimize errors due to reabsorption at high concentrations. The values of γ^{DPAS} and γ^{DBAS} entered in Eqs. 12 and 14 are the values of Y after correction for the effect of the filters on the fluorescence intensity.

The efficiency Φ_{TS} of triplet–singlet energy transfer from acetone to DBAS (Eq. 4) was determined by the time-correlated single-photon counting method (Wilson and Halpern, 1980a,b; Wilson *et al.*, 1984). The fluorescence decay curves of aqueous solutions of acetone and DBAS, following pulse excitation at 300 nm where both acetone and DBAS absorb, were computer-fitted to double-exponential functions of the type

$$I(t) = \alpha\exp(-k_F t) + \exp(-k_{obs}t) \quad (15)$$

predicted by Eqs. 2–10 (without quencher). k_F is the rate constant of DBAS fluorescence decay (Eq. 9) and

$$k_{obs} = k_A + k_{ET}[DBAS] \quad (16)$$

Thus the fast decay component corresponds to the prompt fluorescence of DBAS, excited by direct absorption, while the slow component corresponds to the fluorescence of DBAS sensitized by triplet acetone. The efficiency of this energy transfer is then given by

$$\Phi_{TS} = \frac{R(k_F - k_{obs})}{(1 + \alpha)k_{ET}[DBAS]} \quad (17)$$

where R is the ratio of absorbances of DBAS and acetone:

$$R = \frac{\epsilon_{DBAS}[DBAS]}{\epsilon_A[A]} \quad (18)$$

(see Wilson *et al.*, 1984). In the single-photon counting experiments, all solutions of acetone and DBAS were nitrogen-purged (20 min); the acetone concentration was 0.27 M (7 data points), 0.9 M (4 points), 0.54 or 1.1 M (1 data point), depending on the experiment, and the DBAS concentration was in the range of 30–300 μM. The temperature was 20°C. The single-photon counting instrumentations used in these experiments, as well as in the determination of the fluorescence lifetime of DBAS in water (single-exponential decay), have been described previously (Wilson and Halpern, 1980a; Wilson *et al.*, 1984).

The τ_F value for DPAS was obtained by the method and equipment of Quina and Toscano (1977). Air-equilibrated aqueous solutions of 50 μM DPAS were excited at 360 nm (deuterium lamp with Corning Glass filter CS-7-60) and the observed fluorescence decay curves compared to those obtained with standard fluorescers (anthracene in ethanol, τ_F = 4.5 ns; quinine sulfate in aqueous 0.10 N HCl, τ_F = 19.2 ns; emission beam isolated by Corning Glass filters: CS-7-54 and CS-7-60 and excitation by CS-7-32).

RESULTS

Syntheses

Several attempts to synthesize DBAS and DPAS in high yields by sulfonation of DBA and DPA in dioxane with SO_3 (according to Koeberg-Telder and Cerfontain, 1972) or in acetic anhydride with H_2SO_4.20% SO_3 (Battegay and Brandt, 1923) failed. The procedure reported here, i.e. sulfonation with H_2SO_4.60% SO_3 in nitrobenzene, proved to be more efficient than those reported in the literature. The identity of the reaction product is based on elemental analysis, 400-MHz ^{1}H-NMR, 100-MHz ^{13}C-NMR, UV and fluorescence spectra, and solubility behavior; both compounds are insoluble in benzene and cyclohexane, partially soluble in water and very soluble in methanol. Stock solutions of 260 μM DBAS and 200 μM DPAS in water (maximum solubility at 25°C) are easily prepared by dissolving a given amount of these salts in a minimum volume of methanol, followed by appropriate dilution with water. The preferential sulfonation of DBA and DPA in β-position is expected in view of the steric hindrance afforded by the 9,10-substituents against electrophilic attack in α-position (Suter, 1934).

Absorption and fluorescence data

Figure 1 shows the absorption and fluorescence spectra of DBAS and DPAS in aqueous solutions, at 20°C. These spectra are very similar to those of DBA and DPA in methanol, exhibiting the same

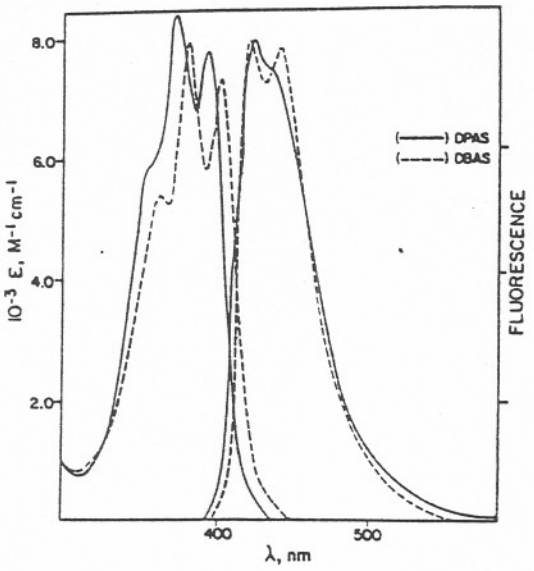


Figure 1. Absorption and fluorescence spectra of DBAS and DPAS in aqueous solution. The fluorescence intensity scale as arbitrary (DBAS or DPAS conc. = 1 μM, λ_{ex} = 310 ± 2 nm). The fluorescence λ_{max} are 426 and 446 nm for DBAS and 428 nm for DPAS.

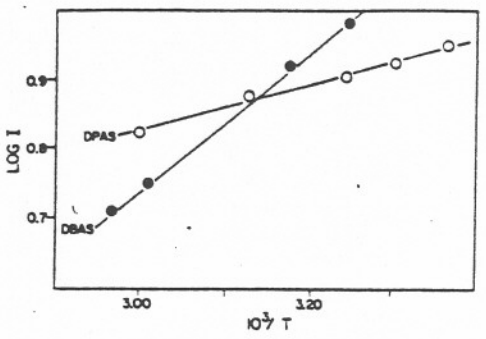


Figure 2. Temperature effect on the quantum yield of DBAS and DPAS in water. DBAS or DPAS conc. = 1 μM; λ_{exc} = 310 ± 2 nm; I is the fluorescence intensity integrated over the whole emission spectrum.

vibrational structure and the expected batochromic shift in the absorption spectra due to the sulfonic substitution. The well-documented negative temperature effect on the quantum yields of DBA and to a smaller extent of DPA (Lim *et al.*, 1966, Kearvell and Wilkinson, 1970; Wilson and Schaap, 1976; Wu and Ware, 1979) is also observed for DBAS and DPAS. The activation energies E_F for these processes were calculated from the slopes of the corresponding Arrhenius plots (Fig. 2). The results are presented in Table 1, which also lists the molar extinction coefficients and the fluorescence quantum yields of DBAS and DPAS in water, as well as corresponding data on DBA and DPA in organic solvents, for comparison.

Energy transfer studies by photoexcitation

The fluorescence decay curves of mixed aqueous solutions of acetone and DBAS could all be fitted to double-exponential functions, as illustrated in Fig. 3. The rate constant corresponding to the prompt decay (directly photoexcited DBAS) was at least 100-fold higher than that of the slow decay component (DBAS excited by energy transfer from triplet acetone). Figure 4 shows the linear dependence of k_{obs} (Eq. 16) on [DBAS]. The slope k_{ET},

Table 1. Photophysical properties of DBA, DPA and their β-sulfonate derivatives

Fluorescer/solvent	ϵ/λ_{max} (M^{-1} cm^{-1}/nm)	Φ_F (20°C)	E_F kJ mol^{-1}	E_F (kcal mol^{-1})	τ_F* (ns)
DBAS/water	7750/405 7950/384 5000/364	0.42	−18.8	(−4.5)	7 ± 1† 7.7 ± 0.1‡
DPAS/water	8050/397 8400/377 6100/361	0.87	−6.7	(−1.6)	10 ± 1†
DBA/acetonitrile		0.16	−20.1	(−4.8)	2.0 ± 0.1‡
DBA/decalin		0.20	−19.2	(−4.6)	1.8 ± 0.1‡§
DBA/benzene		0.20	−18.8	(−4.5‖)	3.8 ± 0.1‡
DPA/benzene		0.90¶	−3.8	(−0.9)	9.2 ± 0.2‡

*In degassed solution.
†By the method of Quina and Toscano (1977).
‡By time-correlated single-photon counting at 20°C.
§Value obtained in cyclohexane instead of decalin.
‖Wilson and Schaap (1971).
¶Hamai and Hirayama (1983).

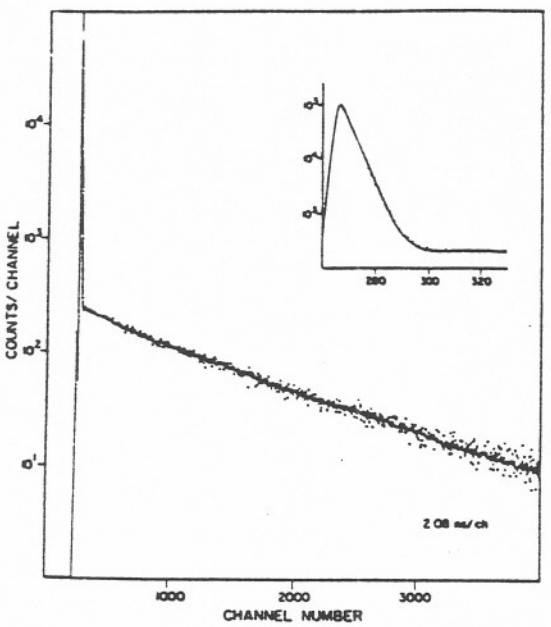


Figure 3. Fluorescence decay curve of a nitrogen-purged aqueous solution of acetone (0.9 *M*) and DBAS (104 μ*M*) at 20°C, λ_{exc} = 305 nm. Fluorescence monitored at 433 ± 20 nm. Inset: same curve, expanded between channels 260 and 320 nm. The full line is the convoluted "best fit" double exponential function corresponding to Eq. 15 with the following parameters: k_F = 1.31 × 10⁸ s⁻¹, k_{obs} = 5.12 × 10⁵ s⁻¹, α = 717 (χ^2 = 1.9).

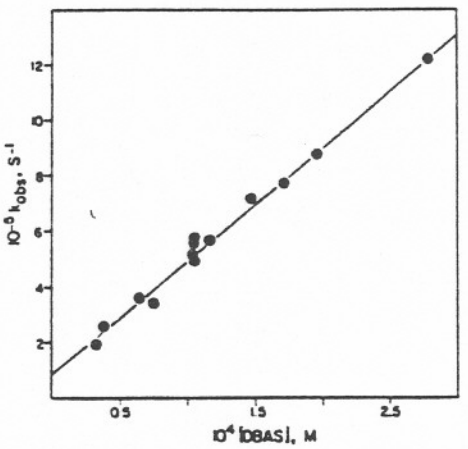


Figure 4. Linear dependence of k_{obs} on DBAS (Eq. 16), in nitrogen-purged aqueous solutions of acetone. Range of acetone conc.: 0.27–1.1 *M* (see Materials and Methods); other experimental conditions as for Fig. 3.

which represents the sum of all bimolecular quenching processes of triplet acetone by DBAS, is 4.1 × 10⁹ M^{-1} s^{-1}. The lifetime of triplet acetone in deaerated water, equal to the reciprocal of the *Y* intercept, is 13 ± 2 μs (Eq. 16). The values of k_{obs} seem not to depend on the concentration of acetone, which was varied between 0.27 and 1.1 *M*. The efficiency of energy transfer, Φ_{TS}, from triplet acetone to DBAS was 0.09 ± 0.01 (Eq. 17). In the absence of acetone, the fluorescence of DBAS decays as a single exponential; two determinations gave τ_F = 7.7 ± 0.1 ns for a 104-μ*M* aqueous solution of DBAS at 20°C.

TMD thermolysis in aqueous medium

In view of the scarcity of information on dioxetanes in water, TMD was investigated with regard to (i) the kinetics of its thermolysis, (ii) the quantum yields and spin-multiplicities of excited products, (iii) the quenching of chemically generated triplet acetone. All solutions contained 10–100 μ*M* EDTA, in order to prevent the non-chemiluminescent decomposition of TMD catalyzed by traces of metal contaminants possibly present in water or buffers (Wilson *et al.*, 1973).

1. *Activation energy for TMD thermolysis.* This was determined by two methods, the "temperature drop" method and the Arrhenius plot of the rate of decomposition of TMD. In the first, the activation energy E_a was calculated from the decrease in chemiluminescence intensity from solutions of TMD (300 μ*M*), containing either DPAS or DBAS (100 μ*M*), when the temperature was rapidly reduced by *ca.* 15°C (see Wilson and Schaap, 1971; Wilson, 1976). In order to obtain the correct value of E_a, the negative temperature coefficient of the fluorescer quantum yield −18.83 kJ mol^{-1} (−4.5 kcal mol^{-1}) for DBAS, and −6.69 kJ mol^{-1} for DPAS, Table 1) was subtracted from the observed activation energy. Table 2 contains the E_a values for TMD thermolysis in water and, for comparison, the corresponding data obtained in decalin and acetonitrile with DBA as fluorescer. Figure 5 shows the Arrhenius plot of the observed chemiluminescence decay rates of TMD thermolysis. The line is taken from Wilson *et al.* (1976) and corresponds to E_a = 115.5 kJ mol^{-1}; it was based on measurements in three solvents (benzene, xylenes and acetonitrile).

2. *Ratio $^3\Phi/^1\Phi$ from TMD.* Figure 6 shows the double reciprocal plots of the DBAS and DPAS sensitized chemiluminescence intensities of TMD thermolysis in aqueous medium *vs* fluorescer concentration. From Eq. 12 the ratio $^3\Phi/^1\Phi$ was found to be *ca.* 10^3, assuming Φ_{ss} = 1. In the case of DBAS, the product $k_{ET}\tau$ (Eq. 14), in which τ is the lifetime of the energy donor, here triplet acetone, was found to be 7.4 × 10^3 M^{-1} at 42°C.

3. *Sorbate quenching.* Quenching studies were carried out in water using sorbate ion as quencher and triplet acetone from TMD thermolysis as donor; triplet acetone was monitored by DBAS, as described in Materials and Methods. Stern–Volmer plots were established at 35°C; the concentrations of TMD and DBAS were 200 and 10 μ*M* respectively. The values of the product $k_q\tau_A$ (Eq. 11) so obtained were 4.7 × 10^3 M^{-1} in aerated solution and 3.4 × 10^4 M^{-1} in nitrogen-purged solution. Note that τ is slightly larger than τ_A since τ_A is determined in the presence of a low concentration of DBAS; see Methods.

Table 2. Activation energy for TMD thermolysis based on temperature-drop experiments

Solvent	Fluorescer	Temperature range	E_a kJ mol^{-1}	E_a (kcal mol^{-1})
Water	DBAS (100 μM) or DPAS (120 μM)	37–76°C	116.3 ± 2.0	(27.8 ± 0.5)
Acetonitrile	DBA (70 μM)	31–80°C	113.8 ± 2.0	(27.2 ± 0.5)
Decalin	DBA (100 μM)	31–50°C	112.5 ± 2.0	(26.9 ± 0.5)

*Water contained 500 μM EDTA and the organic solvents were saturated with Na_2EDTA. The concentration of TMD was 300 μM in water, 230 μM in acetonitrile and 300 μM in decalin.

DISCUSSION

DBAS and DPAS as water-soluble fluorescers

Sulfonation of 9,10-dibromoanthracene results in a water-soluble fluorescer which, like DBA, proves to be an especially useful probe for the investigation of aqueous reaction systems in which triplet excited states are generated; this is due to its ability to accept triplet energy and re-emit it as prompt fluorescence. Lacking heavy-atom substituents, DPAS, like DPA, is essentially an acceptor of singlet energy in energy transfer processes. Nevertheless, because of its high fluorescence quantum yield, the addition of DPAS results in an enhancement of the weak chemiluminescence emitted by singlet excited carbonyl species which have very low quantum yields of fluorescence. Therefore the pair of fluorescers DBAS/DPAS extends to aqueous medium the well-established usefulness of DBA and DPA for recognizing the generation of triplet products and estimating the ratio of triplet to singlet excited species generated by a chemical or enzymatic reaction. It should be kept in mind, however, that DPAS and DPA are quite good electron donors and therefore might play an active catalytic role in some chemiluminescence systems, as recognized by McCapra (1977), Zaklika *et al.* (1979) and, especially, Schuster and coworkers (1980, 1982). This possible complication in the interpretation of results obtained with DPA (or DPAS) has been discussed recently (Wilson, 1985).

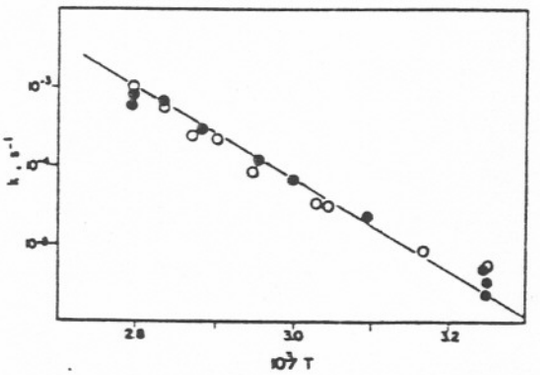


Figure 5. Arrhenius plot of the first-order rate constant k (Eq. 5) of TMD thermolysis, monitored by the decay of chemiluminescence intensity in aqueous solution with (●) or without (○) 100 μM DBAS. All solutions contained 10 μM EDTA; in addition, the bidistilled water was treated with Chelex to remove all possible traces of metal impurities. Without Chelex pretreatment the values of k are much higher and scattered. The line is the fit to data previously obtained in benzene, xylenes and acetonitrile (Wilson *et al.*, 1976).

Like that of DBA, the fluorescence quantum yield of DBAS has a negative temperature coefficient. The corresponding activation energy E_F is interpreted as the difference between the lowest vibrational level of S_1 and a higher vibrational level from which intersystem crossing to a higher triplet state (T_2 or T_3) can rapidly occur (see Wu and Ware, 1980; Gillispie and Lim, 1979 and references therein). The value of E_F obtained here for DBAS in water is similar to the E_F values for DBA in acetonitrile and decalin (Table 1) as well as to literature values in other solvents (Schmidt *et al.*, 1978; Kearvell and Wilkinson, 1970; Wu and Ware 1980). The fluorescence quantum yield of DBAS in water is about twice as large as that of DBA in the three organic solvent studied here; since the E_F are about the same, this would seem to indicate that intersystem crossing is slower in DBAS than in DBA at 20°C, in keeping with the longer fluorescence lifetime of DBAS (Table 1).

TMD thermolysis in aqueous medium

The mean literature value of the activation energy for the thermolysis of TMD in organic solvents (aliphatic, aromatic and hydroxilic) is 110.9 ± 5.4 kJ mol^{-1} (Adam and Zinner, 1982; 5 of the 10 literature values fall between 113 and 116 kJ mol^{-1}). Table 2 and Fig. 5 show that the values of E_a measured here in water, in acetonitrile and in decalin, all fall in the same range. Thus the activation energy required for the cleavage of this dioxetane is remarkably independent of the solvent properties.

The same seems true also of the ratio $^3\Phi/^1\Phi$.

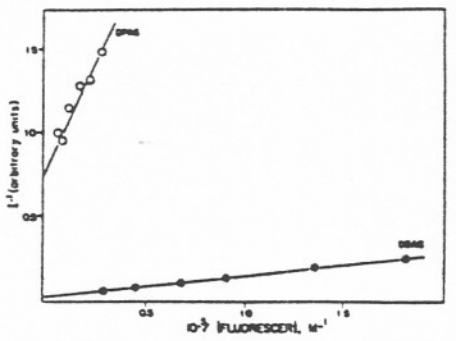


Figure 6. Effect of DBAS or DPAS concentration on the initial intensity of chemiluminescence from TMD thermolysis in aqueous solution, at 42°C. TMD conc. = 80 μM, in bidistilled water containing 100 μM EDTA.

Within the assumptions of the DBAS/DPAS method and the uncertainties attached to the determination of the intercepts of the plots of Fig. 6, it appears that in water TMD generates two orders of magnitude more excited triplet acetone than excited singlet acetone, very much as it has been found for most dioxetanes in organic solvents by several methods (Adam, 1982; Wilson, 1976 and references therein). This clearly remains one of the most fascinating aspects of dioxetane chemistry.

Lifetime of triplet acetone in water

From the time-correlated single-photon counting experiments with DBAS, the lifetime of triplet acetone in deaerated water was found here to be 13 ± 2 μs at 20°C. Although shorter than the value of Porter *et al.* (1973) of 20 μs at 23°C, it is significantly longer than three more recent values, which are as follows: 4.6 μs, at 25°C (Encinas *et al.*, 1980); 5 μs, temperature not indicated (Kasama *et al.*, 1982); 4.2 μs at 27°C (Leigh and Scaiano, 1983). In Porter's experiments, triplet acetone was created by flash excitation and its decay followed in emission (acetone phosphorescence). In contrast, the three other studies used pulsed-laser excitation and triplet–triplet absorption to monitor the transient decay. Commenting on the discrepancy between their 4.6-μs lifetime and the much longer lifetime obtained by Porter, Encinas and coworkers speculated that in their own work, "the high laser power and small reaction volume might have induced some triplet–triplet annihilation in the case of the long-lived triplet state of acetone". This complication may also explain the short lifetimes reported by Kasama *et al.* and by Leigh and Scaiano. In the work reported here, bimolecular events between excited molecules can safely be disregarded. This is one of the advantages of the single-photon counting method.

Two series of experiments with TMD as the source of triplet acetone bear on its lifetime in aqueous solution: the study of energy transfer to DBAS, which yields $k_{ET}\tau$ (Eq. 14) and the quenching by sorbate ion, from which the product $k_q\tau_A$ is extracted (Eq. 11). In the latter case, the Stern–Volmer plot obtained in nitrogen-purged solution gave $k_q\tau_A = 3.4 \times 10^4\ M^{-1}$ at 35°C. If the rate of decay of triplet acetone in water increases with temperature with an activation energy of *ca* 4.5 kcal mol^{-1}, as found by Porter *et al.* (1973) in acetonitrile, one may assume that increasing the temperature from 20 to 35°C reduces τ_A from 13 to 9 μs. This then gives $k_q \simeq 3.8 \times 10^9\ M^{-1}\ s^{-1}$ at 35°C, a rate constant smaller than the diffusion-controlled limit but well within the range of literature values for the quenching of triplet carbonyls by dienes (see, for example, Turro and Tanimoto, 1980).

In air-equilibrated solutions, still at 35°C, the sorbate quenching experiments gave $k_q\tau_A = 4.7 \times 10^3\ M^{-1}$, which indicates an oxygen-limited lifetime of triplet acetone of 1.2 μs. Taking the oxygen concentration of water as 0.2 mM, one calculates that the rate of oxygen quenching of triplet acetone in water is *ca.* $3.6 \times 10^9\ M^{-1}\ s^{-1}$ at 35°C, consistent with expectation.

The results of the energy transfer experiments in aerated solutions at 45°C can be treated in the same manner. First, for a deaerated solution, the assumption made above yields $\tau \simeq 7$ μs at 45°C. The presence of oxygen should reduce this to ~1.2 μs. Thus since $k_{ET}\tau = 7.4 \times 10^3\ M^{-1}$, one estimates $k_{ET} \simeq 6.2 \times 10^9\ M^{-1}\ s^{-1}$ at 45°C. This value of k_{ET} compares well with $k_{ET} = 5.8 \times 10^9\ M^{-1}\ s^{-1}$, previously obtained at 20°C in cyclohexane, a solvent of viscosity quite similar to that of water (Wilson and Halpern, 1980b).

CONCLUSIONS

DBAS and DPAS are two water-soluble fluorescers which extend the application of the often-used DBA and DPA to the study of excited carbonyl compounds in aqueous medium. A first example of the application of these sulfonated anthracenes shows that the properties of tetramethyldioxetane appear to be the same in water as in a variety of organic solvents, both with regard to its kinetic parameters and to the high yield of triplet acetone it generates.

In another application of DBAS, the lifetime of triplet acetone in water was determined photophysically to be 13 ± 2 μs, a value within the range of literature data. Triplet acetone is expected to be quenched by both DBAS and the diene sorbate *via* exothermic energy transfer. In water, k_{ET} and k_q are indeed estimated to be close to the diffusion-controlled limit.

Acknowledgements—The authors at São Paulo University wish to thank the Fundacão de Amparo a Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Cientifico e Technologico (CNPq),

the Volkswagenwerk Stiftung and the Organization of American States for their generous support of this research. The work at Harvard was supported by the National Science Foundation (Grant CHE-8209863). Some of the fluorescence lifetime measurements were done at Northeastern University; we thank Professor A. M. Halpern for making his instrument available to us.

REFERENCES

Adam, W. (1982) Determination of chemiexcitation yields in the thermal generation of electronic excitation from 1,2-dioxetanes. In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 115–152. Academic Press, New York.

Adam, W. and K. Zinner (1982) Determination of activation parameters and the thermal stability of 1,2-dioxetanes. In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 153–189. Academic Press, New York.

Battegay, M. and P. Brandt (1923) Les acides anthracenemonosulfoniques. Sulfonation d'hydrocarbures en milieu basique ou neutre. *Bull. Soc. Chim. Fr.* 33, 1667–1678.

Bechara, E. J. H. and T. Wilson (1980) Alkyl substituent effects on dioxetane properties. Tetraethyl-, dicyclohexylidene-, and 3,4-dimethyl-3,4-di-*n*-butyldioxetanes. A discussion of decomposition mechanisms. *J. Org. Chem.* 45, 5261–5268.

Catalani, L. H. and E. J. H. Bechara (1984) Quenching of chemiexcited triplet acetone by biologically important compounds in aqueous medium. *Photochem. Photobiol.* 39, 823–830.

Cheng, C.-C. (1979) M.Sc. thesis, University of Puerto Rico, Rio Piedras, Puerto Rico, USA.

Cilento, G. (1984) Generation of electronically excited species in biochemical systems. *Pure Appl. Chem.* 56, 1179–1190.

Cilento, G., N. Duran, K. Zinner, C. C. C. Vidigal, O. M. M. Faria Oliveira, M. Haun, A. Faljoni, O. Augusto, R. C. de Baptista and E. J. H. Bechara (1978) Chemienergized species in peroxidase systems. *Photochem. Photobiol.* 28, 445–451.

Demas, J. N. and G. A. Crosby (1971) The measurement of photoluminescence quantum yields. A review. *J. Phys. Chem.* 75, 991–1024.

Encinas, M. V., E. A. Lissi and J. C. Scaiano (1980) Photochemistry of aliphatic ketones in polar solvents. *J. Phys. Chem.* 84, 948–951.

Engel, P. S. and B. M. Monroe (1971) Complications in photosensitized reactions. *Adv. Photochem.* 8, 245–313.

Hamai, S. and F. Hirayama (1983) Actinometric determination of absolute fluorescence quantum yields. *J. Phys. Chem.* 87, 83–89.

Kasama, K., A. Takematsu and S. Arai (1983) Photochemical reactions of triplet acetone with indole, purine and pyrimidine derivatives. *J. Phys. Chem.* 86, 2640–2417.

Kearvell, A. and F. Wilkinson (1970) Temperature dependence of non-radiative transitions. *Transitions Non Radiat. Mol., J. Chim. Phys.* (special edition) pp. 125–131.

Koeberg-Telder, A. and H. Cerfontain (1972) Aromatic sulfonation. Part 30. Sulfonation of anthracene with sulfur trioxide–dioxane; slow proton transfer in the formation of anthracene sulfonic acid. *Recl. Trav. Chim. Pays-Bas* 91, 22–32.

Kopecky, K. R. and J. E. Filby (1979) Yields of excited states from thermolysis of some 1,2-dioxetanes. *Can. J. Chem.* 57, 283–288.

Kopecky, K. R., J. E. Filby, C. Munford, P. A. Lockwood and J.-Y. Ding (1975) Preparation and thermolysis of some 1,2-dioxetanes. *Can. J. Chem.* 53, 1103–1122.

Leigh, W. J. and J. C. Scaiano (1983) Photochemistry of acetone in surfactant solutions. *J. Am. Chem. Soc.* 105, 5652–5657.

Lim, E. C., J. D. Laposa and J. M. H. Yu (1966) Temperature dependence of intersystem crossing in substituted anthracenes. *J. Mol. Spectrosc.* 49, 412–420.

Loufty, R. O., R. W. Yip and S. K. Dogra (1977) The triplet state of ketones in solution, quenching of triplet acetone by olefins. *Tetrahedron Lett.* 2843–2846.

McCapra, F. (1976) Chemical mechanisms in bioluminescence. *Acc. Chem. Res.* 9, 201–208.

McCapra, F. (1977) Alternative mechanism for dioxetan decomposition. *J. Chem. Soc. Chem. Commun.* 946–948.

Melhuish, W. H. (1961) Quantum efficiencies of fluorescence of organic substances: effect of solvent and concentration of the fluorescent solute. *J. Phys. Chem.* 65, 229–235.

Mitchell, G. W. and J. W. Hastings (1971) A stable, inexpensive, solid-state photomultiplier photometer. *Anal. Biochem.* 39, 243–250.

Porter, G., S. K. Dogra, R. O. Loufty, S. E. Sugamori and R. W. Yip (1973) Triplet state of acetone in solution. *J. Chem. Soc. Trans. Faraday I* 69, 1462–1474.

Quina, F. H. (1982) Photophysical concepts in condensed media. In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 1–36. Academic Press, New York.

Quina, F. H. and V. G. Toscano (1977) Photophenomena in surfactant media. Quenching of water-soluble fluorescence probe by iodide ion in micellar solutions of sodium dodecyl sulfate. *J. Phys. Chem.* 81, 1750–1754.

Schmidt, R., H.-D. Brauer and H. Kelm (1978) The energy transfer from triplet state acetone to 9-bromoanthracene and 9,10-dichloroanthracene: an investigation under high pressure. *J. Photochem.* 8, 217–231.

Schmidt, R., H. Kelm and H.-D. Brauer (1980) Reply to comments on "The energy transfer from triplet state acetone to 9-bromoanthracene and 9,10-dichloroanthracene: an investigation under high pressure". *J. Photochem.* 14, 261–263.

Schuster, G. B., B. Dixon, J.-Y. Koo, S. P. Schmidt and J. P. Smith (1979) Chemical mechanisms of chemi- and bioluminescence. Reactions of high energy content organic compounds. *Photochem. Photobiol.* 30, 17–26.

Schuster, G. B. and K. A. Horn (1982) Chemically initiated electron-exchanged luminescence. In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento), pp. 229–247. Academic Press, New York.

Suter, C. M. (1934) *The Organic Chemistry of Sulfur*, p. 302. John Wiley, New York.

Turro, N. J. and Y. Tanimoto (1980) Quenching of acetone triplets by 1,3-dienes in fluid solution. *J. Photochem.* 14, 199–203.

Vassil'ev, R. F. (1963) Spin-orbit coupling and intermolecular energy transfer. *Nature* 200, 773–774.

Wilson, T. (1976) Chemiluminescence in the liquid phase, thermal cleavage of dioxetanes. In *Chemical Kinetics* (Edited by D. R. Herschbach), M.T.P. Int. Rev. Sci.: Phys. Chem. Ser. Two. Vol 9, pp. 265–322. Butterworth, London.

Wilson, T. (1985) Mechanisms of peroxide chemiluminescence. In *Singlet Oxygen*, Vol. II (Edited by A. A. Frimer), pp. 37–65. CRC Press, Inc., Boca Raton, FL.

Wilson, T., S. L. Frye and A. M. Halpern (1984) Energy transfer study of a triplet exciplex of cyclohexanone and mesitylene. *J. Am. Chem. Soc.* 106, 3600–3606.

Wilson, T., D. E. Golan, M. S. Harris and A. L. Baumstark (1976) Thermolysis of tetramethoxy- and *p*-dioxenedioxetanes. Kinetic parameters, chemi-

luminescence, and yields of excited products. *J. Am. Chem. Soc.* **98**, 1086–1091.

Wilson, T. and A. M. Halpern (1980a) A kinetic study of sensitized 9,10-dibromoanthracene fluorescence produced by energy transfer from triplet ketones. 1. Acetophenone as donor. *J. Am. Chem. Soc.* **102**, 7272–7279.

Wilson, T. and A. M. Halpern (1980b) A kinetic study of sensitized 9,10-dibromoanthracene fluorescence produced by energy transfer from triplet ketones. 2. Acetone as donor. *J. Am. Chem. Soc.* **102**, 7279–7283.

Wilson, T., M. E. Landis, A. L. Baumstark and P. D. Bartlett (1973) "Solvent effects" on the chemiluminescent decomposition of tetramethyl-1,2-dioxetane. Competitive dark pathways. *J. Am. Chem. Soc.* **95**, 4765–4766.

Wilson, T. and A. P. Schaap (1971) The chemiluminescence from *cis*-diethoxy-1,2-dioxetane. An unexpected effect of oxygen. *J. Am. Chem. Soc.* **93**, 4126–4136.

Wu, K.-C. (1980) Comments on 'The energy transfer from triplet state acetone to 9-bromoanthracene and 9,10-dichloroanthracene: an investigation under high pressure'. *J. Photochem.* **12**, 363–365.

Wu, K.-C. and W. R. Ware (1979) Solvent effects on the photophysics of dibromoanthracene. *J. Am. Chem. Soc.* **101**, 5906–5909.

Zaklika, K. A., T. Kissel, A. L. Thayer, P. A. Burns and A. P. Schaap (1979) Mechanism of 1,2-dioxetane decomposition: the role of electron transfer. *Photochem. Photobiol.* **30**, 35–44.

Anexo VII

Quenching of triplet acetone by mesitylene and durene: exciplex formation or energy transfer? Indig, G.L., Catalani, L.H., Wilson, T. , *J. Phys. Chem.*, **96**, 8967 (1992).

Quenching of Triplet Acetone by Mesitylene and Durene: Exciplex Formation or Energy Transfer?

G. L. Indig,† L. H. Catalani,‡ and T. Wilson*

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 *(Received: July 14, 1992)*

Triplet acetone interacts with mesitylene or durene (in solution at 20 °C) to generate new triplet transients. These transients were followed by time-resolved single-photon counting, using the fluorescence of 9,10-dibromoanthracene as a triplet probe in acetonitrile and in five aliphatic hydrocarbons. Based on kinetic considerations alone, the new transient could be either a triplet exciplex or the triplet of the methylbenzene, excited by energy transfer from triplet acetone (see excited states diagram, Figure 8). Solvent polarity has no effect on the rate of transient formation, which varies by a factor of ≲2 from acetonitrile to cyclohexane, is 2–3 orders of magnitude below the diffusion controlled limit, and correlates only with the polarizability of the solvent. This result and the fact that the lifetimes of the transients are compatible with that of the triplet methylbenzenes favor the energy-transfer interpretation of the data. On the other hand, earlier observations with xylene and benzene, where triplet energy transfer would be significantly endothermic, point toward the exciplex interpretation. Attempts at comparing the absorption spectrum of triplet durene with the spectrum previously assigned to the triplet exciplex of durene and acetone[2d] have so far been inconclusive. Definitive proof of quenching via exciplex or via energy transfer is therefore not available now. Both models are discussed.

Introduction

In a discussion of exchange energy transfer, Turro considered two cases, based on whether the two electrons are exchanged simultaneously or succesively between donor and acceptor.[1] If the process is stepwise, he suggested that it may involve the intermediacy of a stabilized collision complex on the excited-state surface or even an exciplex. Transient species generated when alkanones are excited to their triplet states in the presence of methylbenzenes were recently interpreted as examples of such excited complexes.[2]

This conclusion was based on two lines of evidence. The more direct clue is the appearance of a novel absorption transient in solutions of acetone and durene irradiated at 308 nm in acetonitrile.[2d] The diffusion-controlled quenching of this transient, by dienes for example, established that it is an electronically excited species. More extensive though less direct evidence comes from experiments with a fluorescence probe, 9,10-dibromoanthracene (DBA).[2] The main observations are as follow. When an alkanone is pulse-photoexcited in the presence of DBA (at λ_{ex} = 305 nm, where both DBA and the ketone absorb), DBA(S_1) can be generated via direct excitation or via energy transfer from triplet alkanone, which is an efficient process. As a result, the decay of the blue fluorescence of DBA follows double-exponential functions, with two very different time constants: ca. 2 ns for the prompt fluorescence and up to microseconds for the emission component sensitized by triplet alkanone. However, if a methylbenzene is present in the same degassed solutions, a third decay component of negative amplitude is observed, which reflects the buildup and decay of a new species, heretofore assigned to a triplet exciplex of alkanone and methylbenzene. Since the lifetime of DBA(S_1) is very short, the kinetics of its fluorescence, monitored by time-resolved single-photon counting, follow the kinetics of the triplet energy donors with no significant lag. The data obtained by triplet–triplet absorption and by the indirect DBA method are in agreement regarding the rate of formation of the new transient.[2d]

In all systems studied so far, this rate is 2 or 3 orders of magnitude lower than the diffusion-controlled limit.[2c,d] If the new species is indeed an exciplex, this result is intriguing since singlet exciplexes and excimers generally form at or near the diffusion-controlled rate.[3] The long lifetimes of the transients, indicative of slow rates of exciplex decomposition, are equally surprising.

To check on the validity of the exciplex assignment, we investigated solvent effects. The results, reported here, show no effect of solvent polarity on the rate of formation of the transients; this rate correlates only with the polarizability of the solvent. In all cases, the decay of the transients is so slow that reliable information on the effect of solvent on their lifetime can unfortunately not be obtained.

TABLE I: Effect of Solvent Viscosity on k_d^A and k_{ET}^A [a]

solvent	η, cP	k_d^A, 10^6 s^{-1}	k_{ET}^A, 10^9 M^{-1} s^{-1}
acetonitrile	0.360	0.02	9.2
n-hexane	0.312	1.3	8.0
cyclohexane	0.977	2.4	8.5
n-dodecane	1.503	1.8	6.7

[a] See eq 13. [acetone] = 0.07 M.

TABLE II: Solvent Effects on the Kinetic Parameters Related to the Quenching of Triplet Acetone by Mesitylene (via Exciplex Formation or Energy Transfer)[a]

solvent	k_1^0, 10^6 s^{-1}	k_2^0, 10^6 s^{-1}	k_q (or k_{eq}), 10^6 M^{-1} s^{-1}	k_{EA} (or k_{et}), 10^7 M^{-1} s^{-1}	k_{AE} (or k_{-et}[A]), 10^5 s^{-1}
acetonitrile	1.4	3.1	2.6	3.6	1.5
n-hexane	1.7	3.6	2.4	4.7	3.7
cyclohexane	1.6	2.0	3.2	6.4	*b*
n-dodecane	1.8	1.8	4.2	5.9	0.1

[a] [DBA] = 1.70×10^{-4} M; [acetone] = 0.07 M. [b] Too small to estimate.

These results are not easily understood in the framework of the exciplex hypothesis. At the time of these studies, an alternative interpretation of the data needed reevaluation, namely, that the transients were simply the triplets of the methylbenzenes, excited via energy transfer from the triplet ketones. One of the arguments used earlier for rejecting this possibility had been the belief, based on the literature, that because of self-quenching the lifetimes of triplet methylbenzenes would be too short to be compatible with the microsecond lifetimes of the transients.[2d] This, in fact, turns out to be false, at least in the case of triplet mesitylene and durene whose long lifetimes were recently determined by the DBA technique.[4]

The experimental data reported here, all obtained by the DBA method, will be discussed from two viewpoints: formation of either triplet exciplexes ($^3E^*$) or triplet methylbenzenes ($^3B^*$). Neither interpretation of the data is entirely satisfactory. Nevertheless, the case for triplet exciplexes of alkanones and methylbenzenes is clearly resting on shakier grounds than we previously thought.

The Kinetic Scheme. Following pulse excitation of acetone and DBA at 305 nm in degassed solution, the processes listed in Scheme I need be considered. Depending on whether $^3E^*$ or $^3B^*$ is generated, either eq 4a or eq 4b describes the kinetics. Similarly,

† Present address: Department of Chemistry, Boston University, Boston, MA 02215.

‡ Permanent address: Instituto de Quimica, Universidade de São Paulo, C.P. 20.780, São Paulo 01498, Brazil.

either reactions 7a or 7b, 8a or 8b, 9a or 9b, and 10a or 10b will obtain.

SCHEME I

$$A + h\nu \rightarrow {}^1A^* \xrightarrow{k_{isc}} {}^3A^* \quad (1)$$

$$DBA + h\nu \rightarrow {}^1DBA^* \quad (2)$$

$${}^3A^* \xrightarrow{k_d^A} A \quad (3)$$

$${}^3A^* + B \xrightarrow{k_{EA}} {}^3E^* \quad (4a)$$

$${}^3A^* + B \xrightarrow{k_{\alpha}} {}^3B^* + A \quad (4b)$$

$${}^3A^* + DBA \xrightarrow{\phi_{TS}^A k_{ET}^A} {}^1DBA^* + A \quad (5)$$

$${}^3A^* + DBA \xrightarrow{(1-\phi_{TS}^A)k_{ET}^A} {}^3DBA + A \quad (6)$$

$${}^3E^* \xrightarrow{k_{AE}} {}^3A^* + B \quad (7a)$$

$${}^3B^* + A \xrightarrow{k_{-\alpha}} {}^3A^* + B \quad (7b)$$

$${}^3E^* \xrightarrow{k_d^E} A + B \quad (8a)$$

$${}^3B^* \xrightarrow{k_d^B} B \quad (8b)$$

$${}^3E^* + DBA \xrightarrow{\phi_{TS}^E k_{ET}^E} {}^1DBA^* + A + B \quad (9a)$$

$${}^3B^* + DBA \xrightarrow{\phi_{TS}^B k_{ET}^B} {}^1DBA^* + B \quad (9b)$$

$${}^3E^* + DBA \xrightarrow{(1-\phi_{TS}^E)k_{ET}^E} {}^3DBA + A + B \quad (10a)$$

$${}^3B^* + DBA \xrightarrow{(1-\phi_{TS}^B)k_{ET}^B} {}^3DBA + B \quad (10b)$$

$${}^1DBA^* \xrightarrow{k_F} DBA + h\nu \quad (11)$$

Experimental Section

Durene (Aldrich) was recrystallized from acetonitrile, mesitylene (Fisher) was distilled, and 9,10-dibromoanthracene was recrystallized from benzene and vacuum sublimed. The purity of durene was assessed by ^{1}H NMR spectroscopy (200 MHz) and that of mesitylene by both gas chromatography and NMR.

Acetone (Mallinckrodt, A. R.), acetonitrile, cyclohexane, and isooctane (Baxter, B&J brand, High Purity Solvent), *n*-hexane (Aldrich, "Spectrograde"), *n*-dodecane (Kodak, "Spectrograde"), and cyclopentane (Kodak) were used as received.

For the fluorescence decay measurements (by time-resolved single-photon counting) the samples were degassed by four freeze–pump–thaw cycles. All experiments were performed at 20 °C. The instrument has been described previously;[2c] the decay curves were analyzed by an iterative reconvolution program developed by S. Frye at Northeastern University and run on IBM PS/2. Absorption spectra and extinction coefficients were obtained with a Uvikon 820 (KONTRON) spectrophotometer. The phosphorescence spectra of acetone and durene were obtained with a Perkin-Elmer spectrofluorimeter Model LS-5. An Applied Photophysics laser flash photolysis instrument was used to record the triplet–triplet absorption of durene, following excitation at 266 nm (fourth harmonic of a Nd:Yag Spectron Laser Systems).

Results

I. Solvent Effects on Energy Transfer from Triplet Acetone to DBA, in the Absence of Mesitylene or Durene. Figure 1 shows typical double-exponential decay (eq 12) of the intensity of DBA fluorescence, I_t, upon 305-nm pulse excitation of acetone and DBA in degassed *n*-hexane solution.

$$I_t = \alpha \exp(-k_F t) + \exp(-k_{obs} t) \quad (12)$$

The slow component (k_{obs}) corresponds to sensitization of DBA fluorescence by triplet acetone (eq 5), via the intermediacy of a higher triplet state of DBA (most likely the T_n located 4 kcal above S_1), and intersystem crossing from T_n to S_1.[5] Since A(T_1) is ca. 4–6 kcal above DBA(T_n), this energy transfer, eq 5, is irreversible. In the four solvents studied, the results fit eq 13. The rate

$$k_{obs} = k_d^A + k_{ET}^A[DBA] \quad (13)$$

constants of decay of triplet acetone, k_d^A, and of energy transfer to DBA, k_{ET}^A, are listed in Table I. k_{ET}^A approaches k_{dif} (calculated according to Smoluchowski equation[6]) when the viscosity increases;[7] see Figure 2.

The lifetime of triplet acetone ($1/k_d^A$) in the four solvents is also much as expected, being the longest in acetonitrile and the shortest in cyclohexane (Table I). In these two solvents as well as in *n*-hexane, the values of k_d^A are close to those determined nearly 20 years ago by Porter et al.[8] The relative values of k_d in the three alkanes reflect quenching of $^3A^*$ via H abstraction from solvent.[9]

II. Effect of Mesitylene and Durene on Energy Transfer from $^3A^*$ to DBA; Solvent Effects. a. Determination of the Rate Constants of Scheme I. The presence of the methylbenzenes in concentrations from 0.01 to 0.4 M always brings up a negative component of decay, as predicted by Scheme I. The decay curves (Figure 3) can be looked at as the superposition of the prompt fluorescence of DBA (resulting from direct excitation), upon the DBA emission sensitized by either $^3A^*$, $^3E^*$, or $^3B^*$. The curves show the buildup and decay of a new triplet species, $^3E^*$ or $^3B^*$, which acts as an energy donor to DBA, eq 9a or 9b. The fit of the data to triple exponential functions (eq 14) is excellent in all solvents.

$$I_t = \alpha \exp(-k_F t) - \beta \exp(-\lambda_a t) + \exp(-\lambda_b t) \quad (14)$$

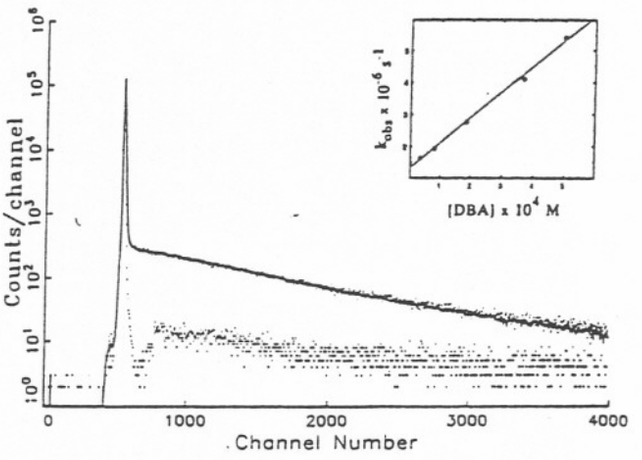


Figure 1. Fluorescence decay curve of a degassed *n*-hexane solution of acetone (0.07 M) and DBA (1.85×10^{-4} M) at 20 °C; λ_{exc} = 305 nm, fluorescence monitored at 433 ± 20 nm. The time scale is 0.418 ns per channel. Also shown is the lamp profile (bottom). The plot shows only one data point in four with even spacing between points. The convoluted "best fit" to eq 12 gives α = 1245, $k_f = 6.29 \times 10^8$ s^{-1}, $k_{obs} = 2.72 \times 10^6$ s^{-1}, and χ^2 = 1.170. Inset: Rate of "slow" decay of DBA fluorescence, k_{obs}, as a function of concentration of DBA.

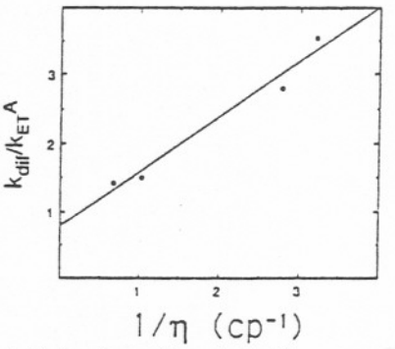


Figure 2. Effect of viscosity on the ratio k_{dif}/k_{ET}.

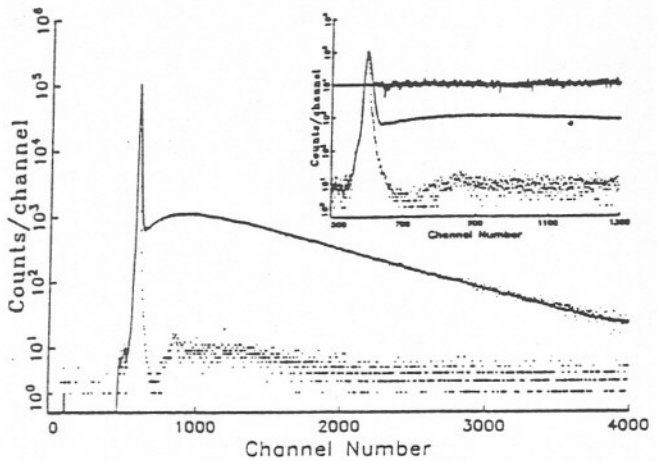


Figure 3. Fluorescence decay curve of a degassed *n*-hexane solution of acetone (0.07 M), DBA (1.70×10^{-4} M), and mesitylene (0.177 M) at 20 °C; λ_{exc} = 305 nm; fluorescence monitored at 433 ± 20 nm. The time scale is 0.418 ns per channel. The solid line is the convoluted best fit to eq 14 with α = 96.4, β = −0.875, $k_f = 5.36 \times 10^8$ s^{-1}, $\lambda_a = 1.05 \times 10^7$ s^{-1}, $\lambda_b = 3.90 \times 10^6$ s^{-1}, and χ^2 = 1.039. Inset shows all data points between channels 500 and 1300.

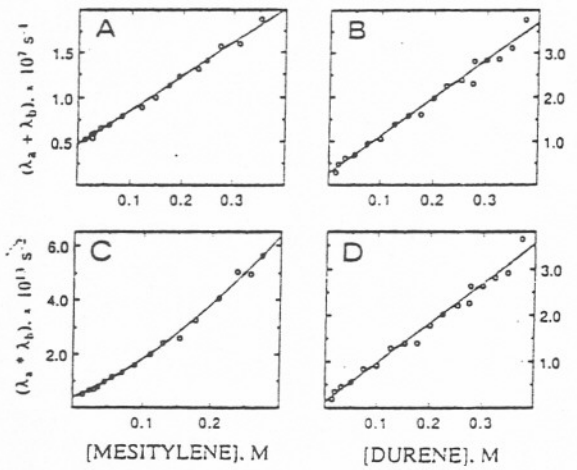


Figure 4. (A and B) Effect of [B] on ($\lambda_a + \lambda_b$). (C and D) Effect of [B] on ($\lambda_a\lambda_b$). All solutions were degassed. The solvents were (A) acetonitrile, (B) dodecane, and (C and D) cyclohexane. Acetone concentration = 0.07 M; DBA concentration = 1.70×10^{-4} M (in mesitylene experiments) or 1.10×10^{-4} M (in durene experiments).

From the rate parameters and amplitudes of a series of decay curves at different concentrations of B, but fixed concentrations of acetone and DBA, one can in principle extract all the rate constants of Scheme I.[2c] The constants k_{EA} of association of triplet acetone with mesitylene or durene (eq 4a), or of energy transfer k_{et} in eq 4b, are the slopes of the plots of ($\lambda_a + \lambda_b$) vs [B] (Figure 4; for a derivation of the equations, see ref 2c). The product of λ_a and λ_b should also be linearly dependent on [B]. This is observed in the case of durene in all solvents except *n*-hexane, where a small upward curvature is observed. With mesitylene, however, the curvature of the product plots is very noticeable in all solvents (Figure 4) and results, most likely, from quenching by ground-state B of either ^{3}E* or ^{3}B* (eq 15a or 15b). This

$$^3E^* + B \xrightarrow{k_q} A + 2B \qquad (15a)$$

$$^3B^* + B \xrightarrow{k_{tq}} 2B \qquad (15b)$$

minor quenching process had been considered previously[2a,d] in the triplet exciplex case; concentration quenching of several triplet methylbenzenes has been reinvestigated recently.[4] When (15) is included in the reaction scheme, the slope of ($\lambda_a + \lambda_b$) vs [B] becomes equal to ($k_{EA} + k_q$[B]).

Other important values are k_{AE}, the rate constant of exciplex dissociation in eq 7a (or k_{-et}[A], in eq 7b), and the pseudo-

TABLE III: Solvent Effects on the Kinetic Parameters Related to the Quenching of Triplet Acetone by Durene (via Exciplex Formation or Energy Transfer)[a]

solvent	k_1^0, 10^6 s^{-1}	k_2^0, 10^6 s^{-1}	k_q (or k_{tq}), 10^6 M^{-1} s^{-1}	k_{EA} (or k_{et}), 10^7 M^{-1} s^{-1}	k_{AE} (or k_{-et}[A]), 10^5 s^{-1}
acetonitrile	1.1	1.9	0	4.3	2
n-hexane	1.8	2.0	0.8	5.9	2
cyclohexane	1.2	0.7	0	8.5	5
n-dodecane	0.8	1.0	0	7.9	*b*
isooctane	1.1	1.4	0	6.4	*b*
cyclopentane	*c*	*c*	0	6.6	*c*

[a] [DBA] = 1.10×10^{-4} M; [acetone] = 0.07 M. [b] Too small to estimate. [c] Not determined.

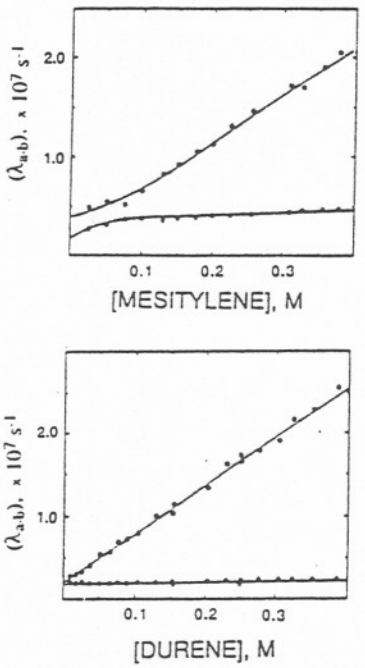


Figure 5. Effect of [B] on λ_a (empty circles) and λ_b (full circles) in degassed *n*-hexane solutions. [acetone] = 0.07 M; [DBA] = 1.70×10^{-4} M (in mesitylene experiments) or 1.10×10^{-4} M (in durene experiments). The solid lines are the best fit to eq 23 by using the iterative SIMPLEX method (see text).

first-order rate constants of decay of ^{3}A* (k_1 or k_3) and of either ^{3}E*(k_2) or ^{3}B* (k_4), as defined by

$$k_1 = k_1^0 + k_{EA}[B] \qquad (16)$$

$$k_2 = k_2^0 + k_{AE} + k_q[B] \qquad (17)$$

$$k_3 = k_1^0 + k_{et}[B] \qquad (18)$$

$$k_4 = k_4^0 + k_{-et}[A] + k_q[B] \qquad (19)$$

with

$$k_1^0 = k_d^A + k_{ET}^A[DBA] \qquad (20)$$

$$k_2^0 = k_d^E + k_{ET}^E[DBA] \qquad (21)$$

$$k_4^0 = k_d^B + k_{ET}^B[DBA] \qquad (22)$$

A SIMPLEX algorithm[10] was used to obtain these rate constants from the fit to eq 23 of the curves of λ_a and λ_b vs [B], minimizing the sum of the χ squares of both curves. Typical fits

$$\lambda_{a,b} = (1/2)(k_1 + k_2) \pm (1/2)[(k_1 - k_2)^2 + 4k_{EA}k_{AE}[B]]^{1/2} \qquad (23a)$$

$$\lambda_{a,b} = (1/2)(k_3 + k_4) \pm (1/2)[(k_3 - k_4)^2 + 4k_{et}k_{-et}[A][B]]^{1/2} \qquad (23b)$$

are shown in Figure 5; the results are listed in Tables II and III. Note that the small values of k_{AE} (or k_{-et}[A]) listed in these tables should be regarded as upper limits only, since the quality of the fit is quite insensitive to lowering the values of this parameter.

b. Effect of Acetone Concentration. If Scheme I represents the formation of triplet exciplexes by eq 4a etc., varying acetone concentration in a series of experiments at constant concentrations of B and DBA should, in first approximation, have no effect on λ_a and λ_b. On the other hand, if the new transient is $^3B^*$ formed via eq 4b etc., then evidently the concentration of acetone should affect the rate of back energy transfer (eq 7b) and thus the values of λ_a and λ_b.

In six experiments in acetonitrile, with 0.1 M durene and 1.0 $\times 10^{-4}$ M DBA, the concentration of acetone was varied 200-fold, from 0.07 to 13.6 M (neat acetone). The values of λ_a and λ_b remained constant (5.3×10^6 s and 1.75×10^6 s^{-1}, ±5%) up to [A] = 1.4 M. At higher acetone concentrations, *both* λ_a and λ_b started increasing. In neat acetone, $\lambda_a = 7.5 \times 10^6$ and $\lambda_b = 2.3 \times 10^6$ s^{-1}.[11]

These results are counterintuitive. If back energy transfer (eq 7b) was significant, it should shorten the lifetime of triplet durene while lengthening that of triplet acetone. With reasonable values of k_d^A and k_d^D (corresponding to triplet lifetimes in the μs range) and k_{-et}[A] = 2×10^5 (Table III), eq 23b gives calculated values of λ_a and λ_b in good agreement with the experimental values, as expected. Decreasing k_{-et}[A] has no effect, while increasing it (thus mimicking a increase in acetone concentration) always rapidly increases λ_a. But the effect on λ_b depends subtly on the values of k_d^A and k_d^B: as [A] increases, λ_b generally *decreases* or, at the most, increases by ≲10%. Clearly the model is an oversimplification that fits well neither the exciplex nor the energy-transfer model. It fails to take into account, for example, the changes in k_d^A and k_{ET}^A accompanying the change in solvent from acetonitrile to acetone, where triplet energy "hopping" from one acetone molecule to the next[12] could complicate the picture. Nor does it allow for a possible change in exciplex stability at high acetone concentration.

c. Efficiency of Energy Transfer to DBA(S_1). The DBA method relies on the efficiency Φ_{TS} of the overall spin-forbidden processes that populate DBA(S_1) via reactions 5 and 9a or 9b in Scheme I. A comparison of Figures 1 and 3 shows the much greater intensity of DBA delayed fluorescence in the presence of 0.18 M mesitylene.[13] Whether $^3E^*$ or $^3B^*$ is the new triplet species formed, it is evident that it has to have a larger Φ_{TS} than $^3A^*$ to account for the characteristic shape of the fluorescence decay curves.

For example, with durene in acetonitrile, on the basis of the exciplex assumption, one calculates $\Phi_{TS}^A = 0.05$ and Φ_{TS}^E or $\Phi_{TS}^B = 0.2$ from the amplitudes and rate parameters of eqs 12 and 14.

d. Validity of Scheme I and Solvent Effects on k_{EA} (or k_{et}). Apart for the need to include a quenching process (eq 15), our results fit the mechanism of Scheme I, to the degree that it predicts triple exponential decay curves and linearity of the plots of ($\lambda_a + \lambda_b$) vs [B]. There is, however, a small but perhaps significant discrepancy in the alkane solvents *n*-hexane, cyclohexane, and dodecane, where corresponding data are available without aromatic (Table I). The values of k_1^0, which represent the rate of decay of triplet acetone at [B] = 0 (eq 20), ought to agree with the values of k_1^0 calculated as the sums of k_d^A and k_{ET}^A[DBA] from the rate constants in Table I, and indeed the agreement is excellent in acetonitrile. But in the alkane solvents, k_1^0 is smaller than anticipated; we have no explanation for this deviation from the predictions of Scheme I.

The easiest rate constants to extract with confidence from the data are ($k_{EA} + k_q$) or ($k_{et} + k_{sq}$), which are the slopes of these plots. We found that these slopes vary surprisingly little from solvent to solvent (a factor of 2 at most). Nevertheless, the effects are clearly systematic inasmuch as the same trends are observed with mesitylene and durene. (With durene in acetonitrile, where k_q (or k_{sq}) = 0, k_{EA} had also been obtained from flash kinetic data; the agreement with the DBA results was excellent.)[2d] As mentioned in the Introduction, the values of k_{EA} (or k_{et}) are 2–3 orders of magnitude lower than k_{dif}. Therefore, one does not expect to see a dependence on viscosity, and indeed none is seen.

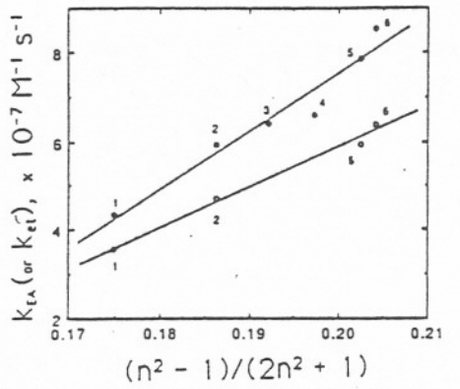


Figure 6. Plot of k_{EA} vs $(n^2 - 1)/(2n^2 + 1)$. Full circles, durene; empty circles, mesitylene. 1, acetonitrile; 2, *n*-hexane; 3, isooctane; 4, cyclopentane; 5, *n*-dodecane; 6, cyclohexane.

Since the rate of photoassociation is usually ≈k_{dif} in the case of singlet excimers and exciplexes, effect of solvent polarity are not observed. Here, in the assumption that an exciplex is formed, one might have expected to see a correlation between the polarity of the solvent and k_{EA}, if charges develop during exciplex formation and some solvent reorganization takes place. This is not observed. k_{EA} (or k_{et}) increases by the same factor from acetonitrile to *n*-hexane as from *n*-hexane to cyclohexane (a factor of 1.3 with mesitylene and 1.4 with durene), even though these last two solvents are equally nonpolar.

In fact, the only solvent bulk property that correlates with k_{EA} (or k_{et}) is its electronic polarizability, i.e., its index of refraction *n* or, for example, the function[14] $(n^2 - 1)/(2n^2 + 1)$ as shown in Figure 6. This effect brings to mind the role of fast solvent relaxation during an electronic transition,[15,16] which results in the red solvent shift of an absorption spectrum as *n* increases.

There is no indication either of a strong effect of solvent polarity on exciplex dissociation, inasmuch as the general shape of the fluorescence decay curves remains unaffected; but the reliability of our determinations of the very low values of k_{AE} is not really adequate to ascertain this.

III. Could the New Transient Be Triplet Methylbenzene? a. Energy Transfer from Triplet Methylbenzene to DBA. Several arguments were previously regarded as militating against the energy-transfer interpretation of the data; among them the endothermicity of the process (especially in the case of xylene and benzene),[2a] the finding of a novel TT absorption spectrum assigned to the exciplex of triplet acetone and durene,[2d] and the long lifetimes of the transients, estimated from either the laser flash or the DBA experiments. In truth, only very scant information on the triplet lifetimes of methylbenzenes was available in the literature and none relative to mesitylene or durene. In the case of *o*-xylene, the literature indicated strong concentration quenching of the triplet,[17] such that this species could not be the transient seen in the cyclohexanone/*o*-xylene/DBA system. It was assumed that triplet mesitylene and durene would be equally sensitive to concentration quenching.

Our "solvent effects", or lack thereof, required reevaluation of the validity of this assumption. In fact, the DBA method is well suited for the purpose. The decay of DBA fluorescence following pulse excitation of degassed cyclohexane solutions of methylbenzene and DBA (*no acetone*), at a wavelength absorbed by both DBA and methylbenzene (280–290 nm), follows triple exponential functions, although here all the amplitudes are positive.[4] The slowest decay component corresponds to DBA sensitization by triplet methylbenzene; its rate parameter is given by

$$1/\tau_3 = k_d^B + k_{sq}[B] + k_{ET}^B[DBA] \qquad (24)$$

with k_d^B, k_{sq}, and k_{ET}^B as in Scheme I and eq 15b. For mesitylene in cyclohexane, $k_{sq} = 6.9 \times 10^6$ M^{-1} s^{-1}. In contrast, the triplet lifetime of durene is not concentration dependent, i.e., $k_{sq} = 0$

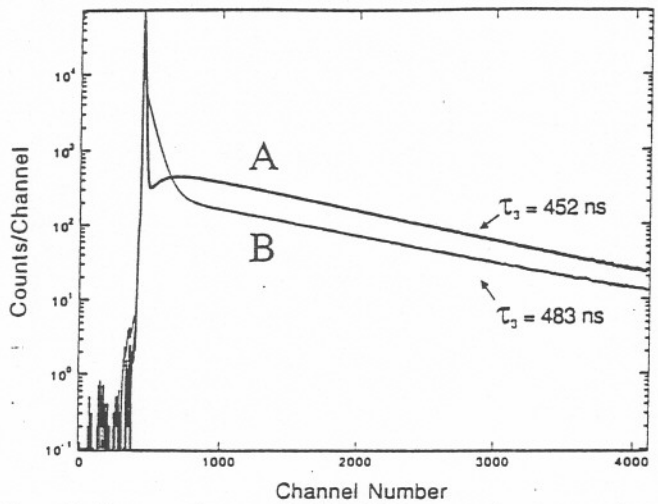


Figure 7. Two experiments with 0.185 M durene and 2.64×10^{-4} M DBA in degassed cyclohexane; λ_{ex} = 285 nm. Solution B contains no acetone; in A the acetone concentration is 0.068 M. At peak channel, the DBA fluorescence intensity was the same in both experiments. At channel 1000, the fluorescence intensity was 150 cts/ch for B, 450 cts/ch for A. This comparison illustrates the significant increase in delayed luminescence brought up by the presence of acetone.

Note that in the acetone experiments we found k_q (or k_{sq}) = 3.2×10^6 M^{-1} s^{-1} with mesitylene and k_q (or k_{sq}) = 0 with durene (Tables II and III).

b. Effect of Acetone on the Durene/DBA System, at λ_{ex} = 285 nm. It is instructive to compare the DBA fluorescence decay curves of two cyclohexane solutions containing the same concentrations of DBA and durene, one with 0.07 M acetone, the other with no acetone, both excited at 285 nm (where most of the light is absorbed by durene). The presence of acetone brings about the typical "exciplex" shape (Figure 7). The third decay component (τ_3) is the same whether acetone is present or not: in both cases it is limited by DBA quenching. The amplitude of this slow component grows in the presence of acetone: i.e., the intensity of the delayed emission is significantly increased, even though, in the experiment of Figure 7, the partial absorbance of acetone represents only 12% of the total absorbance of the solution at 285 nm. Moreover, shifting the excitation wavelength from 285 to 305 nm, where most of the light is now absorbed by acetone, affects only the amplitude α of the delayed emission. The decay curves are simply shifted vertically from one another, but the rate parameters are unchanged.

c. The TT Absorption Transient. Was the absorption transient seen upon laser pulse excitation at 308 nm of acetonitrile solutions of acetone and durene correctly assigned to a triplet exciplex of these solutes,[2d] or was it, in fact, the triplet–triplet absorption spectrum of durene? We have not yet been able to give a definitive answer to this critical question. Only the TT absorption spectra of triplet benzene,[18] and less convincingly that of triplet *o*-xylene,[19] have been reported. In the case of durene, the strong delayed luminescence resulting from TT annihilation is a major problem, particularly difficult to overcome. Nevertheless, 5 μs after laser pulse excitation (at 266 nm) of a 1.7 mM acetonitrile solution of durene, a transient absorption spectrum was recorded in the 310–430-nm region, with an apparent maximum at about 330 nm and a long tail extending toward the visible. Whether this 330-nm maximum is genuine, or an artifact resulting from the residual luminescence after 5 μs, is still open to question. Although it is now quite clear that triplet durene absorbs *in the same spectral region* as the transient hitherto assigned to the triplet exciplex of durene and acetone (which shows no absorption peak at $\lambda >$ 300 nm), there is insufficient information to conclude positively that these two transients are, or are not, the same species.[20]

Discussion

Scheme I and our results show that it is not possible to distinguish between the two possibilities, exciplex formation or energy transfer to mesitylene or durene, on the basis of kinetics alone. It is also evident that whether acetone or the methylbenzene is photoexcited first, the end result is the same: formation of either the triplet of methylbenzene or of a triplet exciplex of acetone and the aromatic.

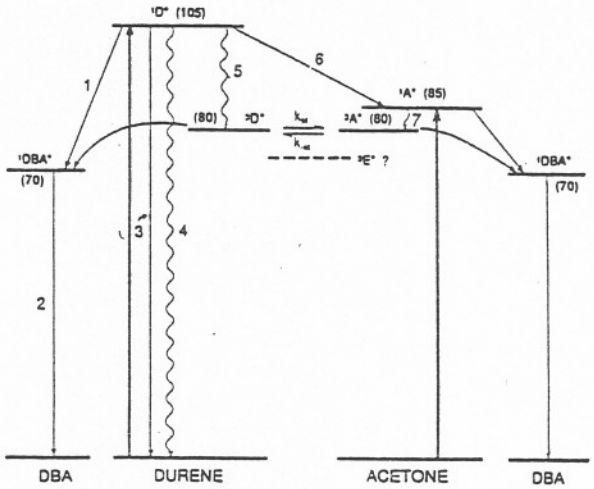


Figure 8. Diagram of excited states of durene, acetone, and DBA. The transitions deactivating $^1D^*$ and $^3D^*$ and their respective rate constants are as follow: 1, $k_{ET}^D = 1.5 \times 10^{10}$ M^{-1} s^{-1}, this work; 2, $k_F^{DBA} = 5 \times 10^8$ s^{-1}, ibid.; 3, $k_F^D = 9.4 \times 10^6$ s^{-1}, 4, $k_{ic}^D = 5.7 \times 10^7$ s^{-1}, and 5, $k_{isc}^D = 2.3 \times 10^7$ s^{-1}, from Quina et al. (Quina, F. H.; Carroll, F. A. *J. Am. Chem. Soc.* **1976**, *98* 6); 6, $k_{SS} \approx 10^{10}$ M^{-1} s^{-1}, the rate constant for SS energy transfer from naphthalene to biacetyl, from Dubois and Cox (Dubois, J. T.; Cox, M. *J. Chem. Phys.* **1963**, *38*, 2538). The fastest transition deactivating $^1A^*$ is 7, $k_{isc}^A = 5 \times 10^8$ s^{-1}, with $\Phi_{isc} = 1$ (Borkman, R. F.; Kearns, D. R. *J. Chem. Phys.* **1966**, *44*, 945).

To the reactions of Scheme I, new processes must be added to describe the photophysics of durene in solution containing DBA and 0.07 M acetone at λ_{ex} = 285 nm, as in the experiment of Figure 7. The rate constants of most of these processes are known (Figure 8). At acetone concentration >0.01 M, the fastest path of deactivation of singlet excited durene ($^1D^*$) is SS energy transfer to acetone. From then on, one is therefore back to Scheme I, where the excitation energy is immediately found in triplet acetone. Therefore, exciting acetone or exciting durene makes no difference except in the efficiency of the reaction, and the question remains open: once triplet acetone is generated, does it form an exciplex with durene or does it transfer its energy to it? In the latter case, the cascade of energy transfer to and from acetone serves to increase the population of triplet durene, as a result of the fast and near 100% efficient intersystem crossing in acetone. This explains the higher amplitude of the delayed DBA emission in the experiment of Figure 7 when acetone is present.

On the other hand, when triplet acetone is formed first (λ_{ex} = 305 nm), as in most of the work reported here, the shape of the fluorescence decay curves requires that the efficiency Φ_{TS} of energy transfer from the new transient to DBA be larger than from triplet acetone: i.e., either $\Phi_{TS}^E > \Phi_{TS}^A$ or $\Phi_{TS}^B > \Phi_{TS}^A$ (see section IIc). One can make a crude estimate of Φ_{TS}^B on the basis of the convoluted fit of the triple exponential decay of DBA fluorescence from solutions containing mesitylene or durene, such as curve B in Figure 7. There are two components of delayed emission, due to energy transfer to DBA from either singlet or triplet methylbenzene. The ratio of the number of photons resulting from energy transfer from $^1B^*$ to those due to energy transfer from $^3B^*$ is given by

$$\beta\tau_2/\tau_3 = k_{SS}[DBA]/k_{isc}\Phi_{TS}^B$$

where the rate constants are as defined in Figure 8 and β is the amplitude of the second decay component. One arrives at rough estimates of Φ_{TS}^B of 0.1–0.15, actually larger than the values of Φ_{TS}^E (or Φ_{TS}^B) estimated from the experiments with acetone, but certainly not incompatible with these results.

In any case, the properties of triplet mesitylene and durene appear to be very similar to that of the hypothetical exciplexes

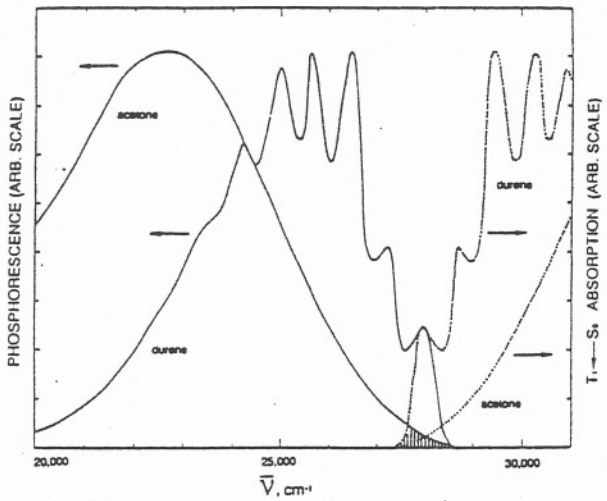


Figure 9. Phosphorescence spectra of durene and acetone (solid lines) and corresponding $T_1 \leftarrow S_0$ absorption spectra (dotted line), drawn as the mirror images of the phosphorescence spectra (superposing the 0–0 transitions in the case of durene and the origins in the case of acetone; see text). The figure shows the overlap area relevant to energy transfer from triplet acetone to durene. For the phosphorescence spectra, the durene and acetone were 0.05 M in EPA glass (ethyl ether:isopentane:ethanol = 5:5:2); λ_{ex} 280 nm, gate time, 1 ms. Excitation slit 10 nm, emission slit 2.5 nm.

in the same solvent: (a) the values of τ_3 in the experiments of Figure 7 are practically the same with or without acetone; (b) the lifetime of the assumed exciplex of triplet acetone and durene is independent of the concentration of durene ($k_q = 0$, Table III), as is triplet durene ($k_{sq} = 0$),[4] while triplet mesitylene and its hypothetical exciplex with acetone are both sensitive to self-quenching. However, in cyclohexane we found k_{sq} to be about two times larger than k_q ($k_{sq} = 6.9 \times 10^6$ compared to $k_q = 3.2 \times 10^6$ M^{-1} s^{-1}, Table II; k_q and k_{sq} are defined in eqs 15a,b).

Going back to the lack of solvent effects, if the transient was a triplet exciplex, we expected its rate of formation and stability to be significantly affected by solvent polarity. This is not the case. The weak dependence of the rate of transient formation on the polarizability of the solvent is more in keeping with triplet–triplet energy transfer to generate triplet mesitylene or durene. Solvent effects of the same order of magnitude have been reported for intramolecular triplet energy transfer.[21]

Literature puts the T_1 state of acetone at 78–80 kcal;[1,22] the phosphorescence spectrum of acetone (Figure 9) does not allow the O–O transition to be located more accurately. The O–O band of durene phosphorescence, at 358 nm, places the T_1 level at 80 kcal, in agreement with the literature.[22] Hence energy transfer between acetone and durene is either isoergic or endothermic by ≤2 kcal. The rate of an exchange-energy-transfer process is proportional to the overlap of the donor emission and acceptor absorption spectra, here the phosphorescence of acetone and singlet–triplet absorption spectrum of durene. Although the latter has not been measured, it is probably safe to assume that it is close to the mirror image of durene phosphorescence emission and that the Stokes shift is small, as between durene absorption and fluorescence. In contrast, with acetone the Stokes shift between $T_1 \leftarrow S_0$ absorption and phosphorescence should be large, as between absorption and fluorescence, if the ketone is bent out of plane in the excited state like formaldehyde.[23] In the transfer of triplet energy from acetone to durene, the relevant spectral overlap between the phosphorescence of acetone and the $T_1 \leftarrow S_0$ absorption spectrum of durene must therefore be small, as illustrated in Figure 9. This is not out of line with a measured rate constant of $(6 \pm 2) \times 10^7$ M^{-1} s^{-1} for this process (taking $k_{et} = k_{EA}$ in Table III). A slight increase in the spectral overlap, when the $T_1 \leftarrow S_0$ absorption of durene shifts to the red as n increases, could account for the small increase in k_{et} shown in Figure 6.

The same argument indicates that the rate of back energy transfer should also be small, again as a consequence of the expected large Stokes shift between acetone phosphorescence and its $T_1 \leftarrow S_0$ absorption spectrum, Figure 9. Even if the back energy transfer (eq 7b in Scheme I) was slightly *exothermic*, its rate constant would be $\ll k_{dif}$. Therefore, the argument that the rate of back energy transfer, k_{-et}, ought to be fast if the cause of the slow forward rate was the small endothermicity of the process is simply not valid. The situation with mesitylene is expected to be essentially the same as with durene, since the E_T of mesitylene is only 0.3 kcal higher than that of durene. Simply put, triplet energy transfer between acetone and mesitylene or durene should be slow in both directions because of the very unfavorable Franck–Condon factors in acetone.[24]

Although none of the arguments discussed here are fully conclusive, the case for the triplet-energy-transfer interpretation of the data should not have been dismissed as casually as it was in our earlier discussions. Definite proof awaits firm spectroscopic identification of the TT absorption spectra of triplet durene and comparison with the acetone/durene transient.

Yet even if triplet durene and mesitylene were indeed the transients responsible for the kinetic data reported here, instead of triplet exciplexes, there remains the fact that "quenching" of triplet alkanones by methylbenzenes is not limited to mesitylene and durene. It occurs also with *o*-xylene, toluene, and even benzene, although no "exciplex peak" is seen in the case of benzene.[2a,b] Loufty and Yip[25] have measured the rate constant for quenching of triplet acetone by benzene, $k_q = 2.8 \times 10^6$ M^{-1} s^{-1}, which is consistent with a lifetime of 17 ns in that solvent.[2a] The triplet state of benzene is ~4 kcal above that of durene. If the quenching of triplet acetone by durene and benzene occurred via "vertical" energy transfer in both cases, then in the case of benzene one would expect

$$k_q = k_{et} \exp(-\Delta_{ET}/RT) = 4.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$$

since $k_{et} = 4.3 \times 10^7$ M^{-1} s^{-1} in the case of durene in acetonitrile (Table III). Clearly vertical energy transfer is inadequate to account for quenching by benzene. The short lifetime of triplet acetone in benzene (E_T = 84 kcal) has long be known[8] and is particularly puzzling, as the following experiment illustrates. In cyclohexane solutions of 0.1 M acetone and 2×10^{-4} M DBA, with or without 4.5 M benzene, the presence of benzene was found to increase not only the rate of decay of the delayed fluorescence of DBA, as expected, but also (by a factor of 6) the amplitude of this decay component, *as if the species sensitizing DBA had a Φ_{TS} value six times higher than that of triplet acetone.*[2a] Assuming that the sensitizer is triplet benzene, generated by endothermic energy transfer from triplet acetone, how would it not be quenched by ground-state acetone (0.1 M) faster than by DBA (2×10^{-4} M), if k_{-et} is $>10^8$ M^{-1} s^{-1}, a minimum value for a process *exothermic* by 4–6 kcal? The spectral overlap for back energy transfer (between benzene $S_0 \leftarrow T_1$ emission and acetone $T_1 \leftarrow S_0$ absorption) should be more favorable than in the case of triplet durene and acetone depicted in Figure 9. Therefore one should not expect to see an increase in the intensity of DBA delayed fluorescence. This argument was the basis for proposing the formation of a triplet exciplex of acetone and benzene in the first place,[2a] in agreement with the earlier conclusions of Loufty and Yip.[25] To this day it seems the most valid interpretation of the data.

The situation is therefore unsatisfactory. On the one hand, the kinetic data obtained with durene and mesitylene can be accounted for reasonably well by the simpler triplet-energy-transfer assumption; on the other hand, as the number of methyl substituents on benzene decreases and its E_T increases, the energy transfer interpretation becomes less and less adequate. One would wish for a mechanism of quenching of triplet alkanones valid for all methylbenzenes and applicable also, for example, to the quenching of benzaldehyde (E_T = 72 kcal) by benzene.[26] At this time, one seems forced to conclude, albeit reluctantly, that there may be no unity of quenching mechanism or else that in all cases, including the quenching of triplet acetone by mesitylene and durene, the first reaction product is an exciplex. The properties of such triplet exciplexes may be so close to that of the uncomplexed triplet

methylbenzene as to make the distinction very difficult indeed.

This interpretation would be entirely in keeping with the suggestion of Turro, who considered the possibility of stabilized exciplexes on the excited-state surface of exchange-energy-transfer processes.[1] The fate of such complexes would depend on the relative excitation energies of the free molecular partners. In the case of triplet acetone and mesitylene or durene, energy transfer is endothermic by at most 2 kcal, which may create ideal conditions for a relatively long-lived exciplex.

Acknowledgment. G.L.I. and L.H.C. are grateful for postoctoral fellowships from the Conselho Nacional de Desenvolvimento Cientifico e Technologico (CNPq, Brazil). The fluorescence lifetime instrumentation was acquired with funds from the National Science Foundation (Grant CHE-8209863). We thank Professor J. W. Hastings for his continuing interest. G.L.I. expresses his gratitude to Professor Frank Quina for enjoyable and useful discussions.

References and Notes

(1) Turro, N. J. *Modern Molecular Photochemistry*; Benjamin/Cummings: Menlo Park, CA, 1978; pp 305–309.
(2) (a) Wilson, T.; Halpern, A. M. *J. Am. Chem. Soc.* **1980**, *102*, 7279. (b) Wilson, T.; Halpern, A. M. *Ibid.* **1981**, *103*, 2412. (c) Wilson, T.; Frye, S. F.; Halpern, A. M. *Ibid.* **1984**, *106*, 3600. (d) Johnston, L. J.; Scaiano, J. C.; Wilson, T. *Ibid.* **1987**, *109*, 1292.
(3) See, for example: Stevens, B. *Adv. Photochem.* **1971**, *8*, 161.
(4) Indig, G. L.; Wilson, T. *J. Photochem. Photobiol. A* **1992**, *63*, 195.
(5) Catalani, L. H.; Wilson, T. *J. Am. Chem. Soc.* **1987**, *109*, 7458.
(6) $k_{dif} = 4000\pi NRD$, with the diffusion coefficient D calculated according to the empirical relation derived by Davis et al. (Davis, H. T.; Tominaga, T.; Evans, D. F. *AIChE J.* **1980**, *26*, 313); Olea and Thomas found that this procedure leads to the best values of k_{dif} (Olea, A. F.; Thomas, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 4494).
(7) In agreement with the conclusions of: Wagner, P. J.; Kochevar, I. *J. Am. Chem. Soc.* **1968**, *90*, 2232.
(8) Porter, G.; Dogra, S. K.; Sugamori, S. E.; Yip, R. W. *J. Chem. Soc., Faraday Trans. I* **1973**, *69*, 1462.
(9) Barltrop, J. A.; Coyle, J. D. *Excited States in Organic Chemistry*; Wiley: New York, 1975; p 194.
(10) Demas, J. N. *Excited States Lifetime Measurements*; Academic Press: New York, 1983; pp 80–89.
(11) We thank Ying-Hua Mei for performing these experiments.
(12) Lechtken, P.; Turro, N. J. *Angew. Chem., Int. Ed. Engl.* **1973**, *12*, 314. Schuster, G.; Turro, N. J. *Tetrahedron Lett.* **1975**, 2261.
(13) The ratio D/P of "delayed counts" to "prompts counts" is estimated by integration of eqs 12 and 14. For Figure 1, $D/P = k_F/(\alpha k_{obs}) = 0.19$; for Figure 2, $D/P = (k_F/\alpha)(\beta/\lambda_a + 1/\lambda_b) = 0.96$. Thus D/P is five times larger in the presence of 0.18 M mesitylene, in otherwise the same conditions.
(14) Bayliss, N. S. *J. Chem. Phys.* **1950**, *18*, 292.
(15) Birks, J. B. *Photophysics of Aromatic Molecules*; Wiley-Interscience: London, 1970; pp 110–116.
(16) Examples: Milder, S. J. *Inorg. Chem.* **1989**, *28*, 868.
(17) Cundall, R. B.; Voss, A. J. R. *J. Chem. Soc., Chem. Commun.* **1969**, 137.
(18) Nakashima, N.; Sumitani, M.; Ohmine, I.; Yoshihara, K. *J. Chem. Phys.* **1980**, *72*, 2226.
(19) Sugawara, T.; Iwamura, H. *J. Am. Chem. Soc.* **1985**, *107*, 1329.
(20) In the presence of acetone, the delayed luminescence of durene ought to be more intense, not less, judging from the experiment described above (IIIb, Figure 7; see also Discussion). The fact that this luminescence was apparently not a major problem in the Johnston–Scaiano[2d] laser flash experiment at $\lambda_{ex} = 308$ nm seems to suggest that the two transients are not the same.
(21) Closs, G. L.; Piotrowiak, P.; MacInnis, J. M.; Fleming, G. R. *J. Am. Chem. Soc.* **1988**, *110*, 2652. These authors reported a change in an intramolecular energy transfer rate of less than a factor of 3 between hexane and acetonitrile as solvents, very similar to our solvent effects on k_{EA}.
(22) E_T of acetone: 80 kcal (Borkman, R. F.; Kearns, D. R. *J. Chem. Phys.* **1966**, *44*, 945); 77.5 kcal (Loufty, R. O.; Loufty, R. O. *J. Phys. Chem.* **1973**, *77*, 336); 78.9 kcal (*Handbook of Organic Photochemistry*; Scaiano, J. C., Ed.; CRC: Boca Raton, FL, 1989; Vol. I, p 376). E_T of mesitylene, 80.1 kcal; E_T of durene, 79.8 kcal (Engel, P. S.; Monroe, B. M. *Adv. Photochem.* **1971**, *8*, 245; also ref 15).
(23) McGlynn, S. P.; Azumi, T.; Kinoshita, M. *Molecular Spectroscopy of the Triplet State*; Prentice-Hall: Englewood Cliffs, NJ, 1969; pp 166–171.
(24) Reference 1, pp 331–333. Mirbach, M. F.; Ramamurthy, V.; Mirbach, M. J.; Turro, N. J.; Wagner, P. J. *Nouv. J. Chim.* **1980**, *4*, 471.
(25) Loufty, R. O.; Yip, R. W. *Can. J. Chem.* **1973**, *51*, 1881.
(26) Giering, L.; Berger, M.; Steel, C. *J. Am. Chem. Soc.* **1974**, *96*, 1974.

Kinetic and Thermodynamic Study of the Hydrolysis of Silicon Alkoxides in Acidic Alcohol Solutions

Jorge Sanchez and Alon McCormick*

Department of Chemical Engineering and Materials Science, University of Minnesota, 421 Washington Ave SE, Minneapolis, Minnesota 55414 *(Received: July 19, 1991; In Final Form: July 22, 1992)*

Though the relevant literature offers little consistency in the kinetic data of the acid-catalyzed hydrolysis of silicon alkoxides, reliable rate constants are essential for the development of kinetic models for sol–gel processing. Si-29 NMR was used in conjunction with numerical simulations to measure hydrolysis rate constants for tetraethoxysilane (TEOS), tetramethoxysilane (TMOS), and hexaethoxydisiloxane. Unlike previous efforts, we have used conditions where the effects of hydrolysis and condensation reactions can be decoupled. We have verified our rate constants using a range of solution compositions. Implications regarding the influence of the synthesis protocol on gel homogeneity are discussed. We have also estimated the enthalpies, entropies, and activation energies for the hydrolysis of TEOS. We find that each subsequent hydrolysis reaction has a higher rate constant, confirming some earlier studies. However, we also find that each hydrolysis step becomes thermodynamically less favorable. These opposing kinetic and thermodynamic trends explain why acid-catalyzed hydrolysis produces a distribution of hydrolyzed intermediates rather than just fully hydrolyzed products. They also suggest that complete and immediate hydrolysis would be difficult to achieve except at very high water concentration.

Introduction

The chemical synthesis of ceramics and glasses by the sol–gel method has attracted much interest.[1] Even though silicon alkoxide systems have been among the most studied sol–gel materials, chemical engineering models remain tentative at best.

Typically, a silicon alkoxide, for example tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS), and a certain amount of water are dissolved in an alcohol in the presence of a catalyst. In acidic solutions, the polymerization of the silicon alkoxide can produce a transparent and porous gel.[1,5] The gel formed is a polymeric network of siloxane bonds (Si–O–Si) with alkoxy or hydroxy side groups. The reactions are generally written

hydrolysis: $Si(OR)_4 + nH_2O \rightarrow Si(OR)_{4-n}(OH)_n + nROH$

alcohol-producing condensation:

$$Si{-}OR + HO{-}Si \rightarrow Si{-}O{-}Si + ROH$$

water-producing condensation:

$$Si{-}OH + HO{-}Si \rightarrow Si{-}O{-}Si + H_2O$$

The actual complexity of the process is not reflected by this simplified representation, though, because many different intermediates are formed during the reaction. The intermediate

Anexo VIII

Quenching of chemiexcited triplet acetone by biologically important compounds in aqueos medium. Catalani, L.H., Bechara, E.J. *Photochem. Photobiol.*, **39**, 823 (1984).

Photochemistry and Photobiology Vol. 39, No. 6, pp. 823–830, 1984
Printed in Great Britain.

0031-8655/84 $03.00+0.00

QUENCHING OF CHEMIEXCITED TRIPLET ACETONE BY BIOLOGICALLY IMPORTANT COMPOUNDS IN AQUEOUS MEDIUM

LUIZ H. CATALANI and ETELVINO J. H. BECHARA*

Department of Biochemistry, Instituto de Química, Universidade de São Paulo, C.P. 20.780, São Paulo, S.P., Brazil

(*Received* 23 *August* 1983; *accepted* 20 *January* 1984)

Abstract—Thermolysis of tetramethyl-1,2-dioxetane is a convenient source of triplet acetone, which can be monitored in aerated solutions by the sensitized fluorescence of 9,10-dibromoanthracene. We have investigated the quenching of chemiexcited triplet acetone in air-equilibrated aqueous solutions containing the 9,10-dibromoanthracene-2-sulfonate ion by five classes of compounds: indoles, tyrosine derivatives, quinones, riboflavin, and xanthene dyes. Quenching rates for indoles, tyrosine and its 3,5-dihalogenoderivatives, and xanthene dyes ($k_q = 10^8$–$10^9\ M^{-1}\ s^{-1}$) are considerably smaller than the diffusion controlled rate, whereas those for quenchers with high electroaffinities, such as quinones (IP = 10–11 eV), approach the diffusion controlled rate ($k_q = 10^{10}\ M^{-1}\ s^{-1}$). Energy transfer for riboflavin probably occurs by a triplet–singlet Förster type process.

A comparison of the present data with previous studies of quenching of enzymically generated triplet acetone (isobutanal/O_2/horseradish peroxidase) by the same classes of quenchers (except riboflavin) reveals that, independent of the nature of the quencher and the deactivation mechanism, the Stern–Volmer quenching constants ($k_q \tau^0$) are systematically about one order of magnitude higher in the enzymatic system. The difference is attributed to a longer lifetime of triplet acetone in the latter case, "protected" in an enzyme cavity against collisions with dissolved oxygen.

INTRODUCTION

The quenching of triplet acetone, produced either by irradiation or *via* thermolysis of tetramethyldioxetane (TMD),† has been extensively studied by several groups, using a large number of techniques (Wagner, 1967; Porter *et al.*, 1973; Steinmetzer *et al.*, 1973; Wilson *et al.*, 1976; Mirbach *et al.*, 1980; Turro and Tanimoto, 1980). Depending upon the electronic and steric characteristics of the donor/acceptor pair and the cage properties of the solvent, collisional triplet–triplet energy transfer, electron exchange (charge transfer complexation), electron transfer, long-range triplet–singlet transfer, triplet–triplet exciton transfer and photoreactions have been proposed to be the operative quenching mechanisms (Loutfy and Yip, 1973; Loutfy *et al.*, 1977; Turro, 1978; Mirbach *et al.*, and ref. therein, 1980; Cilento, 1982). Nevertheless, little attention has been directed towards the investigation of quenching processes in aqueous media and to quenching by biologically related compounds. Noteworthy exceptions include: (i) the study by Porter *et al.* (1973) of the behavior of photoexcited triplet acetone in water, in the absence and presence of electron donors; (ii) the investigation by Encinas *et al.* (1980) of the photochemistry of aliphatic ketones in polar solvents; and, more recently, (iii) a detailed examination by Kasama *et al.* (1982) of the photochemical reactions of triplet acetone with indole, purine and pyridimine derivatives in water.

Recently, triplet acetone was demonstrated to be formed in the horseradish peroxidase (HRP) catalyzed oxidation of isobutanal in phosphate buffer (Bechera *et al.*, 1979). A considerable body of evidence suggests that the excited species is generated within the enzyme, partially protected from quenching by external dissolved oxygen. A large variety of synthetic and natural compounds were shown to act as energy acceptors or quenchers of this enzymically generated triplet acetone. In nearly all cases, Cilento and coworkers (1982) have found apparent Stern–Volmer quenching constants that are at least one order of magnitude higher than those expected for diffusion controlled processes, assuming the lifetime of triplet acetone in aerated water to be 2 μs.‡

In the present work, we describe a study of the quenching of triplet acetone generated by thermolysis of TMD in aqueous solutions ("exposed" triplet acetone) using five classes of biomolecules or derivatives thereof: riboflavin, indole compounds, tyrosine derivatives, quinones and xanthene dyes. The observed quenching constants are compared

*To whom correspondence should be addressed.

†*Abbreviations:* TMD, tetramethyl-1,2-dioxetane; HRP, horseradish peroxidase; DBAS, 9,10-dibromoanthracene-2-sulfonate ion; DMBQ, 2,6-dimethyl-*p*-benzoquinone; DIT, 3,5-diiodotyrosine.

‡Calculated from the $k_{TS}\tau^0$ value [k_{TS} is the rate constant for the overall triplet (acetone)–singlet (DBAS) energy transfer] obtained from the $Y_{intercept}$ slope ratio of a double reciprocal plot of emission intensity *vs* DBAS concentration in an air-equilibrated aqueous solution of 0.2 m*M* TMD. The constant k_{TS}, measured independently in nitrogen-purged deionized water according to Wilson and Halpern (1980), was found to be $3.6 \times 10^9\ M^{-1}\ s^{-1}$ at 20°C.

with those measured for "protected" triplet acetone produced by the isobutanal/O_2/HRP system.

MATERIALS AND METHODS

Sorbic acid, D- and L-tryptophan, and tyrosine were purchased from Sigma Chem. Co.; 3,5-dibromotyrosine, *p*-benzoquinone, tetrabromo-*o*-benzoquinone, and 2,6-dimethyl-*p*-benzoquinone from Aldrich; 3,5-diiodotyrosine, fluorescein and Rose Bengal from BDH; 1,4-naphthoquinone and tetrachloro-p-benzoquinone from Eastman Kodak; eosine and riboflavin from Merck; indole from Baker; N-methylindole from Research Org./Inorg. Chem. Co.; lysyl-tryptophanyl-lysine from Research Org./Inorg. Chem. Co.; lysyl-tryptophanyl-lysine from Research Plus Lab.; and sodium anthraquinone-2-sulfonate from Fisher. These reagents were of the highest grade commercially available and, with the exception of D- and L-tryptophan and the quinones, used without further purification. D- and L-tryptophan were recrystallized from aqueous ethanol; except for sodium anthraquinone-2-sulfonate, the quinones were purified by sublimation. Acetonitrile (Aldrich) and *n*-hexane (Merck) were of spectroscopic grade. *cis/trans*-Decalin was stirred overnight with concentrated sulfuric acid, washed with water, saturated aqueous $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and distilled. The following compounds were synthesized according to published procedures: 3,5-dichlorotyrosine (Bouchilloux, 1955), 2-methyl-1,4-naphthoquinone (Fieser, 1940), and sodium 9,10-dibromoanthracene-2-sulfonate (Battegay and Brandt, 1923). Tetramethyldioxetane, prepared by cyclization of the corresponding β-bromohydroperoxide with freshly prepared anhydrous silver acetate (Kopecky *et al.*, 1975), was isolated as yellow crystals from pentane at −78°C (9% yield) and identified by ^{1}H-NMR spectroscopy and GLPC. Due to the unavailability of concentrated H_2O_2 required for the dioxetane synthesis, an anhydrous ethereal solution of 5 *M* H_2O_2, prepared by extraction of 30% H_2O_2 with ethyl ether (5 times) was employed. The ethereal H_2O_2 extracts were dried over anhydrous $MgSO_4$ and concentrated to one fifth of the initial volume by flushing with dry N_2 at 0°C. CAUTION: *Ethereal solutions of H_2O_2 above 3 M may explode if not handled properly. Bromohydroperoxides and dioxetanes are also unstable!*

Absorption spectra were recorded on a Zeiss DMR 21 spectrometer and the fluorescence measurements carried out on a Hitachi-Perkin–Elmer MPF-4 spectrofluorimeter. GPLC analyses were performed on a CG-Model 20-D chromatograph with thermal conductivity detection. The chemiluminescence from the reaction mixtures was measured on either a Hamamatsu photocounter C-767 equipped with Toshiba filters or a photometer of in-house construction with a 1P28 photomultiplier. The ^{1}H-NMR spectra were recorded on a Varian T-60 spectrometer using TMS as an internal reference and $CDCl_3$ or CCl_4 as solvents.

Tetramethyldioxetane concentrations in stock solutions of acetonitrile were determined by GLPC on the basis of calibration curves obtained with authentic acetone (10% FFAP on chromosorb W, 3 m × 3/16" column; column temperature 120°C, injector 165°C and detector 200°C). Contamination of the dioxetane solution with acetone generated by TMD cleavage during storage was checked by comparing the GLPC traces before and after pretreatment with triphenylphosphine (Bechara and Wilson, 1980), which causes monodeoxygenation of TMD (Bartlett *et al.*, 1973).

The quenching and energy transfer studies were performed in either bidistilled water or phosphate (50 m*M*): pyrophosphate (5 m*M*) buffer, using 0.2 m*M* TMD as the triplet acetone source, at 31–41°C. All quenchers and acceptors were tested over a range of concentration of generally one order of magnitude at a level that did not influence the rate of TMD thermolysis. In the Stern–Volmer type studies, triplet acetone was monitored indirectly by the sensitized fluorescence emission of DBAS (typically 2.5 μ*M*) added to the solutions.

RESULTS

Energy transfer to xanthene dyes

The addition of fluorescein, eosine, and Rose Bengal enhances the luminescence from TMD thermolysis, demonstrating that energy transfer occurs. Neither decomposition of the dye nor alteration of the rate of TMD thermolysis were observed in the reaction mixtures. Figure 1 presents double reciprocal plots of the effect of xanthene dye concentration on the intensity (*I*) of the sensitized dye emission. Appropriate filters were used during measurements to reduce reabsorption artifacts to a minimum.

From the emission intensity (corrected for the filters) extrapolated to infinite dye concentration and the quantum yield of fluorescence (Wade and Spikes, 1971) for fluorescein ($\phi_F = 0.92$), eosine ($\phi_F = 0.19$), and Rose Bengal ($\phi_F = 0.02$), the relative efficiencies of energy transfer from triplet acetone to populate

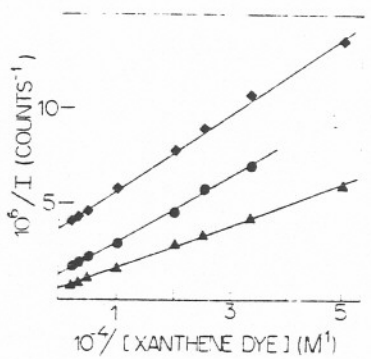


Figure 1. Double reciprocal plots of the effect of fluorescein (▲), eosine (●), and Rose Bengal (■) concentrations on the sensitized emission intensity from 0.2 m*M* TMD, in phosphate (50 m*M*), pyrophosphate (5 m*M*) buffer pH 7.4, at 41°C. The experimental conditions are the same as indicated in Table 1.

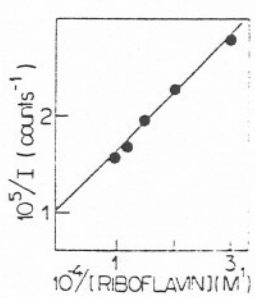


Figure 2. Double reciprocal plot of the effect of riboflavin concentration on the sensitized chemiluminescence intensity from 0.2 m*M* TMD in phosphate (50 m*M*)/pyrophosphate (5 m*M*) buffer pH 7.4, at 37°C.

Table 1. Energy transfer from chemically generated triplet acetone† to xanthene dyes: $k_{ET}\tau^0$ values and relative efficiencies (Φ_{TS}) from double reciprocal plots for the enhanced chemiluminescence

Dye‡	$10^{-3}\ k_{ET}\tau^0$ (M^{-1})	Relative Φ_{TS}
Fluorescein	5.7	1
Eosine	7.8	13
Rose Bengal	18	320

†Triplet acetone from thermolysis of 0.2 m*M* TMD. ‡All experiments were run in buffer (50 m*M* phosphate, 5 m*M* pyrophosphate, pH 7.4), at 41°C. Toshiba filters No. 0–53 (90% cut off below 520 nm), 0–57 (*idem*, 560 nm), and 0–56 (*idem*, 550) were used to remove artifacts due to reabsorption of fluorescein, eosine and Rose Bengal fluorescence, respectively, as well as residual TMD direct chemiluminescence.

the S_1 state of the dye (Φ_{TS}) were calculated to be 1:13:320, respectively. The values of $k_{ET}\tau^0$ (where k_{ET} is the rate constant for the energy transfer and τ^0 the lifetime of triplet acetone in the absence of the acceptor) obtained from the $Y_{intercept}$/slope ratios are similar for the three dyes (Table 1).

Energy transfer to riboflavin

In the presence of 30 μM riboflavin, the emission intensity from aerated solutions of TMD is enhanced by a factor of only 1.5. The double reciprocal plot of intensity *vs* riboflavin concentration is shown in Fig. 2. The intensities were measured with a filter that totally absorbs acetone fluorescence [chemiexcitation yield of singlet acetone from TMD thermolysis is 0.15% according to Wilson *et al.* (1976); λ_{max} acetone fluorescence is about 395 nm]. The $k_{ET}\tau^0$ value, calculated from the $Y_{intercept}$/slope ratio, is $1.8 \times 10^4\ M^{-1}$. Stern–Volmer analysis of the competitive quenching of the sensitized fluorescence of riboflavin fluorescence by the sorbate ion gave $k_q\tau^0 = 1.9 \times 10^3\ M^{-1}$ for the latter; for comparison, with DBAS as monitor for triplet acetone, a value of $k_q\tau^0 = 4.7 \times 10^3\ M^{-1}$ was obtained for the sorbate ion.

Quenching by indole compounds, tyrosine derivatives, and quinones

Triplet acetone produced by TMD cleavage is quenched by indoles, 3,5-dihalogenotyrosines, and quinones. Table 2 summarizes the data for these classes of quenchers obtained from Stern–Volmer

Table 2. Stern–Volmer constants for quenching of DBAS fluorescence[a] and triplet acetone generated by TMD thermolysis[b] monitored indirectly by DBAS sensitized fluorescence

Quencher	Excited species		Ionization potential (eV)
	Singlet DBAS ($k_q\tau^S_{DBAS}$, $\times 10^{-2}\ M^{-1}$)	Triplet acetone ($k_q\tau^T_{acetone}$, $\times 10^{-3}\ M^{-1}$)	
Indole	1.2	4.4	7.9[c]
N-Methylindole	1.0	4.7	
D-Tryptophan	small	2.3	7.8[d]
L-Tryptophan	small	2.2	
Lys-Try-Lys	1.2	2.3	
Tyrosine (pH 6.4)	0.7	0.1	(8.45)[e]
(pH 7.4)	0.7	0.07	
3,5-Dichlorotyrosine (pH 6.4)	1.6	0.9	(8.52)[f]
(pH 7.4)	1.5	0.5	
3,5-Dibromotyrosine (pH 6.4)	2.9	1.3	(8.43[e]–8.69[f])
(pH 7.4)	1.3	1.5	
3,5-Diiodotyrosine (pH 6.4)	1.7	1.9	
(pH 7.4)	1.3	2.6	
p-Benzoquinone (water)	4.8	19	10.8[e]–10.21[f]
(*n*-hexane)	0.7	1.4	
(decalin)	0.7	0.5	
2,6-Dimethyl-p-benzoquinone	4.3	6.5	10.1[g]
Tetrachloro-p-benzoquinone	7.1	13	
Tetrabromo-o-benzoquinone	38	19	
1,4-Naphthoquinone	13	36	10.5[e]
2-Methyl-1,4-naphthoquinone	13	15	9.5[e]
Anthraquinone-2-sulfonate ion	31	4.5	(10.7)[e]

[a]DBAS was excited at 383 nm and the DBAS fluorescence quenching measured at 424 nm. [b]Unless indicated in brackets, the experiments were carried out in bidistilled water at 32–37°C with 0.10–0.25 m*M* TMD. In the case of buffered solutions, 50 m*M* phosphate plus 5 m*M* pyrophosphate was used. [c]Fischer-Hjalmans and Sundbon (1968); [d]Moan (1973); [e]Gayoso *et al.* (1973); [f]Dougherty and McGlynn (1977); [g]Hojer *et al.* (1973). The ionization potential values indicated in brackets were reported for analogous compounds.

type studies using DBAS to monitor triplet acetone. The ionization potentials of the quenchers are also listed in Table 2.

All Stern–Volmer plots were corrected for the direct quenching of singlet DBAS using the expression (Wagner, 1971)

$$I_0/I = [1 + k_q^{DBAS}\tau^S_{DBAS}(Q)][1 + k_q^{acetone}\tau^T_{acetone}(Q)]$$

where (Q) is the quencher concentration, k_q quenching rate constant, τ^S_{DBAS} the lifetime of singlet DBAS and $\tau^T_{acetone}$ the lifetime of triplet acetone, both in the absence of added quencher. The data for direct quenching of DBAS fluorescence by these compounds are also collected in Table 2.

Each of the $k_q\tau^0$ determinations was based on plots with at least six different quencher concentrations, covering a range of about one order of magnitude. The precision of I_0 and I measurements was usually within 5%. Representative examples are shown in Figs. 3–5 for quenching by 2,6-dimethyl-*p*-benzoquinone (DMBQ), Lys-Trp-Lys and 3,5-diiodotyrosine (DIT) at pH 7.4, respectively. 3,5-Dihalogenoderivatives were studied at pH 6.4 and 7.4 in order to examine the contribution of the phenoxide form to the quenching efficiency (pK_a = 6.4; Gemmill, 1955). Also tyrosine (pK_a = 10) was studied at pH 6.4 and 7.4.

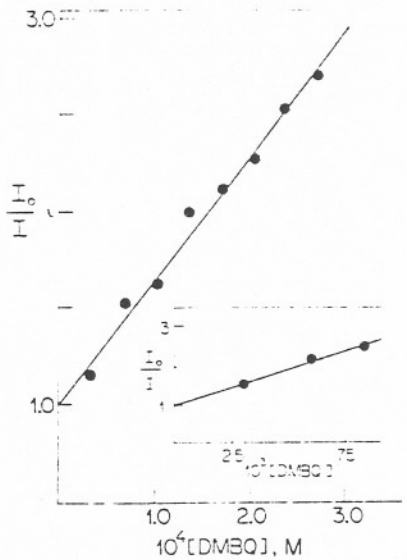


Figure 3. Stern–Volmer plot for the quenching of triplet acetone from TMD thermolysis by 2,5-dimethyl-*p*-benzoquinone, monitored by DBAS (2.5 μ*M*) sensitized fluorescence in bidistilled water. The insert shows the quenching of photoexcited DBAS fluorescence by the same quencher (conditions described in Table 2).

DISCUSSION

Triplet acetone generated from TMD thermolysis in air-equilibrated aqueous solutions has been shown to be intercepted by several categories of synthetic and natural compounds. Table 3 lists these classes of quenchers and fluorescent acceptors, their ionization potentials, and the corresponding Stern–Volmer constants ($k_q\tau^0$ or $k_{ET}\tau^0$) measured in this work and in analogous studies with enzymically generated triplet acetone.

Chemical data

Based on the studies of Mirbach *et al.* (1980), the quenching of "exposed" triplet acetone by quinones is expected to occur at a rate approaching the diffusion controlled limit due to their high electron affinities. Mirbach and coworkers reported that triplet acetone generated from TMD thermolysis in acetonitrile at 56°C is quenched by biacetyl (IP = 9.55 eV), trans-dicyanoethylene (IP = 11.15 eV) and maleic anhydride with rate constants in the range of 10^9–10^{10} M^{-1} s^{-1} and concluded that an interaction π (acetone) → π (acceptor) is operative in these cases. Assuming a lifetime of triplet acetone in normally aerated water of 2 μs[7] (Catalani and Bechara, unpublished), our data indicate k_q values for quenching by quinones in the range of 5 to 20 × 10^9 M^{-1} s^{-1}. In fact, methyl substitution of *p*-benzoquinone and p-naphthoquinone, which

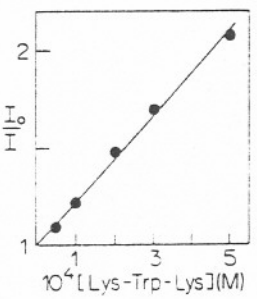


Figure 4. Stern–Volmer plot for the quenching of triplet acetone from TMD thermolysis by Lys-Try-Lys in the presence of 2.5 μ*M* DBAS, in phosphate/pyrophosphate buffer pH 7.4, at 32°C.

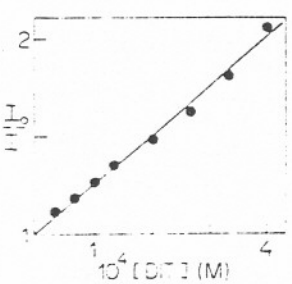


Figure 5. Stern–Volmer plot for the quenching of triplet acetone from TMD thermolysis by 3,5-diiodotyrosine in the presence of 2.5 μ*M* DBAS, in phosphate pyrophosphate buffer pH 7.4 at 36°C.

should decrease the electron affinity, resulted in lower quenching constants. On the other hand, chlorine and bromine substitution did not increase k_q, consistent with quenching at the diffusion limited rate for the remaining quinones. Another evidence for diffusion controlled collisional quenching by quinones is the effect of solvent viscosity: upon changing the solvent from *n*-hexane to decalin (a viscosity increase of a factor of seven), the $k_q\tau^0$ values decreased by a factor of three. The difference in these factors can be attributed to the longer lifetime of triplet acetone in decalin due to the slower diffusion of O_2 in this solvent (the O_2 solubility in both solvents is approximately the same).

The data for the indole compounds, all of which have low ionization potentials, suggest that the quenching processes is dominated by a charge transfer interaction or an electron-transfer step. Indeed, according to Kasama *et al.* (1982), "the quenching processes of triplet acetone by indoles, being diffusion controlled, occur *via* the following paths: triplet–triplet energy transfer, electron transfer, photoaddition of triplet acetone to the 2-carbon atom of the indole ring, and deactivation without a chemical range". As expected, "exposed" triplet acetone does not exhibit chiral discrimination with D- and L-tryptophan, unlike the triplet acetone produced during the aerobic oxidation of isobutanal catalyzed by HPR (Rivas-Suárez and Cilento, 1981).

The quenching of triplet acetone by tyrosine and its 3,5-dihalogenoderivatives, whose ionization potentials are probably somewhat higher than those of indoles (~8 eV, and ~8.5 eV in the case of halogenated phenols; Table 2), may occur (i) by triplet–triplet energy transfer, (ii) by charge-transfer, (iii) by electron transfer or, (iv) in the case of the phenolic form, by H or H^- transfer (Rivas-Suárez *et al.*, 1983). The fact that the $k_q\tau^0$ values are smaller than those expected for a collisional process indicates that the intermediacy of a transient limits the quenching rate. The slight differences in the $k_q\tau^0$ values obtained at pHs 6.4 and 7.4 may signify that an electron-transfer step or hydrogen transfer is not the predominant deactivation mechanism of the triplet acetone.

Since the acetone phosphorescence spectrum overlaps the absorption spectrum of riboflavin and $k_{ET}\tau^0$ measured for triplet acetone-riboflavin is ~10^4 M^{-1}, the emission of riboflavin observed from aqueous solutions of TMD is probably due to a Förster-type long range triplet-singlet transfer process. Nevertheless one cannot exclude a collisional T_1 (acetone)–T_n (riboflavin) transfer, followed by intersystem crossing T_n–S_1 and fluorescence emission.

Finally, the data for xanthene dyes are consistent with a triplet (T_1 of acetone)–upper triplet (T_n of the dye) energy transfer process at a somewhat slower than diffusion controlled rate, followed by intersystem crossing to the dye fluorescent state (Durán and Cilento, 1980). The relative efficiencies of the overall T_1–S_1 transfer from excited acetone to the dyes (320:13:1 for Rose Bengal, eosine and fluorescein, respectively) would thus reflect heavy atom effects (four iodine atoms in rose bengal, four bromines in eosine, and no halogens in fluorescein) on the rate of intersystem crossing from T_n (dye) to S_1 (dye).

Comparison between the chemical and the enzymatic systems

Previous studies by Cilento and coworkers (Cilento *et al.*, 1978; Bechara *et al.*, 1979; Cilento, 1980a; Cilento, 1980b; Cilento, 1982) of the quenching of enzymically (HRP/O_2/isobutanal) generated triplet acetone support the idea that the apoenzyme offers some protection to the nascent excited species against external quenchers, including those described in the present work. Although the quenching and energy transfer experiments provide only $k_q\tau^0$ and $k_{ET}\tau^0$ values, several arguments were presented in favour of long-range processes in most of the cases.

In general, comparison of the chemical and the enzymatic data summarized in Table 3 indicates that the $k_q\tau^0$ and $k_{ET}\tau^0$ values are roughly one order of magnitude higher in the enzymic case, independent upon the nature of the triplet acetone interceptor and, consequently, on the type of deactivation mechanism (e.g. triplet–triplet energy transfer with xanthene dyes and collisional quenching with quinones). In this regard, it should be noted that the lifetime of triplet acetone is ten times longer in deaerated water than in air-equilibrated aqueous solutions (20 μs *vs* 2 μs; Porter *et al.*, 1973, and Catalani e Bechara, unpublished). Thus, the higher quenching constants in the enzymatic system may simply reflect, at least partially, a longer lifetime of triplet acetone in an enzyme "pocket". In addition to the systematic difference of one order of magnitude in the constant values for all classes of quenchers, the observation of the same trends in the chemical and the enzymatic quenching data within classes of quenchers probably means that the essential features of the quenching mechanisms are the same in both cases.

At least three simple hypotheses might, in principle, explain "protection" of triplet acetone by the enzyme against deactivation by collisions with oxygen: (i) the apo-HRP constitutes a barrier against penetration of oxygen to the site of excited acetone generation; (ii) the local oxygen concentration near the site of triplet acetone generation is substantially lower than the bulk value due to local consumption of oxygen by the substrate; and (iii) the microscopic physical properties of the cavity where triplet acetone is formed favor an intrinsically longer lifetime and/or the oxygen solubility in this site is lower due to a more highly polar environment the enzyme pocket.

Hypothesis (i) encounters a parallel in Saviotti and Galley's demonstration (1974) that "buried"

Table 3. Quenching of triplet acetone by biologically related compounds: a comparison between a chemical (TMD) and an enzymatic source (isobutanal/O_2/HRP) of the excited species

Quencher	Quenching constants ($k\tau^0$, M^{-1}) Chemical source	Enzymic source	IP (eV)
Indoles	$2–5 \times 10^3$	$0.8–4 \times 10^4$[a]	8[f]
Tyrosine and its 3,5-dihalogeno derivatives	$0.2–2.0 \times 10^3$	$0.1–1 \times 10^4$[b]	8.5[f]
Quinones	$1–4 \times 10^4$	$0.1–3 \times 10^5$[c]	10–11[f]
Riboflavin	2×10^4	4.5×10^4[d]	—
Xanthene Dyes	$0.6–2 \times 10^4$	$3–10 \times 10^4$[e]	7–7.5[g]

[a]Rivas-Suárez and Cilento (1981); [b]Rivas-Suárez *et al.* (1983); [c]Rivas-Suárez *et al.* (1981); [d]Haun *et al.* (1978); [e]Durán and Cilento (1980); [f]See Table 2. [g]Gouverneur *et al.* (1974).

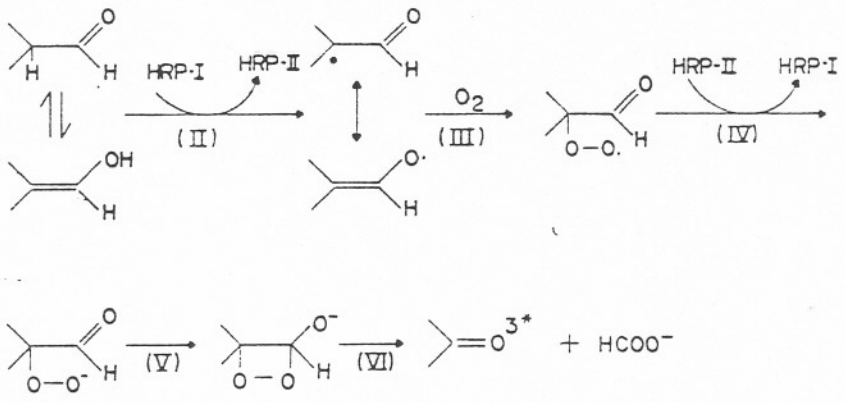
tryptophan residues of proteins can phosphoresce at room temperature in aerated fluid solutions ascribed to protection by the protein barrier against typical bimolecular quenchers, including oxygen (see also Alpert and Lindqvist, 1975; Eflink and Ghiron, 1977; Kishner *et al.*, 1979; Domanus *et al.*, 1980). Turro and coworkers (1978) reported the observation of weak phosphorescence from 1,4-dibromonaphthalene in aerated micellar solutions and, more recently (1982), observed strong protection against oxygen quenching of intramolecular excimer emission in aqueous cyclodextrin solutions. In addition, Rivas-Suárez and Cilento's demonstration (1981) of distinct kinetics for quenching of HRP-generated triplet acetone by D- and L-tryptophan provides strong evidence for the occurrence of collisional events between the excited species and the quencher in a chiral environment. On the other hand, the lifetime of enzyme generated triplet acetone extrapolated to time zero [$(O_2) \sim 0.2$ mM in water at 35°C; Robinson and Cooper, 1970] is shorter than that at complete oxygen depletion time by a factor of only 2.5, i.e. acetone phosphorescence appears as soon as HRP is added to a buffered air-equilibrated solution of isobutanal. Despite this evidence, the "protection" hypothesis suffers from the paradox of requiring that the HRP protein barrier limits oxygen penetration but not that of much larger collisional quenchers, added to the system. More recently, Calhoun *et al.* (1983a) demonstrated that oxygen quenches both the phosphorescence of "buried" tryptophans of liver alcohol dehydrogenase and alkaline phosphatase and the fluorescence of more exposed tryptophans with about the same efficiency, only about 1–1.5 decades slower than the diffusion-limited rate in free solution. Thus, the quenching by oxygen seems to be limited by internal protein diffusion rather than by the entry step. In fact, a number of molecules considerably larger than oxygen were found by Calhoun *et al.* (1983b) to quench protein fluorescence, including acrylamide, ketones and inorganic anions and cations.

The hypothesis of "local depletion of oxygen" can probably be discarded on the basis of the relative rates of production of triplet acetone by the enzyme and of O_2 diffusion from the bulk solution to the active site. The enzymic generation of a triplet acetone molecule requires the following steps: (i) oxygen diffusion to the HRP-isobutanal complex; (ii) hydrogen abstraction from the substrate by HRP Compound I; (iii) addition of oxygen to the substrate-radical yielding a hydroperoxy radical; (iv) reduction of the latter by HRP Compound II to give a hydroperoxy anion; (v) cyclization to a dioxetane intermediate, and (vi) cleavage to formate and triplet acetone (Scheme I; Bechara *et al.*, 1979). Very likely, the time required for so many steps, exceeds that required to reestablish the steady state oxygen concentration at the active site.

Finally, in addition to a slower rate of diffusion of oxygen across the protein and a lower solubility of oxygen in an ionic microenvironment where triplet acetone is formed, it is conceivable that the lifetime of HRP-generated triplet acetone is intrinsically longer within the enzyme. Indeed, oxygen solubility tends to decrease in more polar solvents, being 3 mM in isooctane, 2 mM in benzene, 1.6 mM in 95% ethanol and 0.24 mM in water at 25°C (*Handbook of Photochemistry*, 1973).

Thus, an intrinsic longer lifetime of triplet acetone in the enzyme cavity, together with a lower local oxygen concentration, could explain the relatively high values of $k_q\tau^0$ and $k_{ET}\tau^0$ observed for the isobutanal/O_2/HRP system. In some cases, a static quenching component may also contribute to the high quenching constants in the enzymic system.

Independent of the precise mechanisms of quenching and "protection", the fact that energy transfer from enzymically generated triplet species can efficiently drive photochemical processes in the dark is, in itself, potentially of great importance for living cells (Cilento, 1982). "Protection" of the



excited species against collisional deactivation by oxygen might thus be another biological role of the apo-HRP, in addition to solubilization and protection of hemin—the HRP prostetic group—against autooxidative destruction (Dunford and Stillman, 1976).

Acknowledgements—The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Financiadora de Estudos e Projetos (FINEP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Organization of American States for their generous support of this research. Luiz H. Catalani is a FAPESP predoctoral fellow.

We also thank Dr. Giuseppe Cilento for continuous encouragement and Dr. Frank Quina for reading the manuscript.

REFERENCES

Alpert, B. and L. Lindqvist (1975) *Science* **187**, 836–837.

Bartlett, P. D., A. L. Baumstark and M. E. Landis (1973) *J. Am. Chem. Soc.* **95**, 6486–6487.

Battegay, M. and P. Brandt (1923) *Bull. Soc. Chim. Fr.* **33**, 1667–1678.

Bechara, E. J. H., O. M. M. Faria Oliveira, N. Durán, R. Casadei de Baptista and G. Cilento (1979) *Photochem. Photobiol.* **30**, 101–110.

Bechara, E. J. H. and T. Wilson (1980) *J. Org. Chem.* **45**, 5261–5268.

Bouchilloux, S. (1955) *Bull. Soc. Chim. Biol.* **37**, 255–258.

Calhoun, D. B., J. M. Vanderkooi, G. V. Woodrow III and S. W. Englander (1983a) *Biochemistry* **22**, 1526–1532.

Calhoun, D. B., J. M. Vanderkooi and S. W. Englander (1983b) *Biochemistry* **22**, 1533–1539.

Cilento, G. (1980a) *Photochem. Photobiol. Rev.* **5**, 199–227.

Cilento, G. (1980b) *Acc. Chem. Res.* **13**, 225–230.

Cilento, G. (1982) In *Chemical and Biological Generation of Excited States* (Edited by W. Adam and G. Cilento) pp. 277–307. Academic Press, New York.

Cilento, G., N. Durán, K. Zinner, C. C. C. Vidigal, O. M. M. Faria Oliveira, M. Haun, A. Faljoni, O. Augusto, R. Casadei de Baptista and E. J. H. Bechara (1978) *Photochem. Photobiol* **28**, 445–451.

Domanus, J., G. B. Strambini and W. C. Galley (1980) *Photochem. Photobiol.* **31**, 15–21.

Dougherty, D. and S. P. McGlynn (1977) *J. Chem. Phys.* **67**, 1289–1290.

Dunford, H. B. and J. S. Stillman (1976) *Coord. Chem. Rev.* **19**, 187–251.

Durán, N. and G. Cilento (1980) *Photochem. Photobiol.* **32**, 113–116.

Eftink, M. R. and C. A. Ghiron (1977) *Biochemistry* **16**, 5546–5551.

Encinas, M. V., E. A. Lissi and J. C. Scaiano (1980) *J. Phys. Chem.* **84**, 948–951.

Fieser, L. S. (1940) *J. Biol. Chem.* **133**, 391–396.

Fischer-Hjalmans, I. and M. Sundbon (1968) *Acta Chem. Scand.* **22**, 607–627.

Gayoso, J., A. Boucekkine and H. Bouanani (1973) *J. Chim. Phys. Physicochim. Biol.* **70**, 1643–1650.

Gemmill, C. L. (1955) *Arch. Biochem. Biophys.* **54**, 359–367.

Gouverneur, L., G. Leroy and I. Zador (1974) *Electrochim. Acta* **19**, 215–225.

Handbook of Photochemistry (1973) (Edited by S. L. Murov), p. 89. Marcel Dekker, New York.

Haun, M., N. Durán and G. Cilento (1978) *Biochem. Biophys. Res. Commun.* **81**, 779–784.

Höjer, G., S. Meza and M. E. Ruiz (1973) *Acta Chem. Scand.* **27**, 1860–1874.

Kasama, K., A. Takematsu and S. Arai (1982) *J. Phys. Chem.* **86**, 2420–2427.

Kishner, S., E. Trepman and W. C. Galley (1979) *Can. J. Biochem.* **57**, 1299–1304.

Kopecky, K. R., J. E. Filby, C. Mumford, P. A. Lockwood and J.-Y. Ding (1975) *Can. J. Chem.* **53**, 1103–1122.

Loutfy, R. O. and R. W. Yip (1973) *Can. J. Chem.* **51**, 1881–1884.

Loutfy, R. O., R. W. Yip and S. K. Dogra (1977) *Tetrahedron Lett.* **33**, 2843–2846.

Mirbach, M. F., V. Ramamurthy, M. J. Mirbach, N. J. Turro and P. J. Wagner (1980) *Nouv. J. Chim.* **4**, 471–474.

Moan, J. (1973) *Chem. Phys. Lett.* **18**, 446–450.

Porter, G., S. K. Dogra, R. O. Loutfy, S. E. Sugamori and R. W. Yip (1973) *J. Chem. Soc. Farad. Trans. I* **69**, 1462–1474.

Rivas-Suárez, E. and G. Cilento (1981) *Biochemistry* **20**, 7329–7333.

Rivas-Suárez, E., O. Augusto and G. Cilento (1981) *Photochem. Photobiol.* **33**, 279–282.

Rivas-Suárez, E., N. Durán and G. Cilento (1979) *Photochem. Photobiol.* **30**, 111–115.

Rivas-Suárez, E., L. H. Catalani, E. J. H. Bechara and G. Cilento (1983) *Photochem. Photobiol.* **37**, 93–97.

Robinson, J. and J. M. Cooper (1970) *Anal. Biochem.* **33**, 390–399.
Saviotti, M. L. and W. C. Galley (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4154–4158.
Steinmetzer, H. C., P. Lechtken and N. J. Turro (1973) *Justus Liebigs Ann. Chem.* 1984–2000.
Turro, N. J. (1978) In *Modern Molecular Photochemistry*. Benjamin Cummings, Menlo Park, CA.
Turro, N. J., L. Kou-Chang, M.-F. Chow and P. Lee (1978) *Photochem. Photobiol.* **27**, 523–529.
Turro, N. J., T. Okubo and G. C. Weed (1982) *Photochem. Photobiol.* **35**, 325–329.
Turro, N. J. and Y. Tanimoto (1980) *J. Photochem.* **14**, 199–203.
Wade, M. J. and J. D. Spikes (1971) *Photochem. Photobiol.* **14**, 221–224.
Wagner, P. J. (1967) *J. Am. Chem. Soc.* **89**, 5715–5717.
Wagner, P. J. (1971) *Mol. Photochem.* **3**, 23–34.
Wilson, T. and A. M. Halpern (1980) *J. Am. Chem. Soc.* **102**, 7279–7283.
Wilson, T., D. E. Golan, M. S. Harris and A. L. Baumstark (1976) *J. Am. Chem. Soc.* **98**, 1086–1091.

Anexo IX

Synthesis of trialkylsilylated α-hydroperoxy aldehydes and ketones via ozonolysis of protected allylil hydroperoxides. Adam, W.; Catalani, L.H.; Saha-Möller, C.R.; Will, B. *Synthesis* 121 (1989).

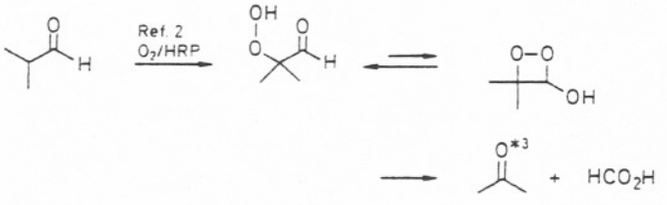
Synthesis of Trialkylsilylated α-Hydroperoxy Aldehydes and Ketones via Ozonolysis of Protected Allylic Hydroperoxides

Waldemar Adam,* Luiz H. Catalani,[1] Chantu R. Saha-Möller, Bernd Will

Institute of Organic Chemistry, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany

α-Silylperoxy aldehydes and ketones **3** have been prepared by ozonolysis of silyl-protected allylic hydroperoxides, followed by subsequent reduction of the latter with dimethyl sulfide. Deprotection of silylperoxy aldehydes **3a–c** with fluoride ion led to decomposition with chemiluminescence.

In the horse radish peroxidase (HRP) catalyzed autoxidation of isobutyraldehyde affording acetone in a triplet excited state, 3-hydroxy-1,2-dioxetanes were postulated as intermediates. Cyclization of the intermediary α-hydroperoxy aldehyde leads



to its cyclic tautomer, which as a labile species is expected to fragment via a chemical excitation into triplet acetone and the formate ion, the former manifesting itself through chemiluminescence.[3]

Since to date no α-hydroperoxy aldehydes appear to have been isolated to substantiate the above mechanistic claim, we attempted to prepare stable derivatives and confirm their role as chemiluminescent precursors. In view of the anticipated labile nature of the α-hydroperoxy aldehydes, we decided to prepare trialkylsilylated derivatives via the sequence outlined below, releasing the free substances by mild desilylation at the moment of need.

The allylic hydroperoxides **1** that are required as starting materials were prepared by conventional photooxygenation[5] and are all known. For the silylation[4,6] to the silylperoxy derivatives **2**, we preferred *tert*-butyldimethylsilyl chloride because of the higher hydrolytic stability of the subsequent products (Table 1). Ozonolysis in methanol at −78 °C followed by dimethyl sulfide reduction led to the α-silylperoxy carbonyl compounds **3** in moderate to good yields (Table 2).

Desilylation by methanol of α-silylperoxy ketone **3e** afforded the known 2-hydroperoxy-2-methylbutanone, which was independently prepared by base-catalyzed autoxidation of 3-methylbutan-2-one.[7] However, for the α-silylperoxy aldehydes **3a–c**,

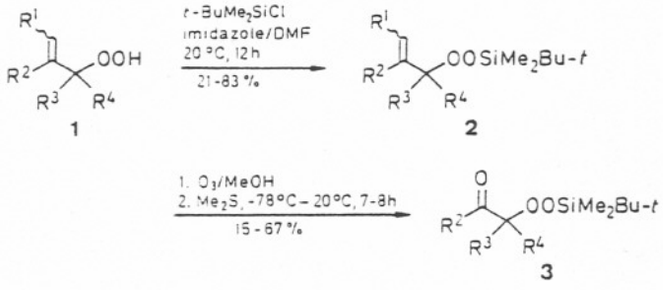
Table 1. Allylic *tert*-Butyldimethylsilyl Peroxides **2** Prepared

Product	Yield[a] (%)	Molecular Formula[b]	IR (neat or CCl_4) ν (cm^{-1})	^{1}H-NMR ($CDCl_3$/TMS)[c] δ, J(Hz)	^{13}C-NMR ($CDCl_3$/TMS)[d] δ
2a	21	$C_{10}H_{22}O_2Si$ (202.4)	3160, 3090, 2960, 2930, 2890, 2860, 1640, 1470, 1460, 1255, 1250 (s, SiOO), 1140, 1050, 990, 870	0.15 (s, 6H, $(CH_3)_2$); 0.92 (s, 9H, $C(CH_3)_3$); 1.25 (d, 3H, J = 6.4); 4.42 (dddq, 1H, J = 6.4, 6.9, 1.0, 1.2); 5.16 (ddd, 1H, J = 10.2, 1.4, 1.0); 5.23 (ddd, 1H, J = 17.2, 1.4, 1.2); 5.83 (ddd, 1H, J = 17.2, 10.2, 6.9)	−5.6 (q); 18.2 (s); 18.25 (q); 26.2 (q); 82.2 (d); 116.8 (t); 138.3 (d)
2b	48	$C_{12}H_{26}O_2Si$ (230.4)	2970, 2935, 2882, 2860, 1670, 1470, 1460, 1365, 1255, 1250 (s, SiOO), 965, 880, 840, 785	0.15 (s, 6H, $(CH_3)_2$); 0.89 (t, 3H, CH_2CH_3, J = 7.5); 0.94 (s, 9H, $(CH_3)_3$); 1.46 (ddq, 1H, CH_ACH_3, J = 15.0, 7.5); 1.71 (ddq, 1H, CH_BCH_3, J = 15.0, 7.5, 5.7); 1.73 (dd, 3H, $C{=}CCH_3$, J = 6.6, 1.7); 4.13 (ddt, 1H, CH−OOSi, J = 0.6, 8.2, 5.7); 5.38 (ddq, 1H, J = 1.7, 15.3, 8.2); 5.7 (ddq, 1H, J = 6.6, 15.3, 0.6)	−5.6 (q); 9.7 (q); 17.8 (q); 18.2 (s); 25.7 (t); 25.9 (q); 87.8 (d); 130.0 (d); 130.2 (d)
2c	72[e]	$C_{11}H_{24}O_2Si$ (216.4)	3090, 2960, 2935, 2900, 2860, 1650, 1470, 1460, 1360, 1255, 1250 (s, SiOO), 1140, 1080, 905	0.15 (s, 6H); 0.94 (s, 9H); 1.31 (s, 6H); 5.06 (dd, 1H, J = 1.2, 11.0); 5.16 (dd, 1H, J = 1.2, 17.7); 6.0 (dd, 1H, J = 11.0, 17.7)	−5.5 (q); 18.3 (s); 24.3 (q); 26.3 (q); 82.2 (s); 113.4 (t); 142.9 (d)
2d	66[e]	$C_{11}H_4O_2Si$ (216.4)	3095, 2965, 2940, 2900, 2865, 1655, 1475, 1465, 1370, 1250 (s, SiOO), 1145, 1080, 910	0.15 (s, 6H); 0.94 (s, 9H); 1.10 (d, 3H, J = 6.5); 1.59 (dd, 3H, $C{=}CCH_3$, J = 1.4, 1.0); 4.26 (q, 1H, CH−OOSi, J = 6.5); 4.73 (m, 1H); 4.78 (m, 1H)	−5.7 (q); 13.4 (q); 17.5 (q); 18.2 (s); 26.2 (q); 84.6 (d); 112.6 (t); 145.1 (s)
2e	83	$C_{12}H_{26}O_2Si$ (230.4)	3100, 2960, 2890, 2860, 1640, 1470, 1460, 1370, 1360, 1255, 1250 (s, SiOO), 1150, 1090, 900	0.12 (s, 6H); 0.92 (s, 9H); 1.30 (s, 6H); 1.78 (m, 3H); 4.83 (m, 1H); 4.89 (m, 1H)	−5.6 (q); 18.7 (s); 24.2 (q); 25.7 (q); 26.3 (q); 83.8 (s); 110.6 (t); 149.4 (s)

[a] Yield of the analytically pure product based on hydroperoxide **1**.
[b] Satisfactory microanalyses obtained: C ± 0.14, H ± 0.22.
[c] Recorded on a Bruker WM-400 (400 MHz) spectrometer.
[d] Recorded on a Bruker AC200 (50 MHz) or WM-400 (100 MHz) spectrometer.
[e] A 1:1 mixture of allyl hydroperoxides **1c** and **1d** was used in the silylation, but the yields are based on 100% of each of the corresponding hydroperoxides.

desilylation with methanol gave the hemiacetals, which were detected by means of ^{1}H-NMR spectroscopy. Attempts to extrude methanol from these led to decomposition. Fluoride ion catalyzed deprotection also gave decomposition, but with chemiluminescence. Presumably the intermediary α-peroxy anion cyclized (precedents are known[8]) to the labile dioxetane and cleavage afforded the electronically excited carbonyl compound with subsequent light emission. Although it was still not possible to isolate stable α-hydroperoxy aldehydes, the results make their intermediacy seem plausible.

Commercial grade reagents and solvents were used without further purification, except when indicated. Infrared spectra were obtained by using Perkin-Elmer 1420 spectrophotometer. Elemental analyses were performed either in-house or at the Microanalysis Laboratories of the University of Giessen (FRG). We thank Prof. Dr. G. Maier and his staff for this service. Mass spectra were recorded on a Finnigan MAT 8200 spectrometer at 80 eV. Melting points and boiling points are uncorrected. The qualitative chemiluminescence experiments of the fluoride ion promoted deprotection of the silylperoxy aldehydes **3a–c** was performed using the apparatus and method described previously.[9]



I–3	R^1	R^2	R^3	R^4
a	H	H	H	CH_3
b	CH_3	H	H	CH_3CH_2
c	H	H	CH_3	CH_3
d	H	CH_3	H	CH_3
e	H	CH_3	CH_3	CH_3

Photooxygenation of Alkenes to Form Allylic Hydroperoxides 1; General Procedure:

The starting alkene (20 mmol) is dissolved in CH_2Cl_2 (20 mL) containing tetraphenylporphin (2–5 mg) as sensitizer. The mixture is irradiated with a sodium street lamp (Phillips G/98/2 SON 150 Watt) at −5 °C, while a slow stream of dry oxygen gas is passed through. The reaction is monitored by ^{1}H-NMR spectroscopy and/or TLC. After completion (2–4 d), the solvent is removed on a rotary evaporator (0 °C/20 Torr), and the residue is fractionally distilled at reduced pressure.

Allylic *tert*-Butyldimethylsilyl Peroxides 2; General Procedure:

In a flame-dried, 50-mL, three-necked, round-bottomed flask containing a solution of *tert*-butyldimethylsilyl chloride (1.8 g, 12 mmol) and imidazole (0.95 g, 14 mmol) in DMF (50 mL, freshly distilled from CaH_2) is added the hydroperoxide **1** (10 mmol) under a nitrogen atmosphere. For **1a** and **1b** imidazole is replaced by diisopropylmethylamine (1.8 g, 12 mmol) and *N,N*-dimethylaminopyridine (6.0 mg, 0.4 mmol). The reaction mixture is stirred at 20 °C for 12 h (for **2c** and **2d** 2h), diluted with ether (50 mL), quickly washed with 1 N aq. HCl (40 mL) and with sat. aq. $NaHCO_3$ (40 mL), and dried (Na_2SO_4). The solvent is removed by rota-evaporation (0 °C/20 Torr) and the residue is purified by flash chromatography (SiO_2, hexane/CH_2Cl_2, 1:1, 20 °C).

2-Oxoalkyl *tert*-Butyldimethylsilyl Peroxides 3; General Procedure:

Silyl peroxide **2** (2 mmol) is dissolved in absolute MeOH (20 mL), cooled to −78 °C. A gentle stream of dry ozone is allowed to pass through the solution until the characteristic blue ozone color persists.

Table 2. α-Silylperoxy Carbonyl Compounds **3** Prepared

Product	Yield[a] (%)	Molecular Formula[b] or MS (DCI, 80 eV) *m/z* (%)	IR (neat or CCl_4) ν (cm^{-1})	^{1}H-NMR ($CDCl_3$/TMS)[c] δ, *J* (Hz)	^{13}C-NMR ($CDCl_3$/TMS)[d] δ
3a	15	222 (100, $M^+ + 18$)	2960, 2930, 2860, 1735 (s, C=O), 1470, 1460, 1375, 1360, 1255, 1250 (s, SiOO)	0.20 (s, 6H); 0.97 (s, 9H); 1.23 (d, 3H, J = 6.1); 4.29 (dq, 1H, J = 6.1, 3.0); 9.80 (d, 1H, J = 3.0)	−5.9 (q); 12.9 (q); 18.1 (s); 26.0 (q); 84.9 (d); 203.1 (d)
3b	23	236 (100, $M^+ + 18$)	2960, 2935, 2890, 2860, 2820, 1740 (s, C=O), 1470, 1460, 1365, 1255, 1250 (s, SiOO), 880	0.18 (s, 3H, $SiCH_3$); 0.20 (s, 3H, $SiCH_3$); 0.94 (s, 9H, $(CH_3)_3$); 1.0 (t, 3H, CH_2CH_3, J = 7.5); 1.66 (dq, 2H, CH_2CH_3, J = 7.5, 6.8); 4.10 (dt, 1H, CH–OOSi, J = 6.8, 3.2); 9.77 (d, 1H, J = 3.2)	−5.9 (q); 9.4 (q); 18.2 (s); 21.5 (t); 26.0 (q); 90.1 (d); 203.5 (d)
3c	24	$C_{10}H_{22}O_3Si$ (218.4)	2960, 2940, 2900, 2860, 1740 (s, C=O), 1475, 1465, 1385, 1365, 1260, 1255 (s, SiOO), 1175	0.16 (s, 6H); 0.92 (s, 9H); 1.27 (s, 6H); 9.70 (s, 1H)	−5.7 (q); 18.2 (s); 19.4 (q); 26.1 (q); 86.5 (s); 203.2 (d)
3d	25	$C_{10}H_{22}O_3Si$ (218.4)	2960, 2930, 2895, 2858, 1725 (s, C=O), 1470, 1460, 1355, 1250 (s, SiOO), 1145, 1090, 862	0.14 (s, 3H); 0.16 (s, 3H); 0.91 (s, 9H); 1.22 (d, 3H, J = 7.0); 2.26 (s, 3H); 4.27 (q, 1H, J = 7.0)	−5.9 (q); 14.7 (q); 18.1 (s); 24.3 (q); 26.0 (q); 86.2 (d); 210.9 (s)
3e	67	$C_{11}H_{24}O_3Si$ (232.4)	2960, 2930, 2890, 2860, 1715 (s, C=O), 1470, 1460, 1375, 1360, 1350, 1255, 1250 (s, SiOO), 1215, 1165, 1130, 845	0.17 (s, 6H); 0.93 (s, 9H); 1.29 (s, 6H); 2.27 (s, 3H)	−5.7 (q); 18.2 (s); 21.4 (q); 23.9 (q); 26.1 (q); 87.9 (s); 211.4 (s)

[a] Yield of the analytically pure product based on **2**.
[b] Satisfactory microanalyses obtained: C ±0.33, H ±0.15.
[c] Recorded on a Bruker WM-400 (400 MHz) spectrometer.
[d] Recorded on a Bruker AC200 (50 MHz) or WM-400 (100 MHz) spectrometer.

The excess ozone is removed by purging the reaction mixture with dry nitrogen gas, and dimethyl sulfide (2 mL) is added at −78 °C. The resulting solution is warmed to 20 °C and stirred for 7–8 h. The solvent is removed on a rotary evaporator (0 °C/20 Torr), and the residue is purified by means of flash chromatography (SiO_2, hexane/CH_2Cl_2, 1:1, −20 °C).

We are grateful to the Deutsche Forschungsgemeinschaft (SFB Nr. 172 "Molekulare Mechanismen kanzerogener Primärveränderungen"), the Sander Stiftung, the A. v. Humboldt Stiftung, the Fritz Thyssen Stiftung, and the Fonds der Chemischen Industrie for generous financial support.

Received: 12 October 1988

(1) A. v. Humboldt postdoctoral fellow 1984–86; present address: Department of Fundamental Chemistry, University of Sao Paulo, Cidade Universitaria, Caixa Postal 20.780, Sao Paulo, Brasil.
(2) Cilento, G. *Pure Appl. Chem.* **1984**, *56*, 1179.
(3) Cilento, G., Adam, W. *Photochem. Photobiol.* **1988**, *48*, 361.
(4) Clark, G.R., Nikaido, M.M., Fair, C.K., Lin, J. *J. Org. Chem.* **1985**, *50*, 1994.
(5) Frimer, A.A. (ed.) "Singlet Oxygenation", CRC Press, Boca Raton, Florida, 1985.
(6) Corey, E.J., Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190.
(7) Cubbon, R.C.P., Hewlett, C. *J. Chem. Soc.* **1968**, 2978.
Sawaki, Y., Ogata, Y. *J. Am. Chem. Soc.* **1975**, *97*, 6983.
(8) Richardson, W.H., Hodge, V.F., Stiggall, D.L., Yelvington, M.B., Montgomery, F.C. *J. Am. Chem. Soc.* **1974**, *96*, 6652.
(9) Adam, W., Cueto, O. *J. Am. Chem. Soc.* **1979**, *101*, 6511.

Improved Synthesis of Methyl Alkoxyacetylenecarboxylates

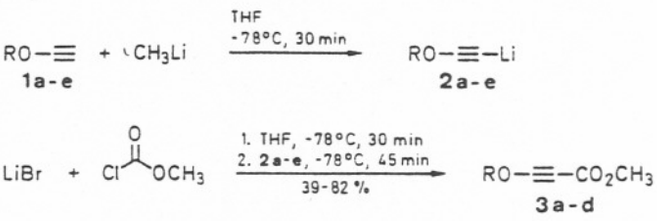
Francisco Camps,* Josep Coll, Amadeu Llebaria, Josep M. Moretó,* Susagna Ricart

Departament de Química Orgànica Biològica, C.I.D. (C.S.I.C), Jordi Girona 18–26, E-08034 Barcelona, Spain

The title compounds **3a–d** can be satisfactorily prepared by the reaction of lithium alkoxyacetylides with methyl chloroformate in tetrahydrofuran at −78 °C, in the presence of a 3.5 molar excess of lithium bromide (based on chloroformate). Moderate to good yields of **3a–d** were obtained according to the product stabilities. However, methyl *tert*-butoxyacetylenecarboxylate (**3e**) was too unstable to be isolated. The putative dual role of the lithium salt and the formation of substantial amounts of carbonates, observed in its absence, are discussed.

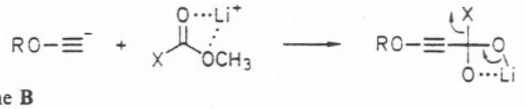
In connection with a current study in our laboratory on the nickel tetracarbonyl promoted cycloaddition of allyl halides with polarized acetylenes,[1] we anticipated that alkyl alkoxyacetylenecarboxylates, bearing substituents with opposite electronic demands, might be suitable models for such a study. However, a literature search revealed that these compounds were obtained in low yields, and described uncompletely due to their reported high instability.[2–7]

In this context, we have found that the synthesis of methyl alkoxyacetylenecarboxylates can be satisfactorily achieved by the reaction of lithium alkoxyacetylides with an alkyl chloroformate in the presence of an excess of lithium bromide in tetrahydrofuran (3.5 molar based on chloroformate). The acetylide solution **2** was prepared *in situ* by direct metalation of the corresponding alkoxyethyne, preferably with methyl lithium[8] (Scheme **A**).



Scheme **A**

We envisage the beneficial role played by the lithium bromide as dual: by enhancement of the electrophilicity of the chloroformate through metal chelation of the oxygen sites,[9] and by partial chlorine-bromine atom exchange[10,11] (Scheme **B**).



Scheme **B**

This activation allows very low reaction temperatures (−78 °C), thus avoiding undesirable side reactions[6] that render the isolation of the thermally labile final products difficult.

It is noteworthy that in the absence of an excess of lithium salts we observed the formation of substantial amounts of carbonates derived from alkoxyacetylide decomposition, which were very difficult to remove from the reaction product.

As shown in the Table, moderate to good yields of diverse methyl alkoxyacetylenecarboxylates were obtained, except in the case of the *tert*-butyl derivative **3e**, where even under these mild reaction conditions, a β-elimination process might be expected.[12]

Surprisingly, and contrary to previous reports, these compounds exhibited a good stability for extended periods of time, provided they were kept pure at low temperatures under inert atmosphere; and in the most favorable cases, they could even be distilled. Thus, methyl ethoxyacetylenecarboxylate (**3b**) was bulb-to-bulb distilled (oven temperature 85 °C/25 Torr), although with extensive decomposition. A pure sample was collected and kept unchanged for months at −18 °C under argon. However, in other cases attempted distillation led to complete decomposition. In fact, the yields paralleled the stabilities of the products. Thus, although methyl isopropoxyacetylenecarboxylate (**3d**) was obtained in moderate yield only, and spectroscopically characterized (through short time experiments), it was not possible to purify satisfactorily a sample to secure correct microanalytical data.

As expected, the great differences in the values of ^{13}C-NMR chemical shift for both acetylenic carbon atoms suggest a highly dipolar triple bond, pointing out that these compounds should be appropriate for dipolar cycloaddition condensations.

THF was freshly distilled from sodium benzophenone ketyl under nitrogen atmosphere. LiBr was dried by heating at 120 °C/1.5 Torr for 6 h. Methyl chloroformate was distilled prior to use under N_2. Ethoxyethyne (**1b**) was prepared from chloroacetaldehyde diethylacetal.[13] *n*-Propoxy- (**1c**), *n*-butoxy- (**1a**), *i*-propoxy- (**1d**), and *tert*-butoxyethyne (**1e**) were prepared from the corresponding vinyl ethers.[14] IR spectra were recorded with a Perkin Elmer 399B spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with WP-80-SY, AM-100, and

Anexo X

Are dioxetanes chemiluminescente intermediates in lipoperoxidation ? Di Mascio, P.; Catalani, L.H.; Bechara, E.J.H. *Free Rad. Biol. Med.* **12**, 471 (1992).

Free Radical Biology & Medicine, Vol. 12, pp. 471–478, 1992
Printed in the USA.

0891-5849/92 $5.00 + .00

Original Contribution

ARE DIOXETANES CHEMILUMINESCENT INTERMEDIATES IN LIPOPEROXIDATION?

PAOLO DI MASCIO,*† LUIZ H. CATALANI,* and ETELVINO J. H. BECHARA*

*Instituto de Química, Universidade de São Paulo, C.P. 20780, 01498, São Paulo, SP, Brazil; and †Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstr. 5, D-4000 Düsseldorf, Germany

(*Received* 25 *July* 1991; *Revised* 18 *December* 1991; *Accepted* 27 *January* 1992)

Abstract—Ultraweak chemiluminescence arising from lipoperoxidation has been attributed by several authors to the radiative deactivation of singlet oxygen and triplet carbonyl products. The latter emitters have been suggested to come from annihilation of RO˙ and ROO˙ radicals as well as from the thermolysis of dioxetane intermediates formed by (2 + 2) cycloaddition of $^1O_2$ to polyunsaturated fatty acids. This article questions possible dioxetane intermediacy in lipoperoxidation, as the literature clearly states that addition of $^1O_2$ to alpha-hydrogen-containing alyphatic olefins yields only the corresponding allylic hydroperoxides. These compounds may undergo dark thermal or Lewis acid-assisted decomposition to the same product obtained from dioxetane cleavage. Here, reexamining the chemiluminescence properties of dioxygenated tetramethylethylene and linoleic acid and comparing them with those of tetraethyldioxetane, a hindered dioxetane, we corroborate the literature information that only steric hindrance leads to dioxetane formation upon singlet oxygen addition to electron-poor olefins, albeit in very low yields. Proton nuclear magnetic resonance (^{1}H-NMR) analysis, quenching by dioxygen and energy transfer studies to 9,10-dibromoanthracene, as well as gas chromatography (GC) analysis of triphenylphosphine-treated and untreated photo- and chemically dioxygenated olefins support our final conclusion that dioxetane formation during lipoperoxidation can be safely excluded on the basis of the data presently available.

Keywords—Singlet molecular oxygen, Linoleic acid, Triplet ketones, Dioxetanes, Chemiluminescence, Free radicals

INTRODUCTION

Lipid peroxidation has been pointed out as a key chemical event of the oxidative stress associated with several inborn and acquired disorders. Disruption of organelle and cell membranes together with calcium homeostasis alterations are the main supramolecular events linked to lipid peroxidation.[1] Not clarified yet, however, is whether the phenomenon constitutes an early, triggering step of the clinical manifestations of the diseases or a terminal, consequent process.

The low-level chemiluminescence which accompanies the peroxidation of polyunsaturated fatty acids (PUFA) has been used as a tool in kinetic and mechanistic studies of biological samples to estimate the extent of the reactions and even to indicate tissue damage promoted by pro-oxidants injected in experimental animals.[2] Triplet carbonyls and singlet oxygen formed in the annihilation of intermediate alkylperoxyl radicals (ROO˙) have been identified as the chemiluminescence emitters.[3] The disproportionation of alkoxyl radicals (RO˙) is also a putative source of triplet ketones.[4]

Several authors have suggested parallel formation of electronically excited carbonyls in lipoperoxidation via the thermal cleavage of dioxetane intermediates supposedly formed by (2 + 2) cycloaddition of singlet oxygen ($^1O_2$) to PUFA.[2,5–7] However, it has long been known that 1,2-addition of $^1O_2$ to alkenes yields dioxetanes only in the case of electron-rich olefins lacking alpha-hydrogens or rigid olefins.[8] We thus decided to reexamine the reaction of $^1O_2$ with appropriate olefins—tetramethylethylene (TME), tetraethylethylene (TEE), and linoleic acid (LA)—with the aim of verifying the possible involvement of dioxetane intermediates in the chemiluminescence associated with lipoperoxidation. Singlet oxygen was generated both photochemically and by the thermolysis of 1,4-dimethylnaphthalene-1,4-endoperoxide ($DMNO_2$).[9]

Address correspondence to Prof. Dr. Etelvino J. H. Bechara, Instituto de Química, Departamento de Bioquímica, Universidade de São Paulo, C.P. 20780, 01498, São Paulo, SP, Brazil.

MATERIALS AND METHODS

Reagents

Reagents were purchased from the following sources: 1,4-dimethylnaphthalene (1,4-DMN), methylene blue, TEE, tetraphenylporphine (TPP), hexanal, and triphenylphosphine (Ph_3P) were from the Aldrich Chemical Co. (Milwaukee, WI). LA was from Sigma Co. (St. Louis, MO). Other chemicals and solvents were from Merck (Darmstadt, Germany). All solvents used were of spectroscopic grade. $CHCl_3$ was filtered over basic alumina (Merck) to remove proton and metal traces. DMN, LA, TME, and TEE were analyzed by ^{1}H-NMR and infrared (IR) spectroscopy.

Synthesis of $DMNO_2$

1,4-Dimethylnaphthalene-1,4-endoperoxide was prepared by a modification of the method described previously by Wasserman and Larsen.[10] In a dry 200-mL glass tube, 2.5 g (16 mmol) of purified DMN and 30 mg of methylene blue were dissolved in 20 mL of dry reagent grade methylene chloride. The solution was bubbled with O_2 under irradiation with a 500-W white lamp for 2 h at 20°C. The reaction was followed by thin-layer chromatography (TLC). The final reaction mixture was filtered through an activated alumina-cooled column, followed by removal of the solvent and recrystallization of the residue from pentane-ether (1:1), resulting in 1.2 g (40% yield) of pure colorless crystals of $DMNO_2$. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.79 (s, 6 H), 6.60 (s, 2 H), 7.15–7.95 (m, 4 H).

Synthesis of tetramethyl-1,2-dioxetane (TMD)

Tetramethyl-1,2-dioxetane was prepared according to Kopecky et al.[11] ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.44 (s, 12 H).

Photooxygenation of TME

Two g (24 mmol) of TME was dissolved in ca. 20 mL of CH_2Cl_2 containing 10–20 mg of TPP. The final solution was irradiated at 0°C with a 500-W white lamp under continuous O_2 bubbling, for about 8 h. The final solution was dried over anhydrous Na_2SO_4, the solvent was removed by rota-evaporation, and the ene-hydroperoxide was distilled under reduced pressure (1 mm Hg, 40°C) resulting in 2.2 g (80%) of pure 3-hydroperoxy-2-methyl-1-butene. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.36 (s, 3 H), 1.81 (s, 6 H), 5.00 (m, 2 H), 7.50 (b, 1 H).

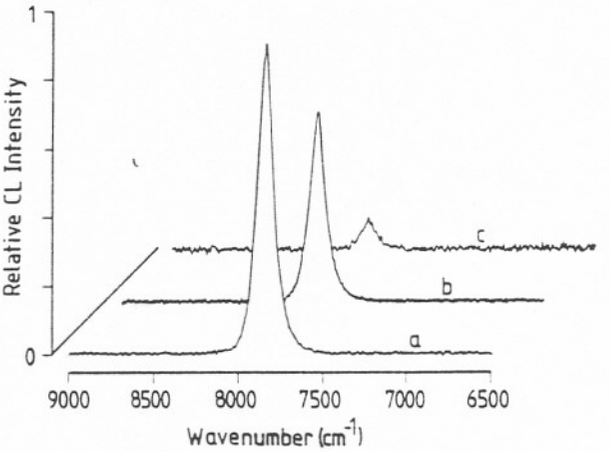


Fig. 1. Monomol light emission spectrum of singlet oxygen generated by the thermodissociation of $DMNO_2$: (A) 71 mM $DMNO_2$ plus (B) 1 mM LA or (C) 25 μM beta-carotene. Conditions: $DMNO_2$-containing chloroform solutions heated at 40°C on an FT-Raman apparatus equipped with an InGaAs NIR detector.

Photooxygenation of TEE

Fifty milligrams (0.36 mmol) of TEE were dissolved in 0.5 mL of CCl_4 containing a few crystals of TPP (< 1.0 mg). This solution was irradiated at 0°C with a 500-W white lamp under continuous O_2 bubbling. The consumption of TEE was followed by GC, which was complete after 15 min of irradiation. The resulting solution was purified by silica gel (about 2 g) column chromatography and eluted with CH_2Cl_2. The fractions containing the ene-hydroperoxide were combined and the solvent was evaporated resulting in 43 mg (69%) of pure 4-hydroperoxy-3,4-diethyl-2-hexene. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 0.77 (t, 6 H), 1.02 (t, 3 H), 1.60 (q, 4 H), 1.70 (d, 3 H), 2.09 (q, 2 H), 5.42 (q, 1 H), 6.80 (b, 1 H).

Photooxygenation of LA

The photooxygenation of LA was performed by two different methods: (1) reaction with singlet oxygen generated by photosensitization with TPP, and (2) reaction with $^1O_2$ generated during the thermodissociation of $DMNO_2$.

Method 1. Ten milligrams (0.036 mmol) of linoleic acid was dissolved in 0.4 mL $CDCl_3$ containing a few crystals of TPP. This solution was irradiated at 0°C with a 500-W white lamp under continuous O_2 bubbling. The consumption of LA was followed by ^{1}H-NMR and was complete after 75 min of irradiation.

Method 2. Ten milligrams (0.036 mmol) of LA was dissolved in 0.5 mL of $CDCl_3$ together with either 56 mg (0.3 mmol) or 5.6 mg (0.03 mmol) of $DMNO_2$. LA, as expected, reduces the $^1O_2$ monomol emission signal (Fig. 1B); that this is indeed $^1O_2$ emission is

corroborated by a dramatic quenching by beta-carotene (Fig. 1C). The spent reaction mixture was kept at 20°C for 24 h prior to use. Observation: If any dioxetane were formed during $^1O_2$ treatment, it should have a thermolysis rate constant at the order of 10^{-7} s^{-1} at 20°C (estimated from thermolysis of 3,4-dimethyl-1,2-dioxetane.[12,13] Knowing that $DMNO_2$ decomposes into DMN plus $^1O_2$ with a rate constant of 3.9×10^{-5} s^{-1} at 20°C, dioxetane accumulation would be expected to be observed if formed. For the sake of simplicity, the resulting solutions of Method 1 and Method 2 are hereafter referred to as LA/TPP and LA/$DMNO_2$ solutions, respectively. For all purposes, the solutions obtained by methods 1 and 2 were used without further purification.

Low-level chemiluminescence measurement

Low-level chemiluminescence was traced using one of the following systems: (a) PCS photometer: an "in-house" constructed cell compartment connected to a Thorn-EMI FACT MK-III cooled housing (Middlesex, England) equipped with a Thorn-EMI 9852 photomultiplier and a Princeton Instruments amplifier-discriminator (Princeton, NJ); (b) OMA photometer: a Jobin-Yvon CP-200 polychromator (Longjumeau, France) equipped with a 200 grooves/nanometer holographic grating and a multichannel detector from Princeton Instruments (IRY-700 S/B) having 760 intensified pixels; and (c) near infrared monomol emission spectrum of $^1O_2$ (Fig. 1A) obtained using a Fourier transform (FT) spectrophotometer (Bomen-DA3) (Quebec, Canada). In the latter case, the emission was measured with a liquid nitrogen cooled Bomen InGaAs detector developed to be used for FT-Raman experiments in the NIR, and the spectral resolution was set to 10 cm^{-1}.[9]

Gas chromatography and ^{1}H-NMR analysis

Gas chromatography analysis of the *n*-hexanal formed during oxidation of LA by $^1O_2$ was performed with a Hewlett Packard Mod 5890 GC system equipped with a 5-m HP-1 Megabore column (apolar). The conditions for *n*-hexanal detection were as follows: nitrogen flow 1 mL/min; column, detector, and vaporizer temperatures were 100, 250, and 250°C, respectively, resulting in a 1.8-min retention time for *n*-hexanal. ^{1}H-NMR analyses were performed on either a Bruker Mod. AM-200 (200 MHz) or a Varian Mod. XL-100 (100 MHz) instrument.

RESULTS

Photooxygenation of TME and TEE

The photooxygenation of TME and TEE sensitized by TPP apparently yielded the corresponding allylic hydroperoxides as sole products in both cases, as identified and quantified by ^{1}H-NMR. However, when a sample of the photooxygenated solution of TEE was heated at 90°C in a photometer, both direct and 9,10-dibromoanthracene (DBA)-enhanced emission were detected and 3-pentanone was identified as the main product of thermolysis. The rate of chemiluminescence decay was around 1.5 times faster than that observed with purified tetraethyldioxetane (TED).[14] The activation energy of this process (E_{CL}) determined by the "temperature-drop method"[15] agrees well with that of TED,[14] within the limits of experimental error (Table 1). On the other hand, no chemiluminescence was detected when heating the photooxygenated solution of TME.

Table 1. Activation Energy Values for TED Thermal Cleavage and for the Thermolysis of TEE Photooxygenated Solution[a]

Compounds	k_1[b] at 90° (s^{-1})	E_{CL} (DBA) (kcal mol^{-1})	E_{CL} (DPA) (kcal mol^{-1})
TEE + $^1O_2$	6×10^{-4}[c]	27 ± 1	30 ± 2
TED	4×10^{-4}	27.4 ± 0.3	31.2 ± 0.2

[a] Determination by the temperature-drop method[15]; in xylenes, between 54 to 100°C; [DBA] and [DPA] = 4×10^{-3} M; [TED] = 1×10^{-4} M.

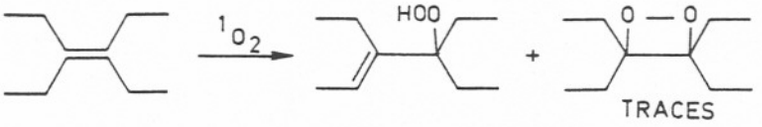
[b] Rate constant of the DBA-sensitized chemiluminescence decay.

[c] Nonpurified dioxetane solutions tend to exhibit high *k* values due to metal contaminations.[15]

At first sight, these results might be interpreted as due to the presence of TED in the TEE photooxygenated solution formed by rearrangement of the corresponding allylic hydroperoxide, followed by its thermal cleavage to 3-pentanone plus light. This possibility was discarded when the same solution showed a similar intensity of light emission after extensive ene-hydroperoxide extraction with aqueous NaOH 2N.[11] This indicated that the emission does not come from the ene-hydroperoxide formed, but instead from traces of TED formed directly during the photooxygenation of TEE (estimated as 0.2%, calculated from the intensity of light emission in the presence of DBA according to Wilson and Schaap[16]) (Scheme I). On the other hand, the thermolysis of the alkaline extract (brought to neutral pH) produced 3-pentanone in a nonchemiluminescent process, probably through a Hock-cleavage mechanism.[17]

Photooxygenation of LA monitored by ^{1}H-NMR

The ^{1}H-NMR analysis of LA/TPP solution clearly showed a decrease of the signal at 2.70 ppm (proton *a* of 1; Scheme II), with a concomitant increase in the

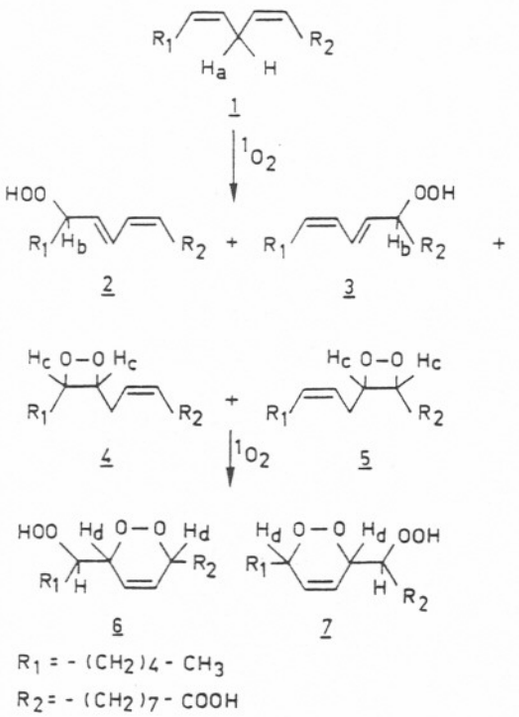


Scheme I. Tetraethylethylene photooxygenation.

signal at 4.29 ppm (protons *b* of 2 and 3). After 60 min of irradiation, about 85% of the starting material was consumed, generating most probably compounds 2 and 3. This yield was calculated taking into consideration that alpha-carbonyl protons of compounds 1–7 have the same chemical shift at 2.28 ppm.

After 75 min of irradiation, the signal at 2.70 ppm had disappeared completely with new signals showing up in the 4–5 ppm region, most probably due to absorption of double oxygenation products 6 and 7 (protons *d*). A similar behavior was observed for the LA/$DMNO_2$ solution. Likewise, we observed a decrease of the signal at 2.70 ppm with appearance of a new signal at 4.29 ppm.

The assignment of protons *b* through *e* is in accordance with chemical shifts of protons of similar structures.[12,13,18,19] However, one cannot discard the possibility of traces of dioxetanes 4 and 5 being formed during this process based on ^{1}H-NMR results alone. The expected position for the absorption of protons *c* is 5.0–5.5 ppm and could be obscured by olefinic protons.[12,13]



Scheme II. Linoleic acid photooxygenation.

Chemiluminescence from a photooxygenated LA solution

The photooxygenated LA/TPP solution developed a faint chemiluminescence upon heating. Figure 2 shows a typical temporal profile of this emission observed in a PCS photometer, where a two-phase decay can be observed. The second decay, which accounts for most of the light output (ca. 95%), showed a first-order kinetics. The rate constant of this decay was calculated, and its temperature dependence showed an activation energy value of 9 ± 1 kcal/mol in the range of 30–57°C (Arrhenius plot; not shown). When $DMNO_2$ is the $^1O_2$ source, the time profile observed is similar to that of Figure 2, lacking, however, the first phase. Addition of either DBA or 9,10-diphenylanthracene (DPA) (10^{-4} to 10^{-3} M) to this solution had no effect on the intensity of light emission, as expected if excited singlet and triplet carbonyls were being formed.[16]

When the chemiluminescence of LA/TPP was measured with an OMA photometer, the spectral distribution observed has basically the shape of TPP fluorescence emission (not shown). The time dependence of the emission at 660 nm of the LA/TPP solution presents a two-phase decay, in keeping with the profile observed when total light emission was monitored (Fig. 2).

The total light emission was calculated using a Hastings-Weber light standard.[20] The total quantum yield calculated was ca. 1×10^{-7} photons per LA mole-

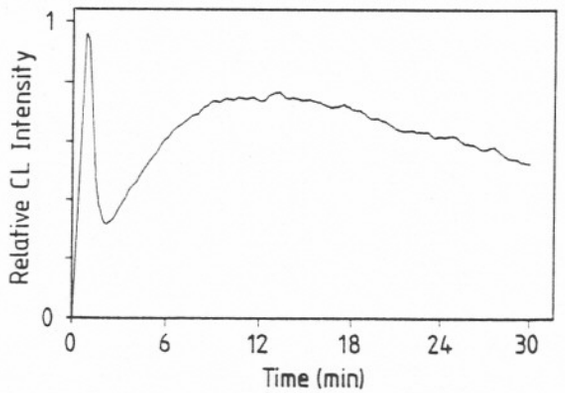


Fig. 2. Time course of the emission from photooxygenated LA in the presence of TPP. Conditions: LA (0.6 mM)/TPP (0.01 mg) in chloroform solution heated at 40°C in a PCS photometer.

Table 2. GC Analysis of *n*-Hexanal from Oxygenated LA Solutions[a]

$^1O_2$ Source	Time (h)	*n*-Hexanal (%)[b]
Photooxygenation (TPP)		
$LA/^3O_2$	3	
$LA/^3O_2/h\nu$	1:15	6
$LA/^3O_2/h\nu$	3	63
$LA/^3O_2/h\nu + Ph_3P$	3	25
Thermodissociation ($DMNO_2$)		
$LA/DMNO_2$	8	22
$A/DMNO_2 + Ph_3P$	8	3

[a] Experimental conditions: [LA] = 89 mM; [$DMNO_2$] = 0.2 M, 10% excess of Ph_3P.
[b] Percent relative to the starting LA.

cule, for an LA/TPP solution irradiated for 75 min (i.e., 100% consumption of LA). On the other hand, when $DMNO_2$ was the source of $^1O_2$, the quantum yield dropped to ca. 2×10^{-9} photons per LA oxygenated molecule. Note that the former solution contains TPP, which might act as a fluorescer, while the latter has DMN, which cannot sensitize triplet carbonyls.

It is noteworthy that this emission was not enhanced upon oxygen removal by argon purging. This would be expected if triplet carbonyls were being formed by dioxetane thermolysis, a higher fraction of triplet species decaying by radiative deactivation in the absence of the oxygen quencher.[21] Furthermore, the light intensity was not altered upon addition of 0.05 mL of H_2SO_4, as it would be expected if the LA 1,4-endoperoxide (6 and 7 in Scheme II) underwent Lewis acid-assisted rearrangement to a dioxetane, followed by its thermolysis.[22]

n-Hexanal detection by gas chromatography

Chromatographic analysis of photo- or chemically oxygenated solutions of LA injected onto a GC column showed the presence of *n*-hexanal as the main product in the respective solutions. When the spent reaction mixture obtained from the oxidation of LA was treated with 10% excess of Ph_3P 30 min prior to GC analysis, decreases of 60% and 87% in *n*-hexanal production were observed using LA/TPP and LA/$DMNO_2$ solutions, respectively (Table 2). These decreases in *n*-hexanal may be due to the reduction of hydroperoxides to the respective alcohols.

DISCUSSION

In the 1960s, Vassil'ev described the chemiluminescence from the autooxidation of alkyl-substituted aromatic hydrocarbons,[23] attributed to the generation of triplet species. The weak chemiluminescence associated with peroxidation of PUFA has also long been known and was proposed to be due to two types of emitters: excited triplet carbonyls and singlet oxygen.[2,5–7] The former excited species (n, π^*) can emit phosphorescence in the visible (blue-green) region and the latter in the red and infrared region. In aerated solutions, triplet carbonyls are short lived due to their quenching by ground state oxygen;[24] singlet oxygen was suggested to be formed in this process.[25,26]

$$\text{>C=O}^{3*} \rightarrow \text{>C=O} + h\nu\ 450\text{–}550\ \text{nm} \quad \text{(phosphorescence)} \qquad (1)$$

$$O_2(^1\Delta_g) \rightarrow O_2(^2\Sigma_g^-) + h\nu\ 1270\ \text{nmp} \quad \text{(monomol emission)} \qquad (2)$$

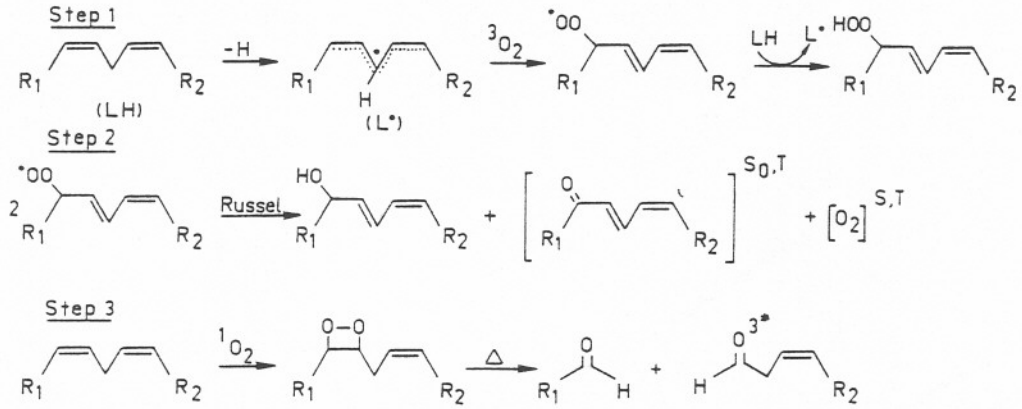
$$2\ O_2(^1\Delta_g) \rightarrow 2\ O_2(^3\Sigma_g^-) + h\nu\ 634 \text{ and } 703\ \text{nm} \quad \text{(dimol emission)} \qquad (3)$$

$$\text{>C=O}^{3*} + O_2(^3\Sigma_g^-) \rightarrow \text{>C=O} + O_2(^1\Delta_g) \quad \text{(quenching)} \qquad (4)$$

The weak direct emission from lipoperoxidation has been demonstrated to be enhanced by addition of fluorescers such as DBA, 9,10-dibromoanthracene-2-sulfonate ion, xanthene dyes, chlorophyll, and europium complexes.[27–29] Both direct and sensitized chemiluminescence have been used as tools for analytical and kinetic purposes when studying lipoperoxidation and lipoperoxides.[30,31] Although the nature of the excited species in lipoperoxidation (Step 1 in Scheme III) can be safely described, the chemical mechanisms underlying their formation are still an open question. Boveris et al.,[2] Lissi et al.,[5] Cadenas,[6] and, more recently, Prat and Turrens,[7] for example, have suggested the annihilation of alkylperoxyl radicals (Step 2) and the thermolysis of dioxetane intermediates (Step 3) as sources of triplet carbonyls during lipoperoxidation, the dioxetanes being formed by (2 + 2) cycloaddition of singlet oxygen to the PUFA (Step 3).

In fact, several lines of evidence support the generation of singlet oxygen by the Russell reaction (Step 2 in Scheme III) and, perhaps, by triplet-singlet energy transfer from excited carbonyls (Eq. 4). In turn, triplet carbonyls can be formed by the Russell reaction,[3] annihilation of alkoxyl radicals,[4] and thermal cleavage of dioxetanes with simple substituents such as alkyl, alkoxyl, acyl, and phenyl (that is, groups with high oxidation potentials[32–34]).

The proposal by those authors that cycloaddition of singlet oxygen to PUFA can lead to dioxetanes lacks theoretical and experimental bases. The bulk of data from the 1960s and 1970s had already established that (2 + 2) cycloaddition of singlet oxygen to

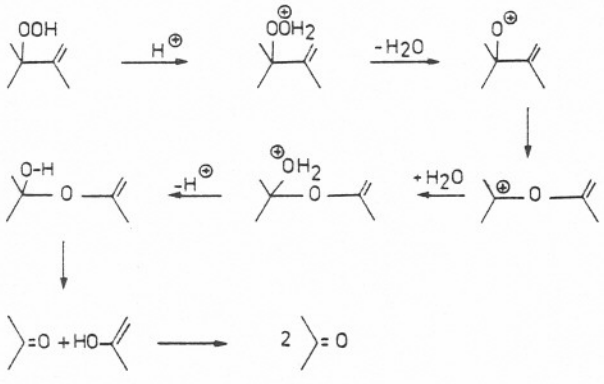


Scheme III. Lipoperoxidation, Russell reaction, and thermolysis of hypothetical dioxetane intermediate.

alkenes, yielding a dioxetane, occurs only with either "electron-rich" olefins lacking alpha-hydrogen atoms or with alkenes with rigid substituents.[35] This is the case for tetramethoxyethylene,[36] *bis*-(*N*-methyl)-acrylidene,[37] and bisadamantylidene,[38] for example.

It is well documented that bulky groups with higher oxidation potentials make the dioxetanes more stable (e.g., bisadamantylidene dioxetane) due to the intermediacy of a short-lived diradical in their thermolysis and, like other alkyl-substituted dioxetanes, they produce mostly triplet species.[32,39] Dioxetanes containing groups with low oxidation potentials probably cleave through an electron-transfer mechanism (chemically initiated electron exchange luminescence; CIEEL) and yield predominantly singlet species.[40]

The main, if not only, product from photooxygenation of alpha-hydrogen-containing alkenes is the corresponding ene-hydroperoxide.[8] It is noteworthy that ene-hydroperoxides, when treated with Lewis acids, undergo the so-called Hock cleavage,[17,41] yielding the same carbonyl fragments as those obtained from the thermolysis of a dioxetane, but in the ground state (Scheme IV). On the other hand, there are only a



Scheme IV. Hock cleavage mechanism.

few examples of dioxetanes that can be generated by rearrangement of Lewis acid-treated 1,4-endoperoxides, formed by 1,4-cycloaddition of single oxygen.[22,42]

The question we address in this article is whether a dioxetane could be involved as an intermediate to explain in part the chemiluminescence accompanying lipoperoxidation. This was approached by a reexamination of the reaction of TME and of LA with chemi- and photoexcited singlet oxygen; for comparison, a hindered olefin with alpha-hydrogen atoms—TEE—was also studied. $DMNO_2$ was used as a chemical source of singlet oxygen and $TPP/O_2/h\nu$ to carry out the olefin photooxygenation.

As expected, 2,3-dimethyl-3-hydroperoxy-1-butene (the ene-hydroperoxide) was the only product obtained from photooxygenation of TME. The spent reaction mixture is nonluminescent under heating, and its products analyzed by GC revealed the presence of only acetone. Pretreatment of the spent reaction mixture with Ph_3P, which converts the hydroperoxide to the corresponding alcohol, quenches the acetone peak in the GC trace, as expected.[14,43]

The photooxygenation of TEE also produced the ene-hydroperoxide as the main product. Injection of the reaction mixture in the GC gave only 3-pentanone—the expected product from thermolysis of TED and the Hock cleavage of 3,4-diethyl-4-hydroperoxy-2-hexene. However, as opposed to the TME case, the reaction mixture is chemiluminescent under heating. Exhaustive extraction of the reaction mixture with aqueous NaOH, which should remove the ene-hydroperoxide,[11] did not affect the intensity of light emission. In addition, the direct chemiluminescence was ehanced by both DPA and DBA, and the ratio I_{DBA}/I_{DPA} is very high (ca. 10^5) as observed for all alkyldioxetanes reported in the literature.[16] Using the "temperature-drop method," the activation energy for the DBA-sensitized chemiluminescence was

found to be similar to that of TED (Table 1).[44] This behavior is compatible with the assumption that TED (0.2% yield, estimated from the I_{DBA} and $\phi^3 = 0.60$; see Ref. 14) was indeed formed during photooxygenation of TEE. Steric hindrance by bulky (not rigid) substituents on the singlet oxygen reaction with olefins would somehow also facilitate the (2 + 2) cycloaddition.

In addition to steric requirements for (2 + 2) cycloaddition, one must take into account the type of reaction which would be most favorable for PUFA. Hence, three properties of the double bonds present will favor the ene-reaction. First, the presence of hydrogens alpha to the double bond is, in itself, reason enough to observe such a reaction. In the absence of steric hindrance and electron-donating substituents, hydrogen abstraction will be the dominant path. Second, abstractable hydrogens in the *cis* configuration will speed up the ene-reaction due to the known "*cis* effect," where both hydrogens assist the $^1O_2$ approach to the double bond.[45,46] Finally, the bridge hydrogens (the hydrogens at carbon 11) of LA undergo double "activation" from the two neighboring double bonds, resulting in an energy gain from conjugation in the product, which makes them more prone to abstraction. These three conditions would be enough to expect ene-reaction as the dominant path.

Indeed, in $CDCl_3$, the addition of the first equivalent of $^1O_2$ led to total disappearance of the bridge hydrogen signal, as observed by ^{1}H-NMR. Moreover, extensive photooxygenation seems to promote (4 + 2) cycloaddition on the conjugated products (see Scheme II) leading to endoperoxides. This is in accordance with previous studies of Frimer.[35]

The only remaining argument to propose dioxetanes as intermediates in lipoperoxidation is the accompanying chemiluminescence. A close look at this event, using LA as a model, shows the following: First, the total amount of light observed is extremely low, with quantum yields of the order of 10^{-7} to 10^{-9} photons per LA molecule. Such low light levels can be attributed to side reactions, including recombination of free radicals formed during the experiment.[5] Second, this luminescence could not be enhanced by either DBA or DPA, or by removal of oxygen, attesting to its nontriplet character. Third, no proton-catalyzed rearrangement of LA 1,4-endoperoxide to a dioxetane seemed to have occurred. Note that alkyl dioxetanes are known to produce huge amounts of triplets.[32,39] Third, the activation energy found for the chemiluminescence from dioxygenated LA (ca. 9 kcal/mol) could hardly be attributed to a dioxetane, for which the corresponding values are usually in the range of 20–30 kcal/mol (Table 1).[39]

Moreover, when discussing the intermediacy of dioxetanes in biological systems, one must consider that, superposed on their normal chemiluminescent decomposition, there is a "dark" pathway catalyzed by adventitious metals. Thus, Wilson et al.[15] reported that the rate of TMD decomposition in methanol is lowered by two orders of magnitude when the solvent is pretreated with Chelex 100 or with 5×10^{-4} M ethylenediaminetetraacetic acid (EDTA).

In conclusion, the present data reinforce that it is not plausible that dioxetanes are intermediates in lipoperoxidation as a result of singlet oxygen 1,2-cycloaddition to PUFA, and to attribute the visible region emission to triplet species formed by those intermediates.

Acknowledgements — The authors are indebted to the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, the Interamerican Development Bank—University of São Paulo scientific program for financial support, and the Fundação de Amparo a Pesquisa do Estado de São Paulo for research grants. They also thank Dr. Ann M. Gunzalez and Frank Quina for reading this manuscript and Dr. Joel C. Rubim for allowing them to use the OMA photometer and the FT-Raman equipment (Instituto de Química, University of São Paulo, São Paulo).

REFERENCES

1. Gutteridge, J. C.; Halliwell, B. Iron toxicity and oxygen radicals. *Ballière's Clin. Haematol.* **2**:195–256; 1989.
2. Boveris, A.; Cadenas, E.; Chance, B. Ultraweak chemiluminescence: A sensitive assay for oxidative radical reactions. *Federation Proc.* **40**:195–198; 1981.
3. Russel, G. A. Deuterio-isotope effects in the autoxidation of aralkyl hydrocarbons. Mechanism of the interaction of peroxy radicals. *J. Am. Chem. Soc.* **79**:3871–3877; 1957.
4. Phillips, D.; Anissimov, V.; Karpukhin, O.; Shliapintokh, V. Energy transfer from chemiluminescent species in polymers. *Nature* **215**:1163–1165; 1967.
5. Lissi, E. A.; Cáceres, T.; Videla, L. A. Visible chemiluminescence from rat brain homogenates undergoing autoxidation. II Kinetics of the luminescence decay. *Free Rad. Biol. Med.* **4**:93–97; 1988.
6. Cadenas, E. Biochemistry of oxygen toxicity. *Ann. Rev. Biochem.* **58**:79–110; 1989.
7. Prat, A. G.; Turrens, J. F. Ascorbate- and hemoglobin-dependent brain chemiluminescence. *Free Rad. Biol. Med.* **8**:319–325; 1990.
8. Frimer, A. A.; Stephenson, L. M. The singlet oxygen "ene" reaction. In: Frimer, A. A.; ed. *Singlet O_2*. Vol. 2. Boca Raton: CRC Press; 1985:67–91.
9. Di Mascio, P.; Bechara, E. J. H.; Rubim, J. C. Dioxygen NIR FT-emission ($^1\Delta_g \rightarrow {}^3\Sigma_g^-$) and Raman spectra of 1,4-dimethylnaphthalene endoperoxide, a source of singlet molecular oxygen. *Appl. Spectro.* **46**:236–239; 1982.
10. Wasserman, H. H.; Larsen, D. L. Formation of 1,4-endoperoxides from the dye-sensitized photo-oxygenation of alkylnaphthalenes. *J. Chem. Soc. Chem. Commun.* 253–254; 1972.
11. Kopecky, K. R.; Filby, J. E.; Mumford, C.; Lockwood, P. A.; Ding, J.-Y. Preparation and thermolysis of some 1,2-dioxetanes. *Can. J. Chem.* **53**:1103–1122; 1975.
12. Adam, W.; Baader, W. J. Effects of methylation on the thermal stability and chemiluminescence properties of 1,2-dioxetanes. *J. Am. Chem. Soc.* **107**:410–416; 1985.
13. Baumstark, A. L.; Dunams, T.; Roskamp, P. C.; Wilson, C. E. Thermolysis of dioxetanes: Activation parameters for *cis*-/

trans-3,4-dialkyl-1,2-dioxetanes. *J. Org. Chem.* **48**:261–263; 1983.
14. Bechara, E. J. H.; Baumstark, A. L.; Wilson, T. Tetraethyldioxetane and 3,4-dimethyl-3,4-di-*n*-butyl-1,2-dioxetane. High ratio of triplet to singlet excited products from the thermolysis of both dioxetanes. *J. Am. Chem. Soc.* **98**:4648–4649; 1976.
15. Wilson, T.; Landis, M. E.; Baumstark, A. L.; Bartlett, P. D. "Solvent effects" on the chemiluminescent decomposition of tetramethyl-1,2-dioxetane. Competitive dark pathways. *J. Am. Chem. Soc.* **95**:4765–4766; 1973.
16. Wilson, T.; Schaap, A. P. The chemiluminescence from *cis*-diethoxy-1,2-dioxetane. An unexpected effect of oxygen. *J. Am. Chem. Soc.* **93**:4126–4136; 1971.
17. Hock, H.; Schrader, O. Der mechanismus der autoxydation einfacher kohlenwasserstoffe als beitrag zur autoxydation von brennstoffen. *Angew. Chem.* **49**:565–566; 1936.
18. Hall, G. E.; Roberts, D. G. A study by infrared and proton magnetic resonance spectroscopy of the monohydroperoxides of oleate and linoleate esters. *J. Chem. Soc.* 1109–1112; 1966.
19. Mihelich, E. D. Structure and stereochemistry of novel endoperoxides isolated from the sensitized photoxidation of methyl linoleate. Implications for prostaglandin biosynthesis. *J. Am. Chem. Soc.* **102**:7141–7143; 1980.
20. Hastings, J. W.; Weber, G. Total quantum flux of isotropic sources. *J. Opt. Soc. Am.* **53**:1410–1415; 1963.
21. Turro, N. J.; Steinmetzer, H. C.; Yekta, A. Tetramethyl-1,2-dioxetane. Simple procedures for chemiexcitation or photoexcitation of acetone phosphorescence in fluid solution. *J. Am. Chem. Soc.* **95**:6468–6470; 1973.
22. Catalani, L. H.; Wilson, T. Electron transfer and chemiluminescence. Two inefficient systems: 1,4-Dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide. *J. Am. Chem. Soc.* **111**:2633–2639; 1989.
23. Vassil'ev, R. F. Secondary processes in chemiluminescent solutions. *Nature* **196**:668–669; 1962.
24. Catalani, L. H.; Bechara, E. J. A. Quenching of chemiexcited triplet acetone by biologically important compounds in Aqueous medium. *Photchem. Photobiol.* **39**:823–830; 1984.
25. Wu, K. C.; Trozzolo, A. M. Evidence for the production of singlet molecular oxygen from the quenching of excited states of dialkyl ketones by molecular oxygen. *J. Photochem.* **10**:407–410; 1979.
26. Foley, M. B.; Sidebottom, H. W. Reactions of the triplet state of ketones with molecular oxygen. *Comm. Eur. Communities.* [REP] EUR *Phys.-Chem. Behav. Atmos. Pollut.* 165–171; 1982.
27. Schulte-Herbrüeggen, T.; Cadenas, E. Electronically excited states generation during the lipoxygenase-catalyzed aerobic oxidation of arachidonate. *Photobiochem. Photobiolphys.* **10**:35–52; 1985.
28. Cadenas, E.; Sies, H.; Campa, A.; Cilento, G. Electronically excited states in microsomal membranes: Use of chlorophyl-*a* as an indicator of triplet carbonyls. *Photochem. Photobiol.* **40**:661–666; 1984.
29. Sharov, V. S.; Kazamanov, V. A.; Vladimirov, Y. A. Selective sensitization of chemiluminescence resulted from lipid and oxygen radical reactions. *Free Rad. Biol. Med.* **7**:237–242; 1989.
30. Chatterjee, S. N.; Agarwal, S. Liposomes as membrane model for study of lipid peroxidation. *Free Rad. Biol. Med.* **4**:51–72; 1988.
31. Iwaoka, T.; Tabata, F.; Takahashi, T. Lipid peroxidation and lipid peroxide detected by chemiluminescence. *Free Rad. Biol. Med.* **3**:329–333; 1987.
32. Adam, W. Determination of chemiexcitation yields in the thermal generation of electronic excitation from 1,2-dioxetanes. In: Adam, W.; Cilento, G.; eds. *Chemical and biological generation of excited states.* New York: Academic Press; 1982:115–152.
33. Koo, J. Y.; Schuster, G. B. Chemically initiated electron exchange luminescence. A new chemiluminescent reaction path for organic peroxides. *J. Am. Chem. Soc.* **99**:6107–6109; 1977.
34. Koo, J. Y.; Schmidt, P.; Schuster, G. B. Bioluminescence of the firefly: Key steps in the formation of the electronically excited state for model systems. *Proc. Natl. Acad. Sci. USA* **75**:30–33; 1978.
35. Frimer, A. A. The reaction of singlet oxygen with olefins: The question of mechanism. *Chem. Rev.* **79**:359–387; 1979.
36. Wilson, T.; Golan, D. E.; Harris, M. S.; Baumstark, A. L. Thermolysis of tetramethoxy- and *p*-dioxenedioxetanes. Kinetic parameters, chemiluminescence and yields of excited products. *J. Am. Chem. Soc.* **98**:1086–1091; 1976.
37. Lee, K. W.; Singer, L. A.; Legg, K. D. Chemiluminescence from the reaction of singlet oxygen with 10,10'-dimethyl-9,9'-biacridylidene. A reactive 1,2-dioxetanes. *J. Org. Chem.* **41**:2685–2688; 1976.
38. Wieringa, J. H.; Strating, J.; Wynberg, H.; Adam, W. Adamantylideneadamantane peroxide, a stable 1,2-dioxetane. *Tetrahedron Lett.* 169–172; 1972.
39. Adam, W.; Zinner, K. Determination of activation parameters and the thermal stability of 1,2-dioxetanes. In: Adam, W.; Cilento, G.; eds. *Chemical and biological generation of excited states.* New York: Academic Press; 1982:153–189.
40. McCapra, F.; Beheshti, I.; Burford, A.; Hann, R.; Zaklika, K. A. Singlet excited states from dioxetane decomposition. *J. Chem. Soc. Chem. Commun.* 944–946; 1977.
41. Farmer, E. H.; Sundralingam, A. Course of autoxidation reactions in polyisoprene and allied compounds. I. The structure and reactive tendencies of the peroxides of simple olefins. *J. Chem. Soc.* 121–139; 1942.
42. Schaap, A. P.; Burns, P. A.; Zaklika, K. A. Silica gel-catalyzed rearrangement of an endoperoxide to a 1,2-dioxetane. *J. Am. Chem. Soc.* **99**:1270–1272; 1977.
43. Horner, L.; Jurgeleit, W. Die reduktion organischer peroxyde mit tertiären phosphinen. *Justus Liebigs Ann. Chem.* **591**:138–152; 1955.
44. Bechara, E. J. H.; Wilson, T. Alkyl substituent effects on dioxetane properties. Tetraethyl-, dicyclohexylidene-, and 3,4-dimethyl-3,4-di-*n*-buthyldioxetanes. A discussion of decomposition mechanisms. *J. Org. Chem.* **45**:5261–5268; 1980.
45. Orfanopoulos, M.; Gerdina, M. B.; Stephenson, L. M. Site specificity in the singlet oxygen-trisubstituted olefin reaction. *J. Am. Chem. Soc.* **101**:275–276; 1979.
46. Schulte-Elte, K. H.; Muller, B. L.; Rautenstrauch, V. J. Preference for the syn *ene* additions of $^1O_2$ to 1-methylcycloalkenes. Correlation with ground-state geometry. *J. Am. Chem. Soc.* **102**:1738–1740; 1980.

ABBREVIATIONS

CIEEL—chemically initiated electron exchange luminescence
DMN—1,4-dimethylnaphthalene
$DMNO_2$—1,4-dimethylnaphthalene-1,4-endoperoxide
DBA—9,10-dibromoanthracene
DPA—9,10-diphenylanthracene
E_{CL}—activation energy obtained by sensitized chemiluminescence
LA—linoleic acid
$^1O_2$—singlet molecular oxygen
PUFA—polyunsaturated fatty acid
$RO^{\cdot}$—alkoxyl radical
$ROO^{\cdot}$—alkylperoxyl radical
TED—tetraethyl-1,2-dioxetane
TEE—tetraethylethylene
TMD—tetramethyl-1,2-dioxetane
TME—tetramethylethylene
TPP—tetraphenylporphine

Anexo XI

Esterase coupled with the H_2O_2/horseradish peroxidase system triggers chemiluminescence from 2-methyl-1-propenylbenzoate: a potential analytical tool for esterase analysis. Yavo, B.; Campa, A.; Catalani, L.H. *Anal. Biochem.* **234**, 215 (1996).

ANALYTICAL BIOCHEMISTRY 234, 215–220 (1996)
ARTICLE NO. 0075

Esterase Coupled with the H_2O_2/Horseradish Peroxidase System Triggers Chemiluminescence from 2-Methyl-1-propenylbenzoate: A Potential Analytical Tool for Esterase Analysis

Boni Yavo,* Ana Campa,* and Luiz H. Catalani†,[1]

Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, CP 66.083, 05389-970 São Paulo, Brazil; and †Instituto de Química, Universidade de São Paulo, CP 26.077, 05599-970 São Paulo, Brazil

Received September 26, 1995

The hydrolysis of 2-methyl-1-propenylbenzoate catalyzed by esterase produces 2-methyl-1-propenol, which can be subsequently oxidized by the H_2O_2/horseradish peroxidase (HRP) system to yield electronically excited triplet acetone. The level of luminescence elicited by this species is proportional to total esterase used, making it possible to determine as little as 2 pmol of esterase. Yet, its intensity can be enhanced several orders of magnitude by fluorescent acceptors like sodium 9,10-dibromoanthracene-2-sulfonate. The system works as a chemiluminescent reaction triggered by esterase and can be used to elaborate analytical assays to determine its activity. This chemiluminescence is also promoted by HRP conjugates instead of free HRP and, hence, this simple reaction system can also be used to develop sensitive chemiluminescent immunoassays based upon peroxidase activity.

Horseradish peroxidase (HRP)[2] catalyzes the oxidation of several substrates at the expense of peroxides. Some of these reactions are luminescent, with high chemiluminescence quantum yields. The luminescence produced by these reactions has been used to develop important analytical assays (1, 2).

The HRP-catalyzed oxidation of several α-methine carboxylic acids and aldehydes and their ability to form electronically excited products have been studied by Cilento and co-workers (3, 4) since the mid-1970s. Among them, isobutanal (IBAL) was the most extensively studied. This HRP-catalyzed oxidation generates triplet acetone, as attested by its phosphorescent emission at 430 nm (5). A 1,2-dioxetane has been proposed as the key intermediate (Fig. 1). The chemiluminescence can be amplified several orders of magnitude by addition of an appropriate sensitizer, like sodium 9,10-dibromoantracene-2-sulfonate (5) or fluorescein (6).

Like in other peroxidase-catalyzed reactions, HRP compound I initiates and compound II propagates the reaction. In fact, the true substrate of this enzymatic reaction was suggested to be the enol form of IBAL, 2-methyl-1-propenol, present in the keto–enol equilibrium, which is catalyzed by phosphate buffer. This was confirmed by the use of the trimethylsilyl enol ether of IBAL (7) (Fig. 1). Its spontaneous hydrolysis, followed by oxidation by the H_2O_2/O_2/HRP system, produced luminescence identical to, but more intense than, that from IBAL. Moreover, addition of fluoride ion promotes the catalytic hydrolysis of the substrate and enhances this luminescence to a point at which it could be observed in the dark with adapted eyes (8).

The first attempt to use a true substrate, such as dimethyl or disodium phosphate enol esters derived from IBAL, did not produce any substantial amount of light (9). Notwithstanding, phosphate esters have a much slower rate of hydrolysis than their silyl ether counterpart and the authors did not examine the effect of catalyst addition.

The analytical potential of these reactions has never been explored. In this work, we illustrate how enzymatic hydrolysis of appropriate substrates can trigger luminescence from subsequent HRP-dependent reac-

[1] To whom correspondence should be addressed. Fax: (xx55-11) 815 5579; E-mail: catalani@usp.br.

[2] Abbreviations used: CTAB, hexadecyltrimethylammonium bromide; DBAS, sodium 9,10-dibromoanthracene sulfonate; HRP, horseradish peroxidase; IBAL, isobutanal; MPB, 2-methyl-1-propenyl benzoate; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran.

0003-2697/96 $18.00

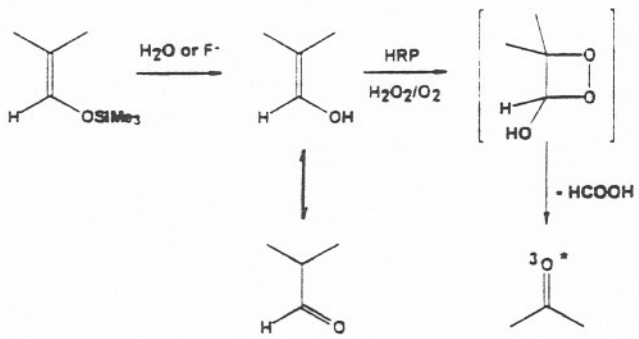


FIG. 1. HRP-catalyzed oxidation of IBAL or its trimethylsilyl enol ether.

tions. We have chosen dog liver esterase as the catalyst and synthesized and tested 2-methyl-1-propenyl benzoate (MPB) as a source of the enol form of IBAL by enzymatic hydrolysis. In addition, we show that HRP can be replaced by an antibody conjugate HRP–IgG.

MATERIALS AND METHODS

Reagents. HRP type I and type VI (EC 1.11.1.7), esterase (EC 3.1.1.1), sodium dodecyl sulfate (SDS), and Triton X-100 were from Sigma. Hydrogen peroxide and hexadecyltrimethylammonium bromide (CTAB) were from Aldrich. IBAL (Merck) was freshly distilled under N_2, triethylamine (Aldrich) was distilled over KOH, while benzoyl chloride (Aldrich) was distilled prior to use. HRP–IgG conjugate (HRP bound to rabbit type G immunoglobulin) was from an Abbott diagnostic kit for HIV-1 EIA recombinant. 9,10-Dibromoanthracene-2-sulfonate (DBAS) was synthesized and purified according to Catalani *et al.* (10).

Synthesis of 2-methyl-1-propenyl benzoate 1. A mixture of 18 g (0.25 mol) of IBAL, 35 g (0.25 mol) of benzoyl chloride, 30 g (0.3 mol) of triethylamine, and 100 mg (0.8 mmol) of 4-dimethylaminopyridine in 80 ml of dried THF was added to a three-neck round flask and refluxed for 8 h. The resulting mixture was washed with 1 N HCl and saturated aqueous $NaHCO_3$ and dried over anhydrous Na_2SO_4, the solvent was removed, and the residue was fractionally distilled at reduced pressure. The fraction distilled at 108–110°C/1 mm Hg contained 84% pure MPB. Further distillation led to 97% purity by GC analysis. Yield was 19 g (43%). MPB is stable at room temperature when free of moisture. Stock solutions can be prepared using acid and water-free solvents. Elemental analysis: C, 72.42%; H, 6.60% (theor 74.98 and 6.86%, respectively). ^{1}H-NMR (in $CDCl_3$; δ/ppm; TMS = 0.00) 1.72 (s, 3H), 1.81 (s, 3H), 7.10 (s, 1H), 7.42 (m, 2H), 7.57 (m, 1H), 8.08 (m, 2H). ^{13}C-NMR (in $CDCl_3$; δ/ppm: TMS = 0.00) 15.5, 18.9, 118.4, 128.2, 129.5, 129.7, 132.9, 133.7, 163.4. MS (EI; m/e) 176 (29.72%), 123 (6.29), 122 (6.54), 105 (100.00), 77 (66.43), 51 (48.46).

Instrumentation. The time course of chemiluminescence was followed in a BioOrbit Model 1251 luminometer and the chemiluminescence spectral distribution and phosphorescence measurements were recorded on a Spex Fluorolog-2 Model FL-111 spectrofluorometer with cooled photomultiplier and phosphorimeter attachments. Oxygen consumption was measured with a Yellow Springs Model 5300 oxygen monitor.

Methods. MPB stock solutions were prepared in absolute ethanol and those of HRP type I and esterase stock contained 374 and 2.4 units/ml, respectively. The standard reaction mixture, in a volume of either 1 ml (for luminometer) or 3 ml (for spectrofluorometer and oxygen consumption monitor), contained 0.48 units/ml esterase, 2.77 units/ml HRP-I and 74 μM H_2O_2 in 0.05 M phosphate buffer at pH 7.4 at 37°C and the reaction was initiated by addition of 1 mM MPB.

Assay protocols. Esterase (0.059 to 1.92 units/ml) was assayed in phosphate buffer, pH 7.4, 1 mM MPB, 2.77 units/ml HRP-I, and 74 μM H_2O_2, at 37°C. HRP type I (1.4 to 8.3 units/ml) and HRP–IgG (0.5 to 120 μl) were assayed in the same conditions with 0.48 units/ml esterase.

RESULTS AND DISCUSSION

The hydrolysis of MPB (compound 1 on Fig. 2) is expected to produce benzoic acid and 2-methyl-1-propenol (compound 2 on Fig. 2). It is also expected that the HRP-catalyzed oxidation of enol 2, which is the enol form of IBAL, elicits luminescence parallel to oxygen consumption.

The substrate MPB was submitted to the H_2O_2/O_2/HRP-I oxidative system in 0.05 M phosphate buffer at pH 7.4, at 37°C. The absence of a specific hydrolytic agent led to a very low level of luminescence and low rate of oxygen uptake. This confirms that the noncatalyzed hydrolysis is very slow. Addition of esterase (0.48 units/ml) to the solution resulted in a great increase of the light intensity, together with an increase in the rate of oxygen consumption, as shown in Fig. 3.

This reaction behaves very much the same way as the oxidation of IBAL by the H_2O_2/O_2/HRP system. In that case, light emission is dependent on O_2 present, which is consumed during the reaction. Likewise, in

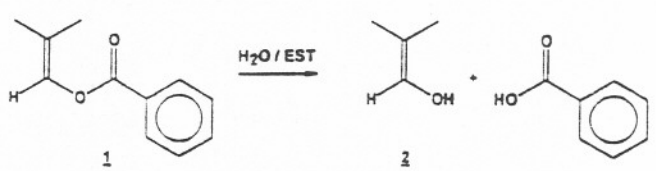


FIG. 2. Hydrolysis of MPB.

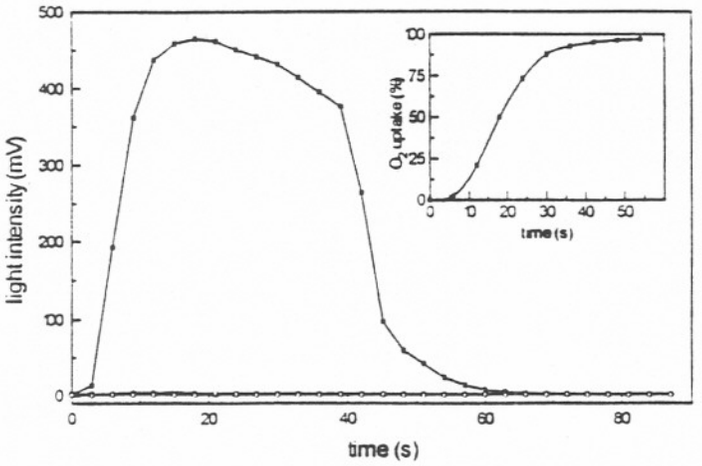


FIG. 3. Light emission kinetics from the MPB/esterase/H_2O_2/O_2/HRP-I system. (■) standard reaction mixture; (●) control without MPB; (♦) control without esterase; (□) control without H_2O_2, and (○) control without HRP. (Inset) Oxygen consumption kinetics of the standard reaction mixture.

the present case light emission rises to a maximum, followed by a fast drop when oxygen is totally depleted. The inset in Fig. 3 shows that oxygen uptake closely follows the kinetics of light emission.

The emission spectrum was obtained by superposition of several wavelength scans taken when the luminescence levels off. The spectral distribution shows a maximum around 450 nm, in conformity with the assumption of triplet acetone as the electronically excited product (5). Figure 4 shows the emission spectrum of the standard reaction and, for comparison, the phosphorescence spectrum of triplet acetone taken in EPA glass (at 77K).

The multiplicity of the excited state arising from the IBAL/O_2/HRP system has been thoroughly explored by Cilento and co-workers (3, 11), and triplet acetone was confirmed as the main excited product by several methods. Aside from small differences in the vibronic structure, the similarity in the shape and position of the two spectra shown in Fig. 4 indicates that the emission elicited by the MPB/esterase/H_2O_2/O_2/HRP-I system is also due to acetone phosphorescence. The question as to why it phosphoresces at room temperature in aerated medium is a point of debate. The protection of the triplet state by the HRP–apoprotein against oxygen deactivation still remains the best explanation (11, 12).

The optimum conditions of pH, buffer, and H_2O_2, HRP, and MPB concentrations were ascertained (Fig. 5). Although the maximum intensity was found with $[H_2O_2] = 148\ \mu M$ (Fig. 5C), we decided to maintain the standard concentration at 74 μM to avoid formation of HRP Compound III. The fall of light intensity at higher concentrations of HRP is probably due to reabsorption, since the Sorret band (λ_{max} 410 nm; ϵ 102 $mM^{-1}\ cm^{-1}$; Ref. 13) of the heme group peaks in the region where triplet acetone emits (see Fig. 4). One cannot, however, exclude quenching of triplet acetone by the apoprotein itself, since it has been shown that triplet acetone abstracts hydrogen from the sugar portion of HRP. A similar effect was observed with the IBAL/O_2/HRP system when monitoring the rate of oxygen uptake (18), indicating that the effect goes beyond pure quenching.

As depicted by Fig. 5E, the peak of light emission depends linearly on the MPB concentration in the 0.05 to 0.6 mM range, then levels off, suggesting solubility limitation. In fact, at higher concentrations the substrate is not totally soluble. Attempts to reach higher levels of emission by the use of surfactants have failed. While Triton X-100 and SDS did not solubilized the substrate, the addition of 1 to 11 mM of CTAB (cmc 0.8 mM; Ref. 14) decreased the intensity of emission, though it increased its solubility. This is not surprising since liver esterase is not expected to act at interfaces, therefore being limited by the solubility of the substrate within the bulk of the aqueous medium.

Phosphate at higher concentrations may have two effects: decrease MPB solubility and increase the ketonization rate of the enol formed (15, 16). Both effects should affect the intensity and the kinetics of light emission. Bohne *et al.* (17) have determined that phosphate is a true bifunctional catalyst in the keto–enol equilibrium of IBAL (the keto form of enol 2). With an equilibrium constant of 1.7×10^{-4} and a ketonization rate constant of 0.5 $M^{-1}\ s^{-1}$, an increase in the phosphate concentration from 0.05 to 0.10 M results in a decrease of enol lifetime from 40 to 20 s.

The effect of pH upon the system arises from a complex combination of the optimum activity of the two enzymes. HRP, known to be active over a broad range of pH for most substrates, shows an optimum at 7.5 for the oxidation of IBAL (18). On the other hand, liver

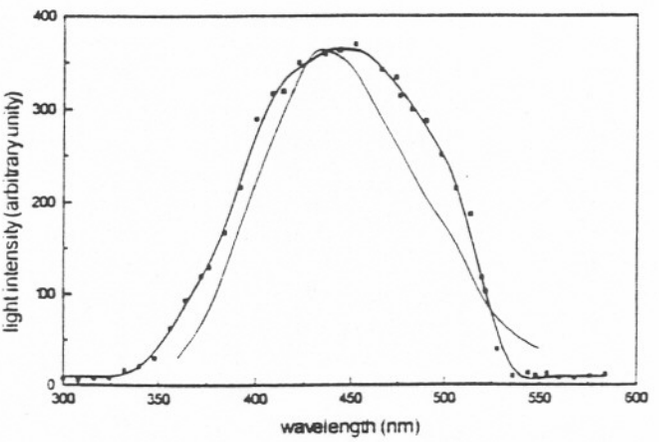


FIG. 4. (■) Spectral distribution of the total system and (solid line) acetone phosphorescence spectrum in EPA glass ($\lambda_{excitation}$ 280 nm).

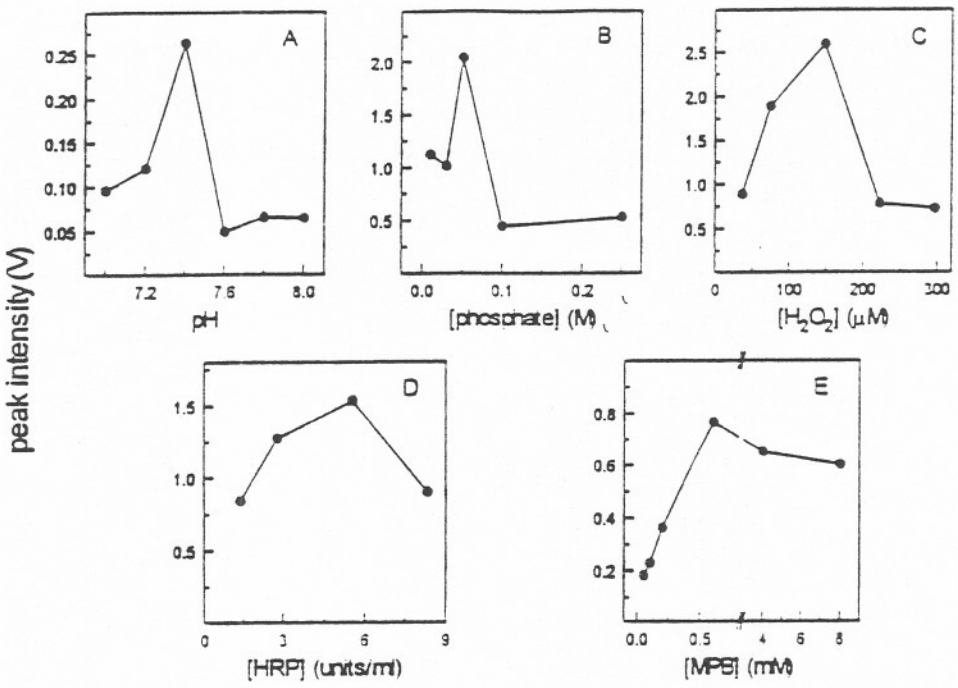


FIG. 5. Dependence of light emission on the mixture components (standard reaction mixture at 37°C otherwise stated).

esterase has an optimum activity at pH 8.0 (19). Since the pK_a of the enol is 11.6 (20), the experimental pH is not expected to have an effect upon the substrate.

A similar behavior is observed when examining the concentration of esterase (Fig. 6). Alteration of esterase concentration led to a lengthening of the emission profile with the reaction span increasing from tens of seconds to several minutes. This is a strong evidence that hydrolysis of the substrate is the limiting step for chemiluminescence. Cilento and co-workers (7, 8) observed a similar effect when the trimethylsi lyl enol ether of IBAL was treated with the H_2O_2/O_2 HRP system. The limiting step for light emission, i that case, was concluded to be the hydrolysis of th enol ether catalyzed by added fluoride ions. Simi larly, addition of a solution of the lithium enolate c IBAL to the reaction mixture (7) was followed by light burst with a $t_{1/2}$ of 5 s.

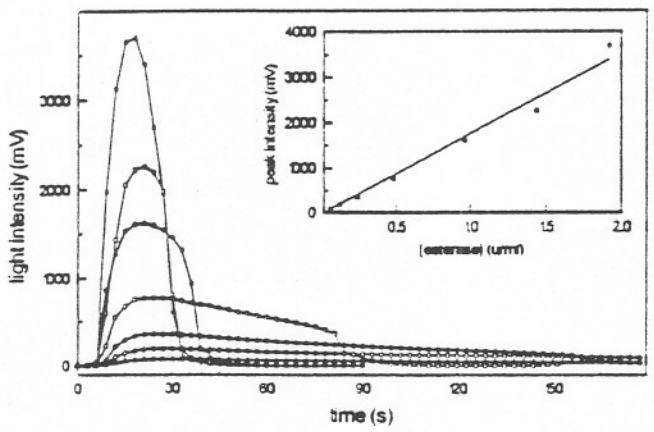


FIG. 6. Effect of esterase concentration on the light emission kinetics. Standard reaction mixture using [esterase] (■) 1.92 units/ml, (□) 1.44 units/ml, (●) 0.96 units/ml, (○) 0.48 units/ml, (♦) 0.24 units/ml, (◇) 0.12 units/ml, and (▲) 0.059 units/ml. (Inset) Peak intensity dependence with esterase concentration.

As shown in the inset of Fig. 6, there is a linea enhancement of the light emission following esteras concentration increase. This fact in itself demonstrate that this reaction can be used as a method for esteras activity determination. The linear range showed goe from 0.06 to 1.9 units/ml, making it possible to deter mine as low as 0.1 μg of this protein (ca. 2 pmol; ester ase MW 60 kDa; Ref. 19). Once the blank intensity i typically 1 mV, the detection limit may be as low a 1.1×10^{-3} units/ml.

Noteworthy is the fact that the MPB-dependen chemiluminescence signal is greatly increased upon ac dition of a fluorescent acceptor like DBAS. Special at tention has been given to this compound as an en hancer of chemiluminescence in several systems wher triplet carbonyls (poor emitters) are formed (10 Hence, addition of ca. 2 μM DBAS to the standard reac tion mixture led to a fivefold increase in the peak inten sity, whereas with only 6 μM DBAS the equipment de tector system reaches saturation (see Table 1). Thi observation further corroborates the triplet nature c the excited product.

Although determination of esterase levels in general is not a routine diagnostic approach, it could be of importance in specific situations like in the evaluation of the total esterase activity in extracts or homogenates of plant and animal tissues, leukocytes, etc. On the other hand, by substituting the radical benzoyl with an appropriate group, determination of specific esterase activity may be achieved. Our laboratory is currently developing specific substrates for lipase in view of its potential importance in diagnosis of several pathologies.

The question whether HRP conjugates or a different mixture of isoenzymes could produce the same results as HRP type I was examined using either HRP type VI or an anti-rabbit HRP–IgG conjugate from a commercial ELISA test. Figure 7 shows the comparative emission profiles for these three mixtures, attesting the generality of the method. As expected, HRP type VI showed similar results but with a shorter reaction time. Note that the amount of HRP-I and HRP-VI used had the same total peroxidase activity, while HRP conjugate used was 140 μl of the commercial conjugate solution, which contains only 1/50 of the peroxidase activity compared with the standard concentration of HRP-I used (determined by the *o*-dianisidine method; Ref. 21). This explains the low level of luminescence obtained with HRP conjugates (Fig. 7).

The remarkable finding that HRP conjugates also oxidize the enol formed in the hydrolysis of MPB, followed by light emission, prompted us to examine the feasibility of the use of this signal to determine its quantity. We have found that the total light intensity is linearly dependent on the concentration of HRP conjugate, with detection limits of the order of 1 μl of the conjugate, considering the signal-to-noise ratio observed. However, this limit may be lowered by sensitization with DBAS. Addition of 9.5 μM DBAS to this mixture resulted in ca. 30-fold enhancement of the peak intensity.

Concerning the use of this system in HRP-based immunoassays, it must be pointed out that the conditions described here are far from optimized. HRP-conjugate preparations are known to include several substances

TABLE 1

Effect of DBAS Addition on the Light Intensity of MPB/Esterase/H_2O_2=HRP-I System[a]

[DBAS] (μM)	Peak intensity (V)
0	0.64
1.9	3.23
3.8	7.06
5.7	9.94

[a] Standard reaction mixture, at 37°C.

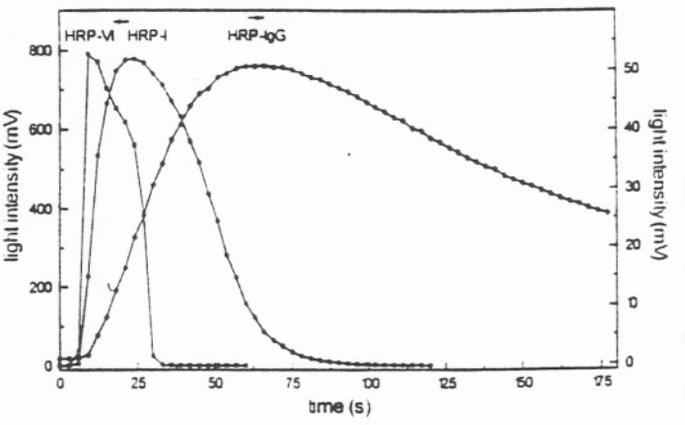


FIG. 7. Light kinetics for different types of peroxidase. Standard reaction mixture using (●) HRP type I, (■) HRP type VI, and (◆) HRP–IgG conjugate. The amount of HRP-VI was equal to HRP-I in enzymatic activity units, while HRP–IgG was 140 μl of the commercial conjugate solution (ca. 1/50 of HRP-I activity units; see text).

connected to the developing method used, including antioxidants and other preservatives, which may quench the chemiluminescence. Moreover, the optimum conditions for free HRP type I (used throughout, unless indicated) may not be the same for HRP conjugates.

CONCLUSIONS

The enol form of IBAL, compound **2**, can be blocked by a benzoyl group to form MPB, compound **1**, precluding its oxidation by the H_2O_2/O_2/HRP-I system. Upon enzymatic hydrolysis, compound **2** is released and light emission is elicited at intensities proportional to the esterase activity. The whole system works as a chemiluminescent reaction triggered by esterase and, thus, can be used to develop an analytical assay for esterases. The esterase used—dog liver esterase—is relatively nonspecific toward the type of substrate used. However, upon exchanging the benzoyl group with an appropriate blocking group, assays to determine the activity of specific esterases or any other hydrolytic enzyme can be developed. We have also found that the peroxidase used—HRP type I—can be substituted by a commercial HRP–IgG conjugate (from a diagnostic kit for HIV-1 EIA recombinant). More than that, the light intensity is also regulated by the amount of the conjugate used, opening the possibility of applying this reaction to develop immunoassays based on chemiluminescent measurements, with potentially much higher sensitivity.

ACKNOWLEDGMENTS

The authors are indebted to the Fundação de Amparo a Pesquisa do Estado de São Paulo, FAPESP, São Paulo, Brazil; the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasília, Brazil; and the Programa de Apoio ao Desenvolvimento Científico

e Tecnológico, PADCT, Brasília, Brazil for support and to Professors E. J. H. Bechara and F. Quina for critically reviewing the manuscript.

REFERENCES

1. De Luca, M., and McElroy, W. D. (1986) *Methods Enzymol.* **133,** 1–649.
2. Campbell, A. K. (1988) Chemiluminescence: Principles and Applications in Biology and Medicine, Ellis Horwood, Chichester.
3. Cilento, G. (1980) *Acc. Chem. Res.* **13,** 225–230.
4. Cilento, G. (1984) *Pure Appl. Chem.* **56,** 1179–1190.
5. Bechara, E. J. H., Oliveira, O. M. M. F., Durán, N., Baptista, R. C., and Cilento, G. (1979) *Photochem. Photobiol.* **30,** 101–110.
6. Durán, N., and Cilento, G. (1980) *Photochem. Photobiol.* **32,** 113–116.
7. Adam, W., Baader, W. J., and Cilento, G. (1986) *Biochim. Biophys. Acta* **881,** 330–336.
8. Baader, W. J., Bohne, C., Cilento, G., and Nassi, L. (1986) *Biochem. Educ.* **14,** 190–192.
9. Gallardo, H., Guillo, L. A., Durán, N., and Cilento, G. (1984) *Biochim. Biophys. Acta* **789,** 57–62.
10. Catalani, L. H., Wilson, T., and Bechara, E. J. H. (1987) *Photochem. Photobiol.* **45,** 273–281.
11. Cilento, G. (1982) *in* Chemical and Biological Generation of Excited States (Adam, W., and Cilento, G., Eds.), pp. 277–307, Academic Press, New York.
12. Catalani, L. H., and Bechara, E. J. H. (1984) *Photochem. Photobiol.* **39,** 823–830.
13. Dunford, H. B., and Stillman, J. S. (1976) *Coord. Chem. Rev.* **19,** 187–251.
14. Mukerjee, P., and Mysels, K. J. (1971) Critical Micelle Concentration of Aqueous Surfactant Systems, National Standard Reference Data System, Washington, DC.
15. Baader, W. J., Bohne, C., Cilento, G., and Dunford, H. B. (1985) *J. Biol. Chem.* **260,** 10217–10225.
16. Dunford, H. B., Baader, W. J., Bohne, C., and Cilento, G. (1984) *Biochem. Biophys. Res. Commun.* **122,** 28–32.
17. Bohne, C., MacDonald, D., and Dunford, H. B. (1987) *J. Biol. Chem.* **262,** 3572–2578.
18. Faria Oliveira, O. M. M., Haum, M., Durán, N., O'Brien, P. J., O'Brien, C. R., Bechara, E. J. H., and Cilento, G. (1978) *J. Biol. Chem.* **253,** 4707–4712.
19. Junge, W. (1983) *in* Methods of Enzymatic Analysis, 3rd ed. (Bergmeyer, H. U., Ed.), Vol. IV, pp. 8–14, VCH, Basel.
20. Chiang, Y., Kresge, A. J., and Walsh, P. A. (1986) *J. Am. Chem. Soc.* **108,** 6314–6320.
21. Becker, L. A. (1977) Worthington Enzyme Manual, p. 66, Worthington Biochemical Corp., Freehold, NJ.

Anexo XII

Chemiluminescence triggered by hydrolase activity in an horseradish peroxidase/H_2O_2 coupled assay. Campa, A.; Andrade, A.C.; Catalani, L.H. *Photochem. Photobiol.* **63**, 742 (1996).

Photochemistry and Photobiology, 1996, 63(6): 742–745

Symposium-in-Print

Chemiluminescence Triggered by Hydrolase Activity in a Horseradish Peroxidase/H_2O_2–Coupled Assay*

Ana Campa[1]†, Andrea de Castro Andrade[1] and Luiz Henrique Catalani[2]

[1]Faculdade de Ciências Farmacêuticas and [2]Instituto de Química, Universidade de São Paulo, São Paulo, Brasil

Received 18 October 1995; accepted 31 January 1996

ABSTRACT

2-Methyl-1-propenyl acetate (MPA) was hydrolyzed by wheat lipase, pancreatic lipase, hydrolases and lipoprotein lipase from serum in a reaction that produces 2-methyl-1-propenol. The latter was subsequently oxidized by horseradish peroxidase (HRP)/H_2O_2/O_2 yielding triplet acetone that emits visible light. The integrated light emission was proportional to the units of lipase or volume of serum present. When pancreatic lipase was assayed, light emission was observed in the presence of bile salt as surfactant and chlorophyll *a* as light sensitizer. The potential application of this reaction in determinations of hydrolase/lipase activities is discussed.

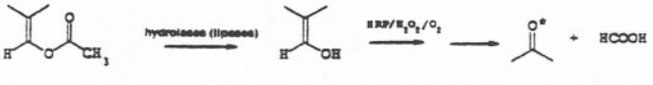
INTRODUCTION

The development of chemiluminescent and bioluminescent assays designed for research and clinical routine purposes are highly desirable given that they are potentially simple, sensitive and that the number of chemiluminometers in laboratories is now increasing (1,2). Many kits of chemiluminescence immunoassays have already been adopted in clinical routines. The development of homogeneous assays that require no separation step has attracted increased attention (3,4). However, chemiluminescent assays for metabolites or enzymes are effectively still to be introduced.

Chemiluminescent assays have improved the determination of several substances of clinical interest. The assays described for the measurements of cholesterol (5), glucose (6), lactate and lactate dehydrogenase (7) set the example. Of further interest is the development of sensitive assays for substances whose current available methodology is impaired. This is the case for lipase (8). Despite its value to clinical medicine and biological research, no simple and efficient methodology is available for its routine determination. The main problems associated with the available methodology are long incubation times, unstable and nonreproducible substrates, requirement of very sensitive devices and lack of discrimination between active and inactive enzyme (9).

Here we introduce a chemiluminescent assay by using specific hydrolases in the production of a substrate of horseradish peroxidase (HRP).‡ The HRP-catalyzed oxidation of several α-methine carboxylic acids and aldehydes and their chemiluminescence have been studied by Cilento and coworkers. Among these, the most extensively studied was the HRP-catalyzed oxidation of isobutyraldehyde (IBAL) (10–15). The HRP cycle initiates when the ferriheme from native HRP either reacts with the peracid, contaminating the substrate solution, or with added H_2O_2 (14). This reaction produces compound I that catalyzes the hydrogen abstraction of the enol form of IBAL (13,14). Phosphate buffer is necessary for the HRP-catalyzed oxidation of IBAL because it accelerates the rate of IBAL enolization (14,15). Compound II formed in the latter step is also catalytically active and regenerates to the enzyme native form. Addition of molecular oxygen to the free radical formed by the HRP cycle yields a supposed 1,2-dioxetane intermediate, which decomposes into triplet acetone and formic acid. Acetone phosphorescence can be detected with high efficiency. Furthermore, the chemiluminescence of this reaction can be enhanced by electronic energy transfer to acceptors with high quantum yield of fluorescence such as sodium 9,10-dibromoanthracene sulfonate (10), xanthene dyes (11) and chlorophyll *a* (12).

Here we use three types of hydrolases (wheat lipase, pancreatic lipase and serum hydrolases enriched or not by lipoprotein lipase, LLP) to hydrolyze 2-methyl-1-propenyl acetate (MPA). The product, which is the enol form of IBAL, is subsequently oxidized by the HRP/H_2O_2/O_2 system, producing chemiluminescence.



MATERIALS AND METHODS

The MPA was synthesized according to Bedoukian (16). The identity and purity was evaluated by ^{1}H-NMR. Hydrogen peroxide.

*This paper is dedicated to the memory of Giuseppe Cilento.

†To whom correspondence should be addressed at: Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, CEP 05508-900, SP, Brasil. Fax: (0055-11) 8132197.

‡*Abbreviations:* HRP, horseradish peroxidase; IBAL, isobutyraldehyde; LLP, lipoprotein lipase; MPA, 2-methyl-1-propenyl acetate; SDS, sodium dodecyl sulfate.

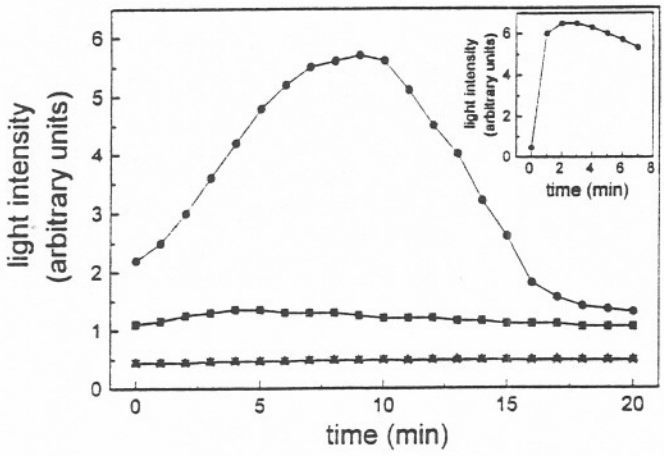


Figure 1. Light emission of the reaction MPA/wheat lipase/HRP/H_2O_2/O_2 (●). The controls without HRP (▲), lipase (■) and substrate (×) are also shown. The reaction conditions were 0.5 *M* phosphate buffer pH 7.4, 134 m*M* substrate, 0.5 *M* ethanol, 0.24 mg wheat lipase, 10 m*M* H_2O_2, 3 μ*M* HRP. Final volume 2.6 mL, temperature 37°C. Inset—Effect of preincubation (4 min) of MPA with lipase.

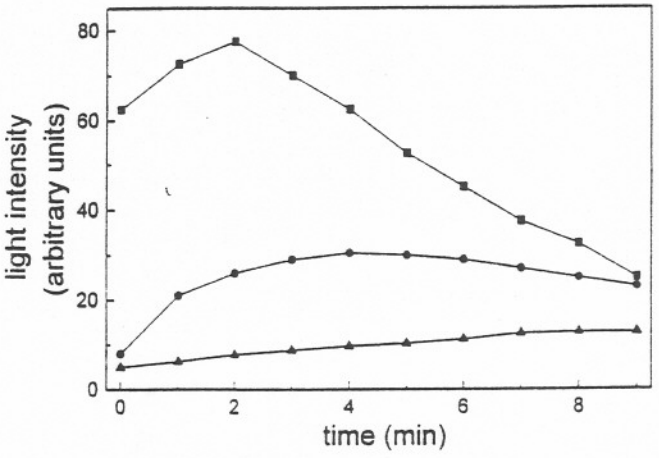


Figure 2. Phosphate buffer molarity effect (▲, 0.5 *M*; ●, 0.3 *M*; ■, 0.05 *M*) upon the reaction MPA/wheat lipase/HRP/H_2O_2/O_2. The conditions were the same as in Fig. 1.

horseradish peroxidase type VI, pancreatic lipase, bile salts and chlorophyll *a* were commercially obtained from Sigma Chemical Co. Wheat lipase was from Worthington Biochemical Corp. and heparin from Roche.

Light emission intensity was observed by using either a Hamamatsu TV C-767 Photon Counter (Figs. 1, 2, 4 and 5) or a commercial Bioorbit luminometer (Fig. 3). Oxygen consumption was followed by a model 53 Yellow Springs oxygen monitor.

Stock solution of MPA was prepared in ethanol immediately before use. When using serum as a source of hydrolases, the experiment was conducted with that obtained from blood collected before and 10 min after an endovenous injection of heparin (100 UI/kg body weight) was applied. The *in vivo* endovenous administration of heparin releases lipoprotein lipase from the endothelial surface, increasing its activity in serum (17–19). The human donor was the same for both preparations.

Unless otherwise stated, the reaction mixture contained MPA as substrate (134 or 0.87 m*M* in, respectively, 0.5 or 0.05 *M* ethanol), a source of hydrolase (0.24 mg wheat lipase, 500 U pancreatic lipase or 200 μL serum), H_2O_2 (10 m*M*) and HRP (3 μ*M*) in phosphate buffer (0.05 *M*), pH 7.4. The final volume was 2.6 mL and the temperature was 36°C. The reaction was triggered by the addition of the hydrolase source.

RESULTS AND DISCUSSION

The hydrolysis of MPA by different hydrolases directly releases the enol form of IBAL. The latter was oxidized by the HRP/H_2O_2/O_2 system in a chemiluminescent reaction. The best conditions for this reaction were analyzed.

Although IBAL is relatively soluble, the acetylation of its enolic form, even with a short carbon chain, led to a great loss of solubility. This is an especially important point for lipase activity because *in vivo* this enzyme acts at interfaces and, consequently, its catalytic activity depends on substrate solubility and the presence or absence of surfactants (17). When using wheat lipase, two limiting substrate concentrations were tested: 134 and 0.87 m*M*. At the highest concentration the substrate was insoluble. Nevertheless it was of the same magnitude as commonly used in reactions where IBAL is the substrate of HRP (14). The solubilization of the substrate by surfactants was evaluated by either adding hexadecyltrimethylammonium bromide, sodium dodecylsulfate (SDS) or Triton X-100 ($0.1–1.10^{-3}$ *M*). Unfortunately, this led to the formation of turbid solutions that impaired any precise light emission measurements. Indeed, with SDS and Triton X-100 a total suppression of light emission was observed.

Under high substrate conditions, maximum intensity chemiluminescence occurred at approximately 9 min (Fig. 1) Controls without HRP, lipase or substrate indicated that emission was dependent on the presence of all reactants. Maximum light emission shifted to shorter times when the substrate was preincubated (4 min) with lipase, prior to the addition of HRP (Fig. 1, inset), which indicates that the hydrolysis of the substrate is the rate-limiting step in the process.

The light emission increased almost one order of magnitude when phosphate buffer molarity was reduced from 0.5 to 0.05 *M* (Fig. 2). This was probably due to: (1) increase of the substrate solubility under low ionic strength conditions and (2) decrease of the rate constant of enol–keto reaction (20). As expected, these effects altered the intensity of light emission.

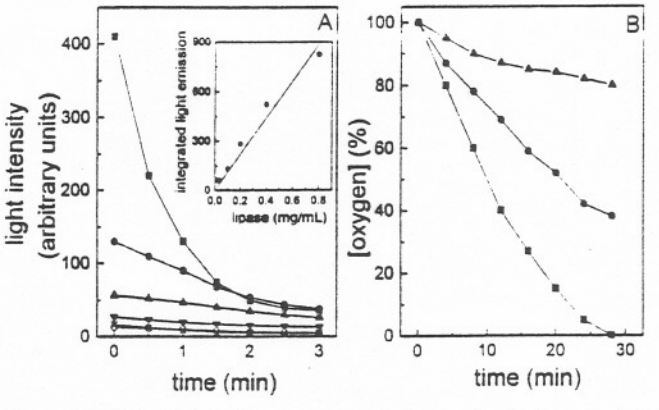


Figure 3. Effect of the lipase concentration on the light emission (A) and oxygen consumption (B) of the reaction MPA/wheat lipase/HRP/H_2O_2/O_2. The conditions of reaction were phosphate buffer 0.05 *M*, pH 7.4, 0.87 m*M* substrate, 10 m*M* H_2O_2, 3 μ*M* HRP, final volume 3 mL, temperature 37°C. Lipase was (×), 0.01; (○), 0.02; (▼), 0.10; (▲), 0.20; (●), 0.40 and (■), 0.80 mg/mL. Inset—Integrated light emission dependence with lipase concentration.

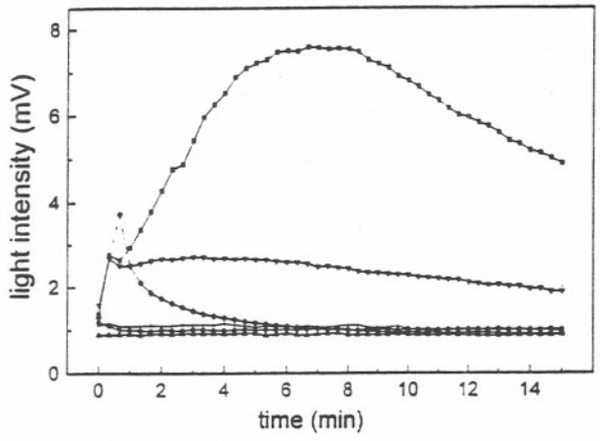


Figure 4. Chlorophyll *a*-sensitized light emission of the reaction MPA/bile salt/pancreatic lipase/HRP/H_2O_2/O_2. The reaction conditions were phosphate buffer 0.065 *M*, pH 7.8, 2 m*M* bile salt, 500 U pancreatic lipase, 118 m*M* MPA, 3 μ*M* HRP, 108 μ*M* H_2O_2, 1.3 × 10^{-5} *M* chlorophyll *a*, final volume 1 mL, temperature 36°C. The complete reaction (■) and the controls without H_2O_2 (▼), MPA (◆), HRP (+), chlorophyll *a* (●) and pancreatic lipase (▲) are shown.

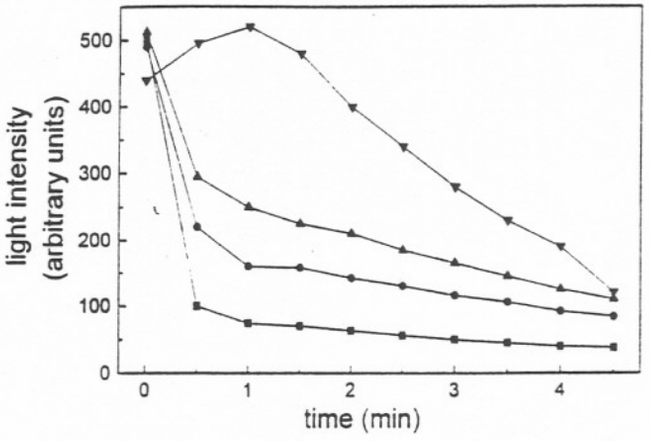


Figure 5. Effect of serum volume (■, 150; ●, 175; ▲, 200 and ▼, 250 μL) upon light emission of the reaction MPA/serum/HRP/H_2O_2/O_2. Control experiments in the absence of substrate, HRP or serum showed no light emission. Conditions were the same as those in Fig. 3.

The pH effect on light emission was evaluated at pH 7.0, 7.4 and 7.8 and for this range there were no significant variations (data not shown). Reduction in temperature from 36°C to 30°C resulted in a significant decrease of light intensity (data not shown). Oxygen uptake could not be determined because under these conditions substrate droplets, formed on the surface of the solution, impaired measurements.

At 0.87 m*M* concentration, the substrate was totally soluble. Decreased substrate concentrations led to profound modifications of kinetics and intensity of the light emission (Fig. 3A). Additionally it allowed for oxygen uptake measurements (Fig. 3B). A fast decay light emission was observed. Within the analyzed range (0.06–2.4 mg of wheat lipase/reaction) there was a strong correlation between integrated light emission and the amount of lipase added (Fig. 3 inset). These results indicate the possible use of this type of assay in determining lipase activity. Although not contemplated herein, light emission might be increased by the addition of appropriate acceptors and thus lead to enhanced sensitivity (10–12).

When pancreatic lipase was used instead of wheat lipase, light emission was observed at high substrate concentrations, in the presence of bile salt as surfactant and chlorophyll *a* as a light sensitizer (Fig. 4). Here a sensitizer was absolutely necessary; light emission could not be observed in the absence of chlorophyll *a*. This molecule has been proposed to be a good triplet acceptor and its fluorescence efficiency is increased when solubilized by surfactants (12). Although it is widely accepted that lipase activity is inhibited by bile salt (21), under the assayed conditions, *i.e.* low bile salt concentration, it is known that it does not inhibit the enzyme (22). Figure 4 shows the importance of bile salt for the observation of chemiluminescence. Bile salt may act like other detergents, increasing the fluorescence quantum yield of chlorophyll *a*.

The determination of lipase activities in biological fluids and tissues is of great importance to medicine and fundamental research. In clinical chemistry, determinations of serum pancreatic lipase are utilized in acute pancreatitis diagnosis and contribute in pancreatic duct obstruction trials (8,23). As lipase inhibition by high bile salt concentration can be reverted by the addition of colipase (21), this chemiluminescence assay might experience significant specificity improvement.

The substitution of purified hydrolases for serum also led to a chemiluminescent reaction. Serum was able to trigger very intense light emission. Correlation between integrated light emission and volume of serum added was identified (Fig. 5). Subsequently we focused on LLP evaluations. This determination is an important test in the investigation of hyperchylomicronemia (type I hyperlipoproteinemia) (23). Capillary endothelial surfaces released LLP to the serum once endovenous heparin was administered *in vivo* (17–19). Total light emission area increased by almost 60% when serum obtained after *in vivo* administration of heparin was used (data not shown). The serum supplies the necessary activators for LLP and the albumin for the enzymatic action (18). Therefore, these results showed that serum hydrolases and LLP catalyze the hydrolysis of the MPA.

CONCLUSIONS

The assay herein described involved two coupled enzymatic steps that were performed under the same conditions. First, MPA was cleaved by wheat lipase to yield the enol form of IBAL. The latter was oxidized by HRP/H_2O_2/O_2 resulting in light emission that was readily quantified by photometry. The assay was easy and results were obtained shortly after injection of the initiator. Optimal condition for this reaction was observed at 0.05 *M* phosphate buffer pH 7.4. This assay can be standardized to determine hydrolase activity, including those important to clinical chemistry among which are pancreatic and lipoprotein lipases.

Finally, this and other HRP-catalyzed reactions based on the formation of triplet acetone deserve further attention. Given that excited triplet species are suppressed by oxygen, excited singlet states are generally responsible for chemiluminescence in solutions. Hence, the phosphorescence of triplet acetone in the peroxidase-catalyzed oxidation of IBAL is thus an exception among chemiluminescent reactions. The

formation of this triplet state probably inside the enzyme protected it from oxygen (24). This ensures a source of triplet species with high yield of phosphorescence in aqueous solutions. On the other hand, because of the longer triplet lifetime these are more prone to energy transfer or reactions. This characteristic is extremely interesting because it offers a unique opportunity for highly efficient triplet species formation to take place in an HRP-catalyzed reaction. Once HRP is commonly used as a conjugate in chemiluminescent immunoassay there is the possibility that such reactions would be useful in the development of a homogeneous assay based on triplet source energy transfer.

Acknowledgements—The authors greatly appreciate the help of Marcia C. Wright (English revision) and thank FAPESP, CNPq and CAPES for financial support.

REFERENCES

1. De Luca, M. and W. D. McElroy (1986) Bioluminescence and chemiluminescence, Part B. In *Methods Enzymol.* **133**, 1–649.
2. Campbell, A. K. (1988) *Chemiluminescence: Principles and Applications in Biology and Medicine.* Ellis Horword, Chichester, England.
3. Patel, A. and A. K. Campbell (1983) Homogeneous immunoassay based on chemiluminescence energy transfer. *Clin. Chem.* **29**, 1604–1608.
4. Jansen, E. H. J. M., R. H. van den Berg and G. Zomer (1990) Horseradish peroxidase as label in chemiluminescent immunoassay. In *Luminescence Immunoassay and Molecular Applications* (Edited by K. Van Dyke and R. Van Dyke), pp 57–75. CRC Press, Boston.
5. Chamoin, M.-C., M. Charbonnier, H. Lafont and J. P. Ternaux (1994) High-sensitive chemiluminescent assay for cholesterol. *Biochim. Biophys. Acta* **1210**, 151–156.
6. Naslund, B., P. Arner, J. Bolinder, L. Hallander and A. Lundin (1991) Glucose determination in sample taken by microdialysis by peroxidase-catalyzed luminol chemiluminescence. *Anal. Biochem.* **192**, 237–242.
7. Tabata, M., M. Totani and T. Murachi (1991) Determinations of lactate and lactate dehydrogenase activity in serum with the flow injection analysis system involving immobilized enzyme column and chemiluminescence. *Anal. Biochem.* **193**, 112–117.
8. Moss, D. W., A. R. Henderson and J. F. Kachmar (1986) Enzymes. In *Textbook of Clinical Chemistry* (Edited by N. W. Tietz), p. 735. W. B. Saunders, Philadelphia.
9. McNeely, M. D. D. (1989) Lipase. In *Clinical Chemistry. Theory, Analysis, and Correlations*, 2nd ed. (Edited by L. A. Kaplan and A. J. Pesce), pp. 930–933. C. V. Mosby, St. Louis.
10. Bechara, E. J. H., O. M. M. F. Oliveira, N. Duran, R. C. Baptista and G. Cilento (1979) Peroxidase catalyzed generation of triplet acetone. *Photochem. Photobiol.* **30**, 101–110.
11. Duran, N. and G. Cilento (1980) Long-range triplet–singlet energy transfer from enzyme generated triplet acetone to xanthene dyes. *Photochem. Photobiol.* **32**, 113–116.
12. Brunetti, I. L., G. Cilento and L. Nassi (1983) Energy transfer from enzymically-generated triplet species to acceptors in micelles. *Photochem. Photobiol.* **38**, 511–519.
13. Dunford, H. B., W. J. Baader, C. Bohne and G. Cilento (1984) On the mechanism of peroxidase-catalyzed chemiluminescence from isobutyraldehyde. *Biochem. Biophys. Res. Commun.* **122**, 28–32.
14. Baader, W. J., C. Bohne, G. Cilento and H. B. Dunford (1985) Peroxidase-catalyzed formation of triplet acetone and chemiluminescence from isobutyraldehyde and molecular oxygen. *J Biol. Chem.* **260**, 10217–10225.
15. W. Adam, W. J. Baader and G. Cilento (1986) Enols of aldehydes in the peroxidase/oxidase-promoted generation of excited triplet species. *Biochim. Biophys. Acta* **881**, 330–336.
16. Bedoukian, P. Z. (1944) A new synthesis of α-bromoaldehydes. *J. Am. Chem. Soc.* **66**, 1325–1327.
17. Baginski, M. L. (1981) Measurement of lipoprotein lipase and hepatic triglyceride lipase in human post heparin plasma. *Methods Enzymol.* **72**, 325.
18. Wang, C.-S., J. Hartsneck and W. J. McConathy (1992) Structural and physiological properties of lipoprotein lipase. *Biochim. Biophys. Acta* **1123**, 1–7.
19. Hultin, M., G. Bengtsson-Olivercrona and T. Olivercrona (1992) Release of lipoprotein lipase to plasma by triacylglycerol emulsion. Comparison to the effect of heparin. *Biochim. Biophys. Acta* **1125**, 17–103.
20. Bohne, C., I. D. MacDonald and H. B. Dunford (1986) Measurements of rates and equilibria for keto–enol tautomerism of aldehydes using horseradish peroxidase compound I. *J. Am. Chem. Soc.* **108**, 7867–7868.
21. Erlanson-Albertsson, C. (1992) Pancreatic colipase. Strucutural and physiologycal aspects. *Biochim. Biophys. Acta* **1125**, 1–7.
22. Morgan, R. G. H. and N. E. Hoffman (1971) The interaction of lipase, lipase cofactor and bile salts in triglyceride hydrolysis. *Biochim. Biophys. Acta* **248**, 143–148.
23. Stein, E. A. (1986) Lipids, lipoproteins, and apolipoproteins. In *Textbook of Clinical Chemistry* (Edited by N. W. Tietz), pp. 860–861. W. B. Saunders, Philadelphia.
24. Catalani, L. H. and E. J. H. Bechara (1984) Quenching of chemiexcited triplet acetone by biologically important compounds in aqueous medium. *Photochem. Photobiol.* **39**, 823–830.

Anexo XIII

Photolysis of a series of α-brominated *ortho*-xylenes in apolar solvents. Rezende, D.B.; Campos, I.P.A.; Toscano, V.G.; Catalani, L.H. *J. Chem. Soc. Perkin Trans.* 1857 (1995).

Photolysis of a series of α-brominated *ortho*-xylenes in apolar solvents

Daisy de B. Rezende, Ivan P. de Arruda Campos, Vicente G. Toscano and Luiz H. Catalani *
Instituto de Química, Universidade de São Paulo, C.P. 26.077, 05599-970, São Paulo, SP, Brazil

The α-brominated *ortho*-xylenes have been subjected to 254 nm irradiation in deaerated benzene, isooctane and benzene–cyclohexene. The product analysis revealed that homolysis of the C–Br bond is followed by a series of hydrogen abstraction and radical recombination reactions resulting in xylenes more and less brominated than the starting compound. The less brominated products are formed with higher quantum yield when cyclohexene is present, due to hydrogen abstraction by the *o*-benzyl radical formed initially, together with cyclohexene dimers. Additionally, the formation of 2-bromo-2,4,4-trimethylpentane is observed when isooctane is the solvent. The quantum yields observed for the photolysis of **1** and **2** are higher in benzene than in isooctane, suggesting sensitization by benzene. A biradical intermediate of the type *o*-quinodimethane was expected in the case of (*a*) photolysis of the *o*-benzyl radical formed (biphotonic process) or (*b*) intramolecular hydrogen abstraction. However, the addition of cyclohexene failed to produce the expected Diels–Alder adduct. The synthesis of the novel α,α-dibromo-*o*-xylene **3** is reported.

Introduction

Since the pioneering work of Zimmerman [1] on the photolysis of several benzyl derivatives, including halides and ammonium salts, a great deal of work has been accomplished in this field. Special attention has been given to the competition between homolytic and heterolytic processes in polar solvents.[2] Conclusions were drawn to provide a general mechanism for the heterolysis of the carbon–heteroatom bond, including the nature of the leading excited state. In contrast, only very few studies were done in apolar solvents.[3]

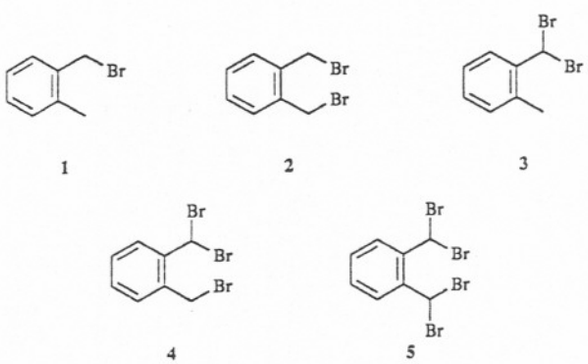
The behaviour of α-halogenated xylenes under UV irradiation is scarcely known. The recent work of Platz and co-workers,[4a] on the search for biradical species from the photolysis of α,α′-dichloro-xylene *ortho* and *meta* isomers remains a landmark. From these compounds the authors reported the preparation of *ortho*- and *meta*-xylylene in a matrix at 77 K, but very few experiments were done in solution with no clear results.

Platz and co-workers [4b] also demonstrated that mesitylylene biradical is produced in the photolysis of matrix-isolated mesitylene through a biphotonic process. Interestingly, the two photons are used to produce the mesitylene monoradical first *via* a T_n state of mesitylene. The nascent hydrogen formed reacts within the matrix cage to form the mesitylylene and H_2, most likely through thermal activation. These findings make α-halogenated *ortho*-xylenes good candidates for the monophotonic generation of xylylenes.

The intermediacy of an *ortho*-xylylene in the production of 1,2-dibromobenzocyclobutene by thermal reduction of α,α,α′,α′-tetrabromo-*o*-xylene **5** was recognized as early as 1957 by Cava *et al.*[5] The importance of *ortho*-xylylenes (or *ortho*-quinodimethanes) in Diels–Alder reactions is attested by the great number of routes for their preparation (including photochemical methods) and of applications in intramolecular cycloaddition reactions as a synthetic approach to polycyclic ring systems.[6]

Finally, Tournier *et al.*[3a] reported high quantum yields of HCl formation when benzyl chloride is photolysed in saturated hydrocarbon solution, and that this yield depends on solvent viscosity. As hydrogen halide photogenerating systems are of great value to photoresist applications, such molecules may be of industrial interest.

We report here the results of a study of the photochemical and photophysical behaviour of a series of α-halogenated xylenes in apolar solvents, where homolysis is expected to be the main event after light absorption. The compounds investigated are α-bromo-*o*-xylene **1**, α,α′-dibromo-*o*-xylene **2**, α,α-dibromo-*o*-xylene **3**, α,α,α′-tribromo-*o*-xylene **4** and α,α,α′,α′-tetrabromo-*o*-xylene **5**. The solvents chosen, benzene, isooctane and benzene–cyclohexene mixture, are representative non-polar solvents of different hydrogen donor abilities.



1 2 3 4 5

Results

Photolysis in benzene

Compounds **1**–**5** were subjected to photolysis in deaerated benzene at 254 nm (low pressure mercury lamp). The reaction mixtures were analysed by GC–MS and the product identification was confirmed by co-injection with authentic samples. The reaction profiles, following either reagent consumption or product formation, are linear up to five hours irradiation, when they start to level out. Table 1 shows the product distribution relative to the total number of moles of the consumed starting compound. These values were calculated from the linear regression of the reaction profiles at 150 min.

The first observation in Table 1 is that the photolysis of these compounds leads to others of similar structure, but with higher and lower degrees of bromination at the α-position. Aldehyde and carboxylic acid functionalities are derived from hydrolysis of geminal dibromo and tribromo derivatives, respectively (most probably as a result of thermal reactions). No ring-

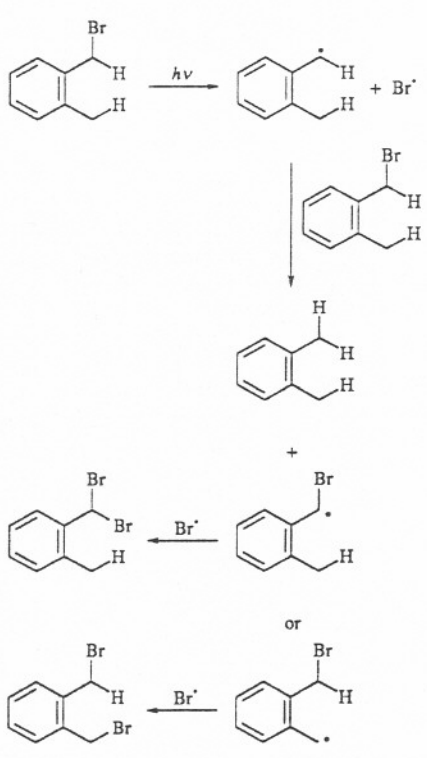
Table 1 Product distribution on the photolysis of **1–5** in benzene[a]

Product	1	2	3	4	5
o-Xylene	38.4	nf[b]	nf[b]	nf[b]	nf[b]
1	—	16.6	5.4	nf[b]	nf[b]
2	11.4	—	3.7	4.0	nf[b]
3	5.1	3.2	—	2.9	nf[b]
4	nf[b]	18.4	22.7	—	6.3
5	nf[b]	nf[b]	nf[b]	12.5	—
9[c]	16.3	—	1.8	6.1	3.5
Other products[c]	9.8 of **10**[d]	5.6 of **11**	18.4 of **12** 5.3 of **13**[d]	3.7 of **12** 11.6 of **13**[d]	12.1 of **14**[d] 2.5 of **15**[d]
Recover[e]	81	43.8	57.3	40.8	24.4
Total conversion[f]	9.4	20.3	18.4	12.0	3.8

[a] In percent relative to the number of moles of reagent consumed after 150 min irradiation. [b] Not found. [c] **9** *o*-tolualdehyde, **10** α-bromo-*o*-tolualdehyde, **11** α-bromo-α′-phenyl-*o*-xylene, **12** *o*-toluic acid, **13** α,α,α-tribromo-*o*-xylene, **14** α-bromo-*o*-toluic acid, **15** α,α-dibromo-*o*-tolualdehyde. [d] Tentative structure (proposed on the basis of MS data; see Experimental section). [e] The sum of all products found (in percent relative to reagent consumed). [f] The percentage of converted starting material.

brominated product was found. It must also be emphasized that this list is restricted by the analytical methods used, which preclude the determination of higher oligomers.

It is expected that in an apolar solvent such as benzene, homolysis of the C–Br bond is the leading photolytic step, forming an *ortho*-substituted benzyl radical (hereafter, called α-xylyl radical) as an intermediate species. As the starting material is the only possible hydrogen atom donor in the system, the 'scrambling' of bromine atoms must occur at its expense, reacting with the nascent α-xylyl radical. This thermal reaction gives rise to a reduced product and a second radical which can then react with the extruded bromine atom producing an *o*-xylene derivative with a higher number of bromo substituents. Moreover, in the non-symmetrical xylenes as **1**, **3** and **4**, there are two different α hydrogens (with different abilities to react with the α-xylyl radical) therefore leading to two different types of radicals (in different proportions). A representation of these processes is shown in Scheme 1 for the case of compound **1**; all other compounds undergo analogous reactions.



Scheme 1

The total concentration of products with higher numbers of bromine atoms is very similar to the concentration of products with a lower number. The apparent exception is **3** and this may be due to its extreme sensitivity towards hydrolysis, which prevented a precise determination of its product distribution. This bromine atom balance is consistent with the observation that Br_2 is not a major product.

Another interesting observation in Table 1 is that the recovery of the photolysed material (that is, the sum of moles of the products found) decreases from **1**→**5**. A possible explanation is that the decrease in the number of abstractable hydrogens in the series might lead to a concurrent reaction of the α-xylyl radical resulting in products not detected by our analytic method, for example, oligomers. One could also argue on energetic grounds that the increase of the number of bromine substituents may provide a lower intrinsic quantum yield due to differences in energy levels. However, (*i*) as we will see below, if the solvent sensitizes the reaction, energy transfer from excited benzene to compounds **1**–**5** is exothermic in all cases (see Table 3); (*ii*) the S_1 and T_1 energy levels measured for compounds **1**, **2**, **4** and **5** are essentially identical; (*iii*) we found that the quantum yield of consumption of **2** is higher than that of **1** in the three solvents tested, including neat isooctane, therefore opposite to the decrease of recovered material.

Photolysis in benzene–cyclohexene mixture

To show that homolysis of the C–Br bond was the primary photolytic process responsible for the products found in benzene, we used other solvents of similar polarity and viscosity, but with enhanced hydrogen donating ability. Our expectation was to reduce the scrambling of bromine by quenching the first intermediate formed, presumably, the α-xylyl radical.

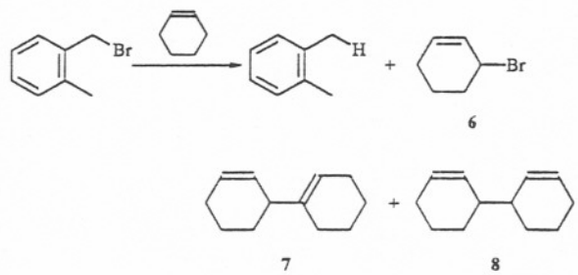
The first approach to reach optimal conditions to carry out this experiment was to exchange only partly the benzene with some solvent of enhanced hydrogen donating ability. Among all possibilities considered, 20% cyclohexene (2.0 mol dm^{-3}) was the best compromise in terms of (*i*) minor change in solvent physical properties, (*ii*) low light absorbance at 254 nm and (*iii*) a cyclohexene concentration competitive with that of the starting reagent (*ca.* 40-fold higher). Furthermore, with cyclohexene present we would have the chance to prove formation of an *ortho*-xylylene through a Diels–Alder type reaction. Although cyclohexene is not an ideal dienophile, it has been used with success as a trapping agent for *o*-xylylene.[7]

Table 2 summarizes the quantum yields in different solvents for (*i*) reagent consumption (Φ_{-RBr}) and (*ii*) formation of the reduced derivative (Φ_{RH}). Comparing benzene to mixed benzene–cyclohexene, the great increase in Φ_{RH} and the

Table 2 Quantum and chemical yields for the photolysis of **1–5**[a]

Reagents	Solvent	Φ_{-RBr}	Φ_{RH}	η_{RH}[b]
1	Benzene	0.33	0.13	0.38
	Benzene–cyclohexene[c]	0.82	0.24	0.29
	Isooctane	0.22	0.08	0.35
2	Benzene	0.52	0.09	0.11
	Benzene–cyclohexene[c]	0.91	0.27	0.29
	Isooctane	0.32	0.07	0.22
3	Benzene	0.49	0.03	0.05
4	Benzene	0.25	0.02[d]	0.41[d]
5	Benzene	0.13	0.01	0.13

[a] Φ_{-RBr} is the quantum yield for consumption of starting material and Φ_{RH} and η_{RH} are the quantum and chemical yields for formation of first reduced product. [b] Determined by GC analysis relative to reagent consumption after 150 min irradiation (molar response corrections were applied in all cases). [c] [Cyclohexene] = 2 mol dm^{-3}. [d] Sum of formation of **2** and **3**.



Scheme 2

observed suppression of scrambling products when cyclohexene is present confirms our hypothesis. Accordingly, we found new products derived from cyclohexene: 3-bromocyclohexene **6**, and the dimers **7** and **8** (Scheme 2). Compound **6** may be a product of radical recombination, while compounds **7** and **8** are probably formed by an addition–elimination reaction of a cyclohexenyl radical on a cyclohexene molecule. We did not find any sign of a Diels–Alder adduct, which shows that formation of *o*-xylylene as an intermediate is very unlikely.

A close look at Table 2 gives rise to two new questions: (*i*) why does Φ_{-RBr} increase so abruptly if cyclohexene is acting in a post-photolytic step? and (*ii*) why is this increment in Φ_{-RBr} not mostly (if not totally) reflected in Φ_{RH}? The answers to these questions are rather connected: in the absence of a good hydrogen donor, recombination of the radicals originally formed may play an important role. In fact, bromine radicals are known to be stabilized in benzene as a π-complex prolonging the lifetime of the radical.[8] Addition of cyclohexene provides an important new pathway for α-xylyl radical deactivation, which is reduction. But it is not the only one possible: polymerization is definitely another path. Although we did not quantify or identify it, we did observe a film deposition of insoluble material onto the quartz wall of the photolysis cell in all three solvents.

Photolysis in isooctane

To test a total exchange of solvent we selected isooctane mainly because it is completely transparent at 254 nm, yet its physical properties are similar to that of benzene [relative permittivity (dielectric constant), $\varepsilon = 1.940$ and viscosity, $\eta = 0.652$ cP for benzene at 20 °C and $\varepsilon = 2.284$ and $\eta = 0.507$ cP for isooctane at 20 °C; ref. 9], which makes it a good solvent for comparison.

Isooctane proved to be a poorer donor of hydrogen atoms than the starting reagent, specially considering its 120-fold higher concentration. The photolysis of **1** and **2** in this solvent showed a set of products similar to that observed in benzene, plus one specific to this solvent, which is 2-bromo-2,4,4-trimethylpentane (data not shown), therefore characterizing the same scrambling of bromine. The role of benzene as sensitizer of the reaction is well represented by the drop in Φ_{RH} (the quantum yield for the formation of the first reduced product) when going from pure benzene to isooctane. In both cases—**1** and **2**— Φ_{RH} in isooctane is *ca.* 1.5 times smaller than in benzene. This is not surprising since most of the incoming light is taken up by the latter.†

Concerning the values of Φ_{-RBr}—the quantum yield for starting material consumption—one consideration must be brought about: in benzene, the starting compound is the major (the only one at time zero) hydrogen donor. For each photon leading to the α-xylyl radical, two molecules of the original xylene are consumed—one by photochemical cleavage of C–Br bond and a second by thermal radical abstraction reaction. Hence, Φ_{-RBr} is overestimated by a factor close to two. In isooctane, hydrogen abstraction from the solvent competes with this process lowering this overestimation. When cyclohexene is present Φ_{-RBr} approaches its 'true' value, once there is no consumption of the starting xylene but by photochemical processes. Nonetheless, Φ_{RH}—the quantum yield for the formation of the first reduced product—is a reliable number for comparison.

Quantum yields and kinetics

The actinometry of our irradiating system was performed using malachite green leucocyanide according to literature.[10] This procedure has two advantages over the classic potassium ferrioxalate system: (*i*) malachite green leucocyanide does not absorb in the visible region, allowing the manipulations to be done without protection from ambient light; (*ii*) it absorbs in the same region as compounds **1–5** avoiding errors deriving from actinometer absorption of higher wavelengths light, not effective in the photochemical system in study, which would cause an apparent lowering in the determination of the quantum yields.

In conditions where all the light is directly absorbed by the reagent and where $\varepsilon_{254}(\text{reagent}) \cong \varepsilon_{254}(\text{product})$ in all cases, we have 'isosbestic' actinometric conditions as described by Bunce.[11] In other words, during the initial phase of the process the system behaves like Bunce's 'photobleaching' and absolute quantum yields are obtained employing eqn. (5) (see Experimental section). When benzene is the sensitizer of the reaction, eqn. (5) still applies and an 'apparent' quantum yield can be calculated if (*i*) most of excited benzene is quenched and (*ii*) the quenching rate constants are similar for reagent and product, conditions which are met here.‡ Fig. 1 represents a kinetic profile of one of the reactions studied here. It is clear that the assumption of linearity is valid in this case. We observed linearity in all the other cases for reaction times up to three hours (or more in some cases). All the quantum yields presented in Table 2 were calculated according to eqn. (5) and they represent its limit to time zero.

Discussion

The photolysis of **1–5** in apolar solvents generated a series of products that can be rationalized in terms of a primary

† Considering the concentrations used and the molar absorption coefficient for compounds **1–5** (of the order of 2000 at 254 nm), 99.9% of the light was totally absorbed in the first 0.15 mm of isooctane solution, while in benzene as solvent or co-solvent ($\varepsilon_{254} = 200$) this layer is of the order of 0.01 mm. The ratio for solvent/solute total absorbance is 11:1.

‡ According to Table 3, all possible energy transfer processes from benzene to compounds **1**, **2**, **4** and **5** are exothermic by ≈8 kcal mol^{-1} (singlet-singlet) or ≈13 kcal mol^{-1} (triplet-triplet). Hence, ET rate constants must be close to diffusion (1×10^{10} dm^3 mol^{-1} s^{-1} for benzene).[12]

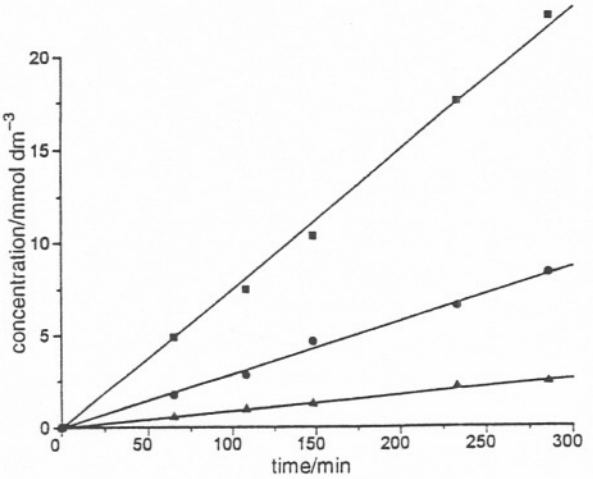


Fig. 1 Time-dependent product distribution for the photolysis of **1** in benzene: (■) $[1]_0 - [1]_t$; (●) [*o*-xylene]$_t$; (▲) [**2**]$_t$

Table 3 Energy levels[a]

Compound	T_1	S_1	T_2
Benzene	84[b]	110[c]	105[b]
Toluene	83[c]	106[c]	—
o-Xylene	82[c]	105[c]	—
1	71	100	—
2	72	100	—
4	70	99	—
5	72	103	—
Benzyl chloride	73[c]	—	—

[a] All measurements were made in EPA matrix; T_1 energies were calculated from the highest energy vibrational band of phosphorescence emission spectra; S_1 energies were calculated from the crossing points of the normalized fluorescence excitation and emission spectra. [b] In crystal (ref. 13). [c] In non-polar solvents (ref. 12).

homolytic cleavage of C–Br bond, followed by thermally allowed radical reactions (Table 1). Our results show that either direct excitation or sensitization by benzene leads to the same type of chemistry. However, comparing the results in benzene and isooctane one sees *ca.* 50% enhancement of the Φ_{RH} quantum yields in the sensitized reaction (Table 2).

In the early 80s, as a result of his studies on the photosolvolysis of benzylic halides and benzylammonium salts, McKenna [2a,b] presented convincing arguments that ion pairs are derived from singlet excited states, while radical formation would come mostly from a triplet species formed from the singlet *via* intersystem crossing. This process should be facilitated by spin-orbit relaxation, enhanced here by the presence of bromine substituents. Using different quenching techniques, Cristol and co-workers [2e,f] showed the existence of two triplet states on benzyl chlorides. Contradicting McKenna, they stated that either an upper short-lived triplet or the first singlet state is responsible for formation of photosolvolysis products. In addition, they consider that T_1 does not lead to products.

In the present case benzene sensitizes the reaction when it is used as a solvent or co-solvent. Considering the low quantum yield of fluorescence and the short lifetime of benzene singlet state in non-polar solvents (6% and 34 ns, respectively) [12] both S_1 and T_1 excited states of benzene could be quenched with sufficient efficiency to produce the sensitized quantum yields observed in Table 2. Benzene's second triplet could also be at play, since its energy is somewhat lower than S_1 (see Table 3), but its lifetime is expected to be extremely short [13] constraining energy transfer from this state.

Many other groups have questioned the factors that regulate homolysis *versus* heterolysis of benzylic systems upon irradiation.[2,3] The simple fact that this competition has been looked at only in nucleophilic (polar) solvents already biases the observations, mainly because of the induced polarization of the carbon–halogen bond. On the basis of *ab initio* MO–Cl and valence bond calculations, Larson and co-workers suggest that the initial triplet species undergoes dissociation into radical pairs 'unless there is an alternate decay mechanism'.[2d] In polar solvents, formation of an ion pair would be accomplished by a fast spin-inversion. When examining the photolysis of alkyl iodides, Kropp and co-workers [14] were convinced that electron transfer, producing ion pairs, is a path of deactivation of the radical pair, competing with diffusion. This became known as the Kropp hypothesis. It should be noted that in our work, should ion pairs be formed, they would not be noticed once they have no other way of reaction but to collapse back to starting reagent. The only evidence we have for the presence of a carbocation is the formation of compound **11** (see Table 1) as a product of electrophilic substitution on a solvent molecule.

As in heterolysis, solvent participation in deactivating the unstable intermediates formed during photohomolysis determines the set of products obtained. The hydrogen donating ability of the solvent could limit or even totally abort the scrambling of bromo radicals, as was the case of benzene–cyclohexene presented here. The only few studies of similar systems in apolar solvents [3] also showed a determining participation of the media towards thermally activated radical chemistry.

We did not observe products coming from an *o*-xylylene intermediate. Even using 20% v/v of cyclohexene in the photolysis of **1** and **2**, the expected Diels–Alder adduct was not detected. Two possible ways for its formation were foreseen: (*i*) extrusion of HBr or Br_2 through a Berson type mechanism as suggested by Platz [4b] and (*ii*) stepwise homolysis of two C–Br bonds by a two-photon process. By the first mechanism, the bromine atom must react with its partner radical abstracting a hydrogen at α' position within the solvent cage. The low viscosity of the solvents used here must have prevented this mechanism from operating. We are presently testing its viability in solvents of very high viscosity. The second possibility was certainly precluded by the low light intensity of our system. Adam and Ouchi [15] observed that photolysis of 1,8-bis(bromomethyl)naphthalene produced acenaphthene by the stepwise homolysis of the two C–Br bonds, followed by intramolecular C–C bond formation. This was possible by using the laser-jet technique [16] in which a high intensity laser beam is concentrated into a very small area. Unfortunately, this technique failed to produce results in the photolysis of **2** in benzene when sensitized by acetophenone.[17]

Experimental

Instruments

All qualitative and quantitative chromatographic analyses were performed on a Shimadzu CG-14-A gas chromatograph, using a splitter injector (43:1; 230 °C), a 25 m × 0.25 mm × 0.22 μm Shimadzu CBP-1 column (polydimethylsilicone), helium as carrier gas (1.6 cm^3 min^{-1}), and a flame ionization detector (260 °C, nitrogen as make-up gas). Oven temperature program: 80 °C (4 min), 30 °C min^{-1} (up to 250 °C), 250 °C (2.7 min). Data were collected with a Shimadzu Chromatopac C-R4A electronic recorder/digital integrator. GC–MS analyses were done either on a Finnigan-MAT model ITD-800 ion trap mass spectrometer coupled to a Finnigan-MAT gas chromatograph with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm), or on a Finnigan-MAT INCOS-50 quadrupole mass spectrometer interfaced to a Varian 3400 gas chromatograph having a similar column, or on a Hewlett-Packard 5988A quadrupole mass spectrometer attached to a

5890 gas chromatograph having a HP1 (12 m × 0.25 mm × 0.25 μm) column; in all cases, the experimental conditions were those described for conventional GC. The high-resolution mass spectrum was obtained with a Finnigan Mat 90 instrument (in direct sample injection mode). ^{1}H NMR (200.13 MHz) spectra were recorded at 20 °C on a Bruker AC-200-F spectrometer. All measurements were performed in 5 mm o.d. tubes, using a deuterium lock. Absorption spectra were obtained on a Hitachi U-2000 spectrophotometer in hexane solutions, at room temperature. Fluorescence and phosphorescence spectra were determined on a SPEX-Fluorolog-2 FL 111 spectrofluorimeter in EPA matrix, at 77 K, with a 1934D phosphorimeter attachment being employed for all phosphorescence emission measurements.

Materials

All solvents employed in irradiations and spectroscopic measurements were of spectrophotometric grade (Aldrich Gold Label), used without further purification. α-Bromo-*o*-xylene[18a] (**1**) was prepared from *o*-xylene (Aldrich) and purified by fractional distillation in a 10 cm Hempel column, under vacuum. α,α′-Dibromo-*o*-xylene[18b] (**2**), α,α,α′-tribromo-*o*-xylene[18c] (**4**) and α,α,α′,α′-tetrabromo-*o*-xylene[18d] (**5**) were also prepared from *o*-xylene (Aldrich) by literature procedures and recrystallized from hexanes (light petroleum, **2**: 60–68 °C and **5**: 80–100 °C; **3**: hexane–benzene, 9:1 v/v), until colourless. *o*-Methylbenzaldehyde was obtained from α-bromo-*o*-xylene, through a Kornblum reaction,[18e] and twice distilled. The final purity attained for each one of these compounds was of, at least, 97% (by GC). Malachite green leucocyanide was prepared from malachite green oxalate (Carlo Erba) according to Calvert and Rechen.[19]

α,α-Dibromo-*o*-xylene (3). Boron tribromide (14.0 cm^3, 37.1 g; 0.15 mol; Aldrich, Gold Label) was added to 8.10 g of *o*-methylbenzaldehyde (0.07 mol), dissolved in 150 cm^3 of anhydrous chloroform, and stirred overnight under argon. The solvent was removed in the absence of oxygen and moisture, and the residual material pyrolysed under vacuum, at 150 °C/10 mmHg, during a bulb-to-bulb distillation. The crude product was then fractionally distilled (through a 5 cm Vigreaux column) and 10.2 g of compound **3** was obtained as a slightly yellowish liquid of bp = 104–106 °C/1 mmHg. Yield: 57%. Purity: 98% (by GC). Meaningful elemental analysis results for this compound could not be obtained, due to its prompt hydrolysis upon exposure to air moisture. High-resolution mass spectrometry, however, afforded a parent peak group with the required isotopic masses and distribution: *m/z* (rel. abundance) Found: 261.899 296 (51.4%), 263.897 292 (100), 265.895 288 (48.6); $C_8H_8Br_2$ requires: 261.899 296 (51.1%), 263.897 292 (100), 265.895 288 (48.9); $\delta_H(CDCl_3)$ 2.38 (s, 3 H), 6.86 (s, 1 H), 7.06 (dm, 1 H, J = 7.7 Hz), 7.18 (m, 1 H), 7.24 (m, 1 H) and 7.80 (dd, 1 H, J = 7.8, 1.2 Hz).

Preparative photolyses

Solutions of compounds **1** and **2** were irradiated using four circular ('doughnut' type) low pressure mercury lamps model PCQ-X1 from Ultra-Violet Products (18 W/lamp; $\lambda_{emission}$ = 254 nm). The solutions (25 cm^3; 0.1 mol dm^{-3}) were placed in a cylindrical quartz vial within the circumference of the lamps and purged with argon throughout the irradiation time. Samples were collected by means of a syringe every 30 or 60 min, depending on the system, and analysed by GC with n-hexadecane as internal standard. All products were identified by GC–MS spectrograms, and their identities confirmed by co-injection with authentic samples. The exceptions are compounds **10**, **13**, **14** and **15**, whose structures are proposed on the basis of the detailed interpretation of their mass spectra. The quantifications were performed by conventional GC, utilizing response factors determined in relation to n-hexadecane.

Mass spectral assignment of 10, 13, 14 and 15

C_8H_7BrO (**10**; 199 g mol^{-1}; on INCOS-50) *m/z* (assignment; rel. int.): 200 ($M^{\bullet+}$ [for ^{81}Br]; 20.08%), 198 ($M^{\bullet+}$ [for ^{79}Br]; 20.75), 119 ($M^{\bullet+}$ − Br$^{\bullet}$; 100.00), 118 [$M^{\bullet+}$ − HBr; 16.78), 91 ([119]$^+$ − CO; 87.62), 65 ([91]$^+$ − HC≡CH; 17.75). $C_8H_7Br_3$ (**13**; 343 g mol^{-1}; on INCOS-50) *m/z* (assignment; rel. int.): 346 ($M^{\bullet+}$ [for $^{81}Br_3$]; 0.30%), 344 ($M^{\bullet+}$ [for $^{81}Br_2{}^{79}$Br]; 0.93), 342 ($M^{\bullet+}$ [for $^{81}Br^{79}Br_2$]; 0.98), 340 ($M^{\bullet+}$ [for $^{79}Br_3$]; 0.35), 331 ([346]$^{\bullet+}$ − $^{\bullet}CH_3$; 0.08), 329 ([344]$^{\bullet+}$ − $^{\bullet}CH_3$; 0.24), 327 ([342]$^{\bullet+}$ − $^{\bullet}CH_3$; 0.25), 325 ([340]$^{\bullet+}$ − $^{\bullet}CH_3$; 0.09), 265 ($M^{\bullet+}$ − Br$^{\bullet}$ [for $^{81}Br_2$]; 54.58), 263 ($M^{\bullet+}$ − Br$^{\bullet}$ [for $^{79}Br^{81}$Br]; 74.01), 261 ($M^{\bullet+}$ − Br$^{\bullet}$ [for $^{79}Br_2$]; 60.51), 184 ([265 or 263]$^+$ − Br$^{\bullet}$; 37.68), 182 ([263 or 261]$^+$ − Br$^{\bullet}$; 35.90), 103 ([184 or 182]$^{\bullet+}$ − Br$^{\bullet}$; 100.00), 91 ($C_7H_7^+$; 18.66), 77 ([103]$^+$ − HC≡CH; 52.92), 63 ([103]$^+$ − MeC≡CH; 20.00). $C_8H_7BrO_2$ (**14**; 215 g mol^{-1}; on ITD-800) *m/z* (assignment; rel. int.): 216 ($M^{\bullet+}$ [for ^{81}Br]; 15.89%), 214 ($M^{\bullet+}$ [for ^{79}Br]; 17.33), 171 ([216]$^{\bullet+}$ − $^{\bullet}CO_2H$; 20.50), 169 ([214]$^{\bullet+}$ − $^{\bullet}CO_2H$; 22.36), 122 ($M^{\bullet+}$ − :CHBr; 27.33), 121 ($M^{\bullet+}$ − $^{\bullet}CH_2Br$; 33.23), 105 ($M^{\bullet+}$ − $^{\bullet}$Br − CH_2O; 64.91), 104 ($M^{\bullet+}$ − HBr − CH_2O; 49.36), 90 ([171 or 169]$^+$ − $^{\bullet}$Br; 20.50), 89 ([171 or 169]$^+$ − HBr; 17.39), 77 ([105]$^+$ − CO; 100.00), 63 ([89]$^+$ − HC≡CH; 24.84). $C_8H_6Br_2O$ (**15**; 278 g mol^{-1}; on INCOS-50) *m/z* (assignment; rel. int.): 280 ($M^{\bullet+}$ [for $^{81}Br_2$]; 1.27%), 278 ($M^{\bullet+}$ [for $^{81}Br^{79}$Br]; 2.41), 276 ($M^{\bullet+}$ [for $^{79}Br_2$]; 1.62), 199 ($M^{\bullet+}$ − Br$^{\bullet}$ [for ^{81}Br]; 46.83), 197 ($M^{\bullet+}$ − Br$^{\bullet}$ [for ^{79}Br]; 58.02), 171 ([199]$^+$ − CO; 6.02), 169 ([197]$^+$ − CO; 6.94), 118 ([199 or 197]$^+$ − Br$^{\bullet}$; 100.00), 90 ([118]$^{\bullet+}$ − CO; 67.29), 89([118]$^{\bullet+}$ − $^{\bullet}$CHO; 96.52), 64 ([90]$^{\bullet+}$ − HC≡CH; 9.58), 63 ([89]$^+$ − HC≡CH; 69.74), 62 ([90]$^{\bullet+}$ − $H_2C{=}CH_2$; 33.77).

The MS spectrum of **13** is similar to that obtained from authentic **4**, the main difference being that only the MS spectrum of **13** shows a multiplet arising from $M^{\bullet+}$ − $^{\bullet}CH_3$. This feature, also present in the MS spectrum of **3** but absent from that of **2**, is a strong evidence in favour of the proposed structure for **13**. Upon aging, *o*-toluic acid, the hydrolysis product of **13**, was identified and confirmed by co-injection of authentic sample. The same was observed for **15**, giving rise to *o*-bis(formyl)benzene.

Actinometry

The quantum yields of these photoprocesses were determined by performing the irradiation of solutions of compounds **1**–**5**, in a spinning merry-go-round apparatus surrounded by the lamps with room for six quartz tubes of 13 × 100 mm. The concentration used was 0.053 mol dm^{-3} for **2** in benzene–cyclohexene mixture, 0.016 mol dm^{-3} for **2** in isooctane and 0.100 mol dm^{-3} in every other case. The solutions were argon purged before being transferred to the tubes under inert atmosphere. The tubes to be analysed (as described above) were removed from the irradiating system at equal intervals, maintaining the lamps on. The photon flux in this irradiation system was determined using malachite green leucocyanide, according to Johns[10a] and the malachite green cation formation was monitored by measuring its light absorption at 618 nm.

These systems may be represented by the kinetic eqn. (1),

$$-V\frac{d[R]_t}{d(I_0 \times t)} = \Phi\frac{[R]_t}{[R]_0} \quad (1)$$

adapted from Bunce,[11] V is the volume of irradiated solution, $[R]_0$ and $[R]_t$ are the concentration of solute at time zero and t respectively, I_0 is the photon flow (in Einstein min^{-1}) of light absorbed and Φ is the quantum yield of the reaction.

When integrated this equation becomes (2) which implies

$$\Phi = \frac{V}{I_0 \times t}[R]_0 \ln\left(\frac{[R]_0}{[R]_t}\right) \quad (2)$$

that the solute is consumed exponentially with time and, therefore, quantum yield varies in the same way. Nevertheless, at sufficiently short time, this curve can be described as a straight line, represented by eqn. (3)

$$[R]_t = [R]_0 - k_0 t \quad (3)$$

which means that

as $$\lim_{[R]_t \to [R]_0} \left\{[R]_0 \ln\left(\frac{[R]_0}{[R]_t}\right)\right\} = k_0 t \quad (4)$$

then $$\Phi = \frac{k_0 V}{I_0} \quad (5)$$

Acknowledgements

We thank Dr Thérèse Wilson (Harvard University) for critically reviewing this manuscript and Dr Günter Ebeling (Universidade Federal do Rio Grande do Sul) for helping with the syntheses of compounds **3** and **4**. This work was supported by FAPESP, FINEP-PADCT II and CNPq.

References

1 H. E. Zimmerman and V. R. Sandel, *J. Am. Chem. Soc.*, 1963, **85**, 915; H. E. Zimmerman and S. Somasekhara, *J. Am. Chem. Soc.*, 1963, **85**, 922.
2 (*a*) D. C. Appleton, B. Brocklehurst, J. McKenna, J. M. McKenna, S. Thackeray and A. R. Walley, *J. Chem. Soc., Perkin Trans. 2*, 1980, 87; (*b*) D. C. Appleton, B. Brocklehurst, J. McKenna, J. M. McKenna, M. J. Smith, P. S. Taylor, S. Thackeray and A. R. Walley, *J. Chem. Soc., Chem. Commun.*, 1977, 108; (*c*) B. Arnold, L. Donald, A. Jurgens and J. A. Pincock, *Can. J. Chem.*, 1985, **63**, 3140; (*d*) J. R. Larson, N. D. Epiotis and L. E. McMurchie, *J. Org. Chem.*, 1980, **45**, 1368; (*e*) S. J. Cristol and T. H. Bindel, *J. Org. Chem.*, 1980, **45**, 951; (*f*) S. J. Cristol and T. H. Bindel, *J. Am. Chem. Soc.*, 1981, **103**, 7287.
3 (*a*) A. Tournier, X. Deglise and J. C. André, *J. Photochem.*, 1983, **22**, 223; (*b*) A.-M. A. Abdel-Wahab, M. T. Ismail, O. S. Mohamed and A. A. Khalaf, *Gazz. Chim. Ital.*, 1985, **115**, 591.
4 (*a*) K. Haider, M. S. Platz, A. Despres, V. Lejeune, E. Migirdicyan, T. Bally and E. Haselbach, *J. Am. Chem. Soc.*, 1988, **110**, 2318; (*b*) K. W. Haider, E. Migirdicyan, M. S. Platz, N. Soundararajan and A. Despres, *J. Am. Chem. Soc.*, 1990, **112**, 733.
5 M. P. Cava, A. A. Deana and K. Muth, *J. Am. Chem. Soc.*, 1959, **81**, 6458.
6 W. Oppolzer, *Synthesis*, 1978, 793.
7 (*a*) J. M. Hornback and R. D. Barrows, *J. Org. Chem.*, 1982, **47**, 4285; (*b*) B. H. Han and P. Boudjouk, *J. Org. Chem.*, 1982, **47**, 751.
8 W. G. McGimpsey and J. C. Scaiano, *Can. J. Chem.*, 1988, **66**, 1474.
9 *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, 1986, 67th edn.; *Beilsteins Handbuch der Organischen Chemie*, Springer Verlag, 4th edn., E IV, **1**, 439.
10 (*a*) H. E. Johns, *Methods Enzymol.*, 1969, **16**, 274; (*b*) Comission on Photochemistry, *Pure Appl. Chem.*, 1989, **61**, 188.
11 N. J. Bunce, *J. Photochem.*, 1987, **38**, 99.
12 S. L. Murov, I. Carmichael and G. L. Hug, *Handbook of Photochemistry*, Marcel Dekker, New York, 1993, 2nd edn.
13 S. D. Colson and E. R. Bernstein, *J. Chem. Phys.*, 1965, **43**, 2661.
14 P. J. Kropp, G. S. Poindexter, N. J. Pienta and D. C. Hamilton, *J. Am. Chem. Soc.*, 1976, **98**, 8135.
15 W. Adam and A. Ouchi, *Tetrahedron Lett.*, 1992, **33**, 1875.
16 R. M. Wilson, K. A. Schnapp, K. Hannemann, D. M. Ho, H. R. Memarian, A. Azadnia, A. R. Pinhas and T. M. Figley, *Spectrochim. Acta, Part A*, 1990, **46**, 551.
17 L. H. Catalani and W. Adam, unpublished results.
18 (*a*) E. F. J. Atkinson and J. F. Thorpe, *J. Chem. Soc.*, 1907, **91**, 1687; (*b*) E. F. M. Stephenson, *Org. Synth. Coll. Vol.*, 1963, **4**, 984; (*c*) J. O. Halford and B. Weissmann, *J. Org. Chem.*, 1953, **18**, 30; (*d*) J. C. Bill and D. S. Tarbell, *Org. Synth. Coll. Vol.*, 1963, **4**, 807; (*e*) N. Kornblum, W. J. Jones and G. J. Anderson, *J. Am. Chem. Soc.*, 1959, **81**, 4113.
19 J. G. Calvert and H. J. L. Rechen, *J. Am. Chem. Soc.*, 1952, **74**, 2101.

Paper 5/01763G
Received 20*th March* 1995
Accepted 7*th June* 1995

Anexo XIV

Real-time determination of ultraviolet degradation kinetics of polymers in solution. Catalani, L.H., Rabello, A.M., Florenzano, F.H., Politi, M.F., Reed, W.F., Int. J. *Polym. Anal. Char.*, no prelo.

Real-time determination of ultraviolet degradation kinetics of polymers in solution

[1]L.H. Catalani, [1]A.M. Rabello, [2]Fabio H. Florenzano [2]M.J. Politi and [3]W.F. Reed
[1]Departamento de Química Fundamental and [2]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, 05599-970, São Paulo, Brazil
[3]Department of Physics, Tulane University, New Orleans, Lousiana 70118

ABSTRACT

Real time monitoring of ultraviolet degradation of polystyrene with varying degrees of methylvinyl ketone (MVK) substitutions was carried out. This automated technique is demonstrated to be rapid, accurate and quantitative, yielding the absolute degradation rate constant in terms of number of bonds broken per second per initial dalton of polymer mass. The MVK substituted heteropolymers yielded degradation rates around two orders of magnitude higher than pure polystyrene, which varied linearly with the percentage of MVK substitution.

Introduction

We have found time dependent total intensity light scattering (TDSLS) to be a useful and efficient means of making real-time determinations of absolute degradation rate constants, and also for making mechanistic and structural deductions about the polymers themselves. Information on degradation rates, mechanisms and associated polymer structure is of fundamental importance in such areas as developing degradable materials, extracting and processing biological molecules, testing the durability of new polymers, etc. The mechanisms, chemistry, stabilization and other aspects of polymer degradation have been extensively reported.[1-3]

While many types of degradation are non-random, such as exocydic enzymatic cleavage and shear degradation, the depolymerization of polymers may often be approximated as random when they are subjected to such agents as acids, bases, enzymes, heat, radiation, etc. The focus in this work is the ultra-violet induced random degradation of modified polystyrene polymers.

The problem of determining fragment distributions for random degradation of long chain polymers was treated by Charlesby [4] and Kuhn[5], and later extended to finite single chains by Montroll and Simha[6]. The basic theory for angular dependent light scattering intensity as a function of random degradation of an initially monodisperse population of single stranded coils was presented in ref. 7, and extensions to include non-ideal effects

and polydispersity were given in ref. 8. Under assumptions of ideality it was possible, in ref. 9, to find a polydispersity-independent degradation rate constant. Ref. 10 developed the characteristic time-dependent scattering 'signatures' for simply branched ('comb') polymers undergoing various types of degradation; sidechain stripping, sidechain random degradation, backbone degradation, and combinations of these. Most recently, a general formalism was presented for determining kinetic rates and making structural determinations about multiply stranded polymers.[11]

Several commercially available biodegradable polymers rely on a photo-oxidative process to start full biological recycling to carbon dioxide and water. Some widely known trade names like E/CO®, Ecolyte®, PolyGrade®, Plastor®, etc, are polyethylene or polystyrene, modified either by copolymerization with a carbonyl derivative, such as carbon monoxide and vinyl ketones, or by addition of photosensitizers, like ferric salts. (G. Scott, *Polym. Deg. and Stab.* **29**, 135-154, 1990). On the other hand, for certain applications, addition of photoantioxidants, like metal thiolates (M.U.Amin & G. Scott, *Europ. Polym. J.*, **10**, 1019, 1974) is used to retard the initial phase of degradation. The serviceable lifetime of these materials for a given application will depend on fine tuning the amount of photodegradation. Moreover, these modifications must preserve the mechanical characteristics of the unmodified materials.

Our long range program involves the development of fine control over the serviceable lifetime of certain types of modified plastics. Specifically, the synthesis of co-polymers of styrene and vinyl ketones and the study of its photo-degradation in the presence of metal oxidants and stabilizers are being carried out. Of interest are the effects of the percent of vinyl ketone co-monomer, amount and type of added metal complexes on degradability parameters like rate of degradation and size of fragments at long irradiation times. Although the rate at which the molecular weight of such materials is reduced is of prime importance, the final mass of the fragments must also be a parameter of control, since microorganism action is inversely proportional to molecular weight

Background on TDSLS

When a polymer is broken into two or more fragments, the light scattered from the fragments will, in general, be less than from the intact polymer. The central theoretical problem of TDSLS is to determine how the intensity of light scattered is related to the number of cuts made on a polymer, to how the cuts are made (e.g. randomly), and to the structure of the polymer. The quantity which is needed to find this relationship is P(q,r), that is, the polymer scattering form factor as a function of both scattering vector q and the average number of cuts made per original polymer r. Once P(q,r) is found from a suitable model then, the scattering can be found for an initially monodisperse population, using the well known Zimm equation for the total scattering I(q,r) (expressed as the absolute Rayleigh ratio, which in the CGS system is in cm $^{-1}$).[12] For an initially monodisperse population of polymers of initial mass M_o= mN, where m is the mass of a monomer,

$$Kc_o/I(q,r) = 1/M_oP(q,r) + 2A_2c_o \qquad (1)$$

where q is the usual scattering wave vector amplitude, given by

$$q = \frac{4\pi n}{\lambda} sin(\theta/2) \tag{2}$$

where n is the pure solvent refractive index, λ the laser vacuum wavelength, and θ is the scattering angle. K is an optical constant given, for vertically polarized light, as

$$K = \frac{4\pi^2 n^2 (dn/dc)^2}{\lambda^4 N_A} \tag{3}$$

where dn/dc is the differential refractive index and N_A is Avogadro's number. Here c_0 is the initial polymer concentration in g/cm^3. It does not change during the course of degradation. A_2 is the second virial coefficient (cm^3-mole/g^2). In principle, A_2 is a function of polymer mass, and hence can change in a complicated way in time. The effects of a changing A_2 on light scattering by single strand polymers were considered in ref. 10. It was found experimentally in this work, that the Kc/I(q,t)vs. t curves for different c_o superpose to within an additive constant of $2A_2c_o$, which justifies taking this latter term as time-independent.

In the case of a polydisperse initial population of polymers, with initial concentration distribution $C_O(M)$ the P(q,r) can be used and eq. 1 becomes

$$Kc_o / I(q,r) = c_o / \int_0^\infty MC_0(M)P(q,r(M))dM + 2A_2c_o \tag{4}$$

where $C_o(M)dM$ is the initial concentration of polymer within the mass interval M to M+dM.

Ref. 11 provides a general formalism for finding P(q,r): Consider r average cuts, which are introduced into a linear polymer in any fashion at all. The monomers on the polymer are labelled from 1 to N. The probability that the polymer segment between monomers i and j is still intact can be represented by W(r,i,j)[11], and then P(q,r) is found by

$$P(q,r) = (1/N^2)\sum_{i=1}^{N}\sum_{j=1}^{N} W(r,i,j) << \exp(-i\vec{q}\cdot\vec{r}_{ij}) >> \tag{5}$$

This procedure weights the double sum over all polymers by the probability that monomers i and j are still connected after r cuts. If they are no longer connected, the resulting fragments are presumed to diffuse away from each other, leaving no phase correlation between monomers on separate fragments.

For random degradation of random coil polymers, as can occur, for example, with ultraviolet radiation, P(q,r) turns out to be the Debye function D(u) whose traditional argument of $u=q^2<S^2>_0$ is translated by r,

$$P(q,r) = D(u) = \frac{2}{u^2}\left[e^{-u} - 1 + u\right], \quad u = q^2 < S^2 >_o + r \tag{6}$$

where $<S^2>_0$ is the initial mean square radius of gyration of the undegraded polymers.

The fragment distribution function resulting from random degradation of a monodisperse polymer of initial mass M_o was determined in refs. 4, 6 and 9.

$$f(r,x) = e^{-r}\,\delta(1-x) + \exp(-rx)[2r - r^2x + r^2] \tag{7}$$

where $x=M/M_o$ and f(r,x)dx is the fraction of fragments in the interval x to x+dx.

For an initially monodisperse population of mass M_o, the number, weight and z-averages, respectively, are found from this to be,

$$M_n = \frac{M_o}{1+r} \tag{8}$$

$$M_w = \frac{2M_o}{r^2}\left[e^{-r} - 1 + r\right] \tag{9}$$

$$M_z = \frac{3M_o\left[\frac{2}{r}\left(e^{-r}\right) - 1\right] + 1 + e^{-r}}{r\left[\frac{e^{-r}}{r} + 1 - \frac{1}{r}\right]} \tag{10}$$

The result that P(q,r) is the Debye function leads to two immediately interesting limiting cases. Namely, if u>3, P(q,r) reaches a linear asymptote such that for random degradation of a single strand ideal coil

$$Kc_o / I(0,\beta) = 1/2M_n + \beta/2 + \gamma q^2/2 + 2A_2c_o \tag{11}$$

where β is the number of cuts per original dalton (or g/mole) of polymer mass, and γ relates $\langle S^2\rangle$ to M via $\langle S^2\rangle = \gamma M$. For many degradation reactions the reaction rate $\dot{\beta}$ will be constant as long as the number of scissile bonds is large compared to r. Then

$$\beta = \dot{\beta}t \tag{12}$$

This means that as long as $\dot{\beta}$ is constant, Kc/I(q,t) vs. t will be linear.. $\dot{\beta}$ is determined simply by the initial linear portion of Kc/I(q,t) according to

$$\dot{\beta} = 2\frac{d(Kc/I)}{dt} \tag{13}$$

The average number of cuts per polymer of any original mass M is just

$$r = \beta M \tag{14}$$

Significantly, $\dot{\beta}$ is independent of initial polydispersity, the virial coefficient and any of the intial mass averages (ref). This limit is reached for random coils for any combination of $q^2\langle S^2\rangle$ and r such that u>3, whereas this limit will apply to *any* linear polymer in the q=0 limit, regardless of shape, excluded volume, etc.; i.e. it suffices to run the reaction until r>3 to reach the limit in this case. This result in for the q=0 limit was pointed out by Thomas and Doty.[13]

The second limiting case is when u<1, which will apply for early degradation times near q=0 for all linear polymers of any size and shape, or at early times at any accesible value of q for small molecules where $q^2\langle S^2\rangle << 1$. This limiting case was termed the 'kinetc Guinier regime' in ref. 11, because it parallels in time what the low-angle Guinier approximation follows in q^2.

$$\frac{Kc_0}{I(q,r)} = \frac{1}{M_{w,0}}\left[1 + \frac{q^2\langle S^2\rangle_{z,0}}{3} + \frac{M_{z,0}}{3}\beta\right] + 2A_2c_0 \tag{15}$$

Hence, in this limit

$$\dot{\beta} = \frac{3M_{w,o}}{M_{z,o}} \frac{d(Kc/I)}{dt} \tag{16}$$

Again, the rate constant is independent of A_2, and polymer conformation and excluded volume effects, but there is a (simple) dependence on the initial polydispersity of the sample.

Since $\dot{\beta}$ is not constant in the case where the number of bonds broken is not much smaller than the total number of scissile bonds, the time dependence of $\dot{\beta}$ must be considered. For the case of random degradation by uv, the polymer bonds will be cut in a first order reaction dependent on the incident light intensity S, so that the total concentration of intact, scissile bonds m, on an original decreases according to,

$$\frac{dm}{dt} = -km \tag{17}$$

where k is a degradation rate constant per scissile bond which depends on the rate of absorption of uv photons and the quantum efficiency for photolyis. Then,

$$m(t) = m_o e^{-kt} \tag{18}$$

where m_0 is the initial total concentration of uncut bonds in solution. The average number of cuts per initial polymer is simply

$$r(t) = m_o[1 - e^{-kt}] \tag{19}$$

where M is the number of original number of scissile bonds in a polymer. Hence, when a degradation reaction is measured over long enough times for non-linearity (downwards curvature) in Kc/I(q,t) vs. t to become apparent, eq. 19 will be appropriate for fitting in conjunction with eq. 4 for Kc/I(q,r).

Finally, it is important to point out that in most previous cases the final fragment sizes of the degradation product were much smaller than the initial polymers themselves, so that the final scattering due to endproducts was negligible. This amounts to neglecting the j=i (self) terms in eq. 4. In this work, however, because the final fragment distribution is governed by the amount of scissile linker (methyl vinyl ketone in this case), the final fragments may still scatter significantly. Under the assumption of equal final fragments small enough that P(q)=1, the following approximation is derivable from eq. 5

$$P(q,r) = \frac{\delta + D(u)}{1+\delta} \tag{20}$$

where the Debye function D(u) and the relation of u to q and r are given in eq. 5. Here δ is the reciprocal of the number of final fragments, given by

$$\delta = \frac{1}{fN+1} \tag{21}$$

where f is the fraction of scissile monomers in the heteropolymer of N total monomers. In this case, determining $\dot{\beta}$ from the initial slope gives

$$\dot{\beta} = \frac{3M_{w,o}}{M_{z,o}(1+\delta)} \frac{d(Kc/I)}{dt} \tag{22}$$

Materials and Methods

TDSLS measurements were made with a Wyatt Technology Dawn-F DSP light scattering photometer. The flow cell was used, permitting the scattering to be measured simultaneously from 16 angles. Data was transferred via an RS-232c line to a microcomputer. One of the authors (WR) wrote software for data acquisition and analysis.

Raw scattering voltages at each angle q and time point t V(q,t), were transformed to absolute time-dependent Rayleigh ratios I(q,t) according to

$$I(q,t) = \frac{V(q,t) - V_s(q)}{V_a(q_r) - V_d(q_r)} N(q) I_a F \tag{23}$$

where $V_s(q)$ is the solvent scattering voltage at wave vector q, $V_a(q_r)$ is the voltage at a reference wavevector q_r (here taken as that corresponding to $\theta=90°$) of the absolute calibration solvent, which in this case was toluene, with a known absolute Rayleigh scattering ratio at T=25°C of $I_a=0.00001408$ cm^{-1}. $V_d(q_r)$ is the photodetector dark count at the reference wavevector. F is a geometrical correction factor which, for longitudinal, cylindrical flow cell of the Dawn F amounts to the ratio of the refractive index of the sample's solvent to that of toluene (and to this factor squared for upright cylindrical 'batch'cells), divided by the total Fresnel reflection losses at the glass/solvent interface. N(q) is the normalization factor for each photodiode which is computed according to

$$N(q) = \frac{V_n(q_r) - V_s(q_r)}{V_n(q) - V_s(q)} \tag{24}$$

where $V_n(q_r)$ is the scattering voltage of the normalisation solution in the sample's solvent at the reference wavevector. The normalisation solution is composed of an isotropic scatterer in the sample's solvent. In this case a solution of PS of M_w=4,000 at 15 mg/ml in THF was used as the normalizing solution.

Toluene, tetrahydrofuran (THF) and methanol were of HPLC grade (Merck). Styrene (Dow Chemical Co.) and methylvinylketone (MVK; Aldrich) were freshly distilled prior to use. 2,2'-azobis-iso-butyronitrile (AIBN) was recrystalized from methanol. The copolymers of styrene and MVK were prepared by radical polymerization (Kato, M., Yoneshige, Y., Makromole. Chemie, 164, (1973) 159-169: Sikkema, K., Hanner, M.J., Brennan, D.J., Smith, P.B., Priddy, D.B., Polymer Degradation and Stability, 38 (1992), 119-124) starting with 5%, 10% and 15% in molar fraction of the ketone to yield

respectively MVK5, MVK10 and MVK15 copolymers, as referred to respectively in this work. For the MVK polymerizations 50% by weight of styrene ** and *** of MVK were added to distilled toluene. The solution was degassed by four freeze-pump-thaw cycles, ampoule sealed under vacuum and heated at 70°C for 48 hours. The product was isolated by addition of methanol, redissolved in toluene and, again, precipitaded with methanol, dried under vacuum to give yields of approximately 90% of the product.

The incorporation of MVK in the polymers were evidenced by the carbonyl absorption at 1712 cm^{-1} in the infrared spectra (Perkin Elmer FT-IR 1750 and Nicolet FT-IR 510) using KBr pellets for the intact heteropolymers and in NaCl pellets for the photoproducts. Elemental analysis of the products (12 samples of each product) showed distinct proportions for MVK incorporation, as shown in Table 1. These measurements were made with Perkin Elmer Elemental Analyzer 2400 CHN, in which the sample is heated to 925°C in presence of pure oxygen, with production of CO_2, H_2O and NO_2, after which these products pass through a separation column and are detected by thermal conductivity. The level of carbon was obtained from CO_2 and the level of oxygen from the H_2O.

M_w and A_2 values were obtained by 'batch'light scattering in upright, cylindrical cells, using the standard Zimm plot method, with concentrations ranging from **-15 mg/ml. The angular variation of the heteropolymers was too slight for accurate determination of mean square radius of gyration. This latter quantity, at any rate, is not important for the degradation kinetics of interest. The value of dn/dc was taken as 0.21 mL/g for polystyrene homopolymer in THF at 20°C (Huglin, M.B, in Polymer Handbook, 3rd Ed. Brandrup, J. Immergut, E.H., Eds., John Wiley, New York, 1989, p. VII/445,446)) and used for all polymers. Table I summarizes the main features of the polymers used, including the extinction coefficient at λ=254nm and the number of final fragments due to MVK substitution, considering a monomer mass of 104 daltons. Unfortunately, no chromatography data were available for assessing the degree of initial polydispersity of the material.

TABLE I. Characteristics of the PS homopolymer and PS/MVK copolymers.

Polymer	MVK%	M_w	A_2	ε(ml/mg-cm)*	# of fragments
PS	0	107,700	7.96e-4	1.73	xx
MVK5	6.1± 0.7	42,700	1.1e-3	2.01	26
MVK10	11.5±1.2	45,200	1.2e-3	1.89	51
MVK15	13.7±0.9	42,100	1.2e-3	2.11	56

* at λ=254nm.

A quartz reaction vessel containing 50ml of the polymer solution (filtered through 0.22μm teflon membrane from Millipore) with magnetic stirring was set inside of three 4 watt circular low pressure mercury lamps (99.5% of emission at 254 nm). An HPLC pump (600E gradient module, Waters) provided a continuous pumping rate of 1ml/min through a closed loop; from the reaction cell the solution was led through a dust filter (2 cm pre-column for GPC, 25 μm of porosity, Phenogel 5 guard column, 50 x 7.8mm) to the Wyatt Dawn-F flow cell and back. The amount of solution circulating through the system exterior to the reaction cell was about 17 ml, giving an initial dead time of close to 1000

seconds before photolysed material enters the scattering detector. The solution was kept at room temperature by forced ventilation.

RESULTS and DISCUSSION

Fig. 1 shows typical ultraviolet degradation curves for the four polymers used. The data shown are raw scattering voltages from $\theta = 90^\circ$ transformed into Rayleigh scattering ratios via eq. 23, and represented as Kc/I(t). No data smoothing was used, and the individual points shown are noise-free enough that they appear as virtually a solid line. The initial 840 seconds of dead time, the period it takes for degrading polymer to first reach the scattering cell from the reaction vessel, has been eliminated in each.

It is immediately striking that the PS/MVK copolymers degrade extremely rapidly compared to pure PS, which is almost a flat line at the bottom of the figure. Furthermore, the initial slopes, which by eq. 16 (and eq. 22) are proportional to the initial scission rates, are seen to increase in the order of increasing MVK substitution. The data for MVK5 were collected over a long enough period that significant curvature is seen. The origin of this is discussed below. For the shorter time scale over which they were measured, MVK10 shows little curvature, and MVK15 is virtually a straight line.

Fig. 2a shows the initial, linear portion of each curve in fig. 1, from which the rate constant $\dot{\beta}(t=0)$ is computed according to eq. 22. We note that the factor δ in $1/(1+\delta)$ is quite small, amounting to 0.038, 0.02 and 0.018, for MVK5, MVK10 and MVK15, respectively. Since no polydispersity data were available, the rates shown *are uncorrected for the polydispersity factor* M_w/M_z in eq. 22. For a typical polymerization with a most probable distribution, the correction factor would be 2/3 times the uncorrected values. These uncorrected rates, averaged over the 16 detection angles, are shown in Table II. There being no angular dependence to the scattering, the rate constant was virtually the same at each angle, and the standard deviation from averaging rates over 16 angles was always less than 1%. It can be seen in these short time curves that there is a short, initial transient curvature which follows the dead time period, probably due to mixing, before each curve becomes unambiguously linear.

Fig. 2b shows the long-term degradation behavior of PS, from which the rate constant in Table II was determined. Strikingly, the rate constants for the PS/MVK polymers range from about 60 to over 100 times higher than pure PS. Hence, in these fits and the following, the degradation rate of the pure PS portions of the copolymers was neglected compared to the MVK portion degradation rate.

Table II. Initial degradation rate constants from linear and non-linear fits

Polymer	PS	MVK5	MVK10	MVK15
$\dot{\beta}(0)$ (cuts/dalton-s), from initial slope, eq. 22	3.37×10^{-10}	1.93×10^{-8}	3.18×10^{-8}	3.88×10^{-8}
$\dot{\beta}(0)$ from non-linear fit and eq. 25	xx	1.88×10^{-8}	2.58×10^{-8}	2.80×10^{-8}

As mentioned, the downwards curvature in Kc/I(t) for MVK5 is quite pronounced, and would undoubtedly have shown up more clearly if the runs for MVK10 and MVK15 had been extended in time. The main reason for the curvature is an exponentially diminishing first order rate constant in time (eq. 19). The existence of finite end products rather than monomers can also contribute to the curvature according to eq. 20. For the polymers in this study, however, this latter effect is expected to be small. In principle, an increasing A_2 with decreasing fragment sizes could also lead to downwards curvature in Kc/I(t). A kinetic run with MVK5 at ten times less concentration, however, was completely superposable on a run at full concentration (10 mg/ml). Hence an increasing A_2 as degradation proceeds is excluded as the cause of the curvature.

Non-linear least squares fits for the three heteropolymer curves of fig. 1 were made to eq.1 using the P(q,r) of eq. 20 and the exponential time-dependence of r(t) from eq. 19. The adjustable parameters taken were k and m_o, although, in principle, this latter is known., The fits are included in fig.1, but run so closely through the points that they can't be seen. Because non-linear fits with several parameters usually will closely match the data to be fit, we do take the extremely close match of the fits in fig. 1 as good evidence for the model, but not as a proof of it.. Table II includes the initial rate constants from these fits, which are obtained from the fitting parameters according to

$$\dot{\beta}(0) = km_o / M_o \tag{25}$$

with $M_o=M_w$ taken from Table I. This latter step introduces additional error since the experimental Zimm plot values for M_w are used. Additionally, initial polydispersity considerations are not taken into account. The agreement for $\dot{\beta}(0)$ for MVK5 between the linear and non-linear fits is excellent, but is less good for MVK10 and MVK15, probably due in part to the fact that these latter curves have very little non-linearity to fit to. Besides the use of multiple parameters in a non-linear fit, it is also necessary to use the value of $2A_2c_o$ in eq. 1, which adds further error. Hence, the preferred method of obtaining the rate constants is by the initial slope, for which the $2A_2c_o$ factor is irrelevant, and for which the only fitting parameter is the slope.

Fig. 3 shows a plot of the initial rate constants $\dot{\beta}(0)$ from both the linear fits and the full non-linear model fit vs. the percentage of MVK substitution. The rate constants are quite linear in percentage of MVK substitution, as expected. Under the experimental conditions used, these rates yield, for a starting polymer of M=50,000 daltons, 0.06, 3.47, 5.72 and 6.98 cuts per hour for PS, MVK5, MVK10, and MVK15, respectively.

Fig. 4 shows a direct plot M_w vs. t for each of the degradations, obtained from the extrapolation at each point to zero angle. Although this is a useful representation for seeing the progress of the degradation, we prefer the Kc/I(t) representation since it immediately yields the rate constants.

An estimate of the number of cuts per absorbed photon (quantum efficiency) was made on the basis of actinometric data. The photon flux was found to be *****. For the extinction coefficients in Table I, and polymer concentration of 0.01 g/ml, it is clear that all the incident uv flux is absorbed in a very thin layer of solution in the reaction vessel. For MVK5, for example, 99% of the incident flux is absorbed in the first 1mm of the solution. **** computation of quantum efficiency******.

SUMMARY

The feasibility of making real-time determinations of absolute degradation kinetics for polymers in solution subjected to ultraviolet has been demonstrated. The technique is automated and rapid, and requires no human intervention. Because the theory predicts, within the approximations discussed, and the data show, that there is no angular dependence in computing the rate constants, it would be sufficient to measure the time-dependent scattering intensity at a single angle. Thus, a full multi-range scattering instrument such as the Dawn-F is not a critical requirement, and much simpler systems, such as a single fixed detector, or even a spectrofluorimeter, could be used.

Although a plausible explanation for the curvature in Kc/I(t) is presented and succesfully fit to, the linear fits to the initial slope of Kc/I(t) provide the simplest and most accurate means of detemrining initial degradation rates. Degradation rates were increased by a factors of 60 to over 100 compared to pure PS, and varied linearly with the percentage of MVK substitution. Future use will include determination of degradation rates for different percentages and sequences of the methylvinyl ketone monomers, and in the presence of catalysts such as oxygen and heavy metals.

Acknowledgements

WR acknowledges support from NSF INT 9101058 and FAPESP. MJP and HC acknowledge.CNPq, FAPESP, CAPES and FINEP.

List of figures

Fig. 1 Kc/I vs. t for the ultraviolet degradation of PS, MVK5, MVK10 and MVK15. Data are from $\theta = 90^{o}$.

Fig. 2a Short time behavior of Kc/I(t) for the data from figure 1, whence the initial degradation rates $\dot{\beta}(0)$ in Table II are determined.

Fig. 2b Long-term degradtion behavior of PS, whence its degradation rate in Table II is determined.

Fig. 3 Initial degradation rates, $\dot{\beta}(0)$ vs. percentage of MVK substitution, from both initial linear fits and full non-linear model.

Fig. 4 Corresponding polymer mass profile vs. t for the same experiments as in fig. 1.

References

1. L. Reich and S.S. Stivala, *Elements of Polymer Degradation* (McGraw-Hill, New York, 1971)

2. G. Scott, *Mechanisms of Polymer Degradation and Stabilisation* (Elsevier Science Pub., Essex, 1990)

3. R.L. Clough and S.W. Shalaby, *Radiation Effects on Polymers*, (ACS Symposium 475, 1991)

4. A. Charlesby, Proc. Royal Soc., **A224**, 120 (1954)

5. W. Kuhn, Ber. der Bunsensgesellschaft, **63**, 1503, (1930)

6. E.W. Montroll and R. Simha, J. Chem. Phys., **8**, 721, (1940)

7. C.E. Reed and W.F. Reed, J. Chem. Phys. **91**, 11, 7193 (1989)

8. C.E. Reed and W.F. Reed, J. Chem. Phys. **93**, 12, 9069 (1990)

9. W.F. Reed, C.E. Reed and L.D. Byers, Biopolymers **30**, 1073 (1990)

10. S. Ghosh and W.F. Reed, Biopolymers, **35**, 435, (1995)

11. W.F. Reed, J. Chem. Phys., **103**, 7576-7584 (1995)

12. B.H. Zimm, J. Chem. Phys. **16**, 1093 (1948)

13. C.A. Thomas and P. Doty, J. Am. Chem. Soc., 78,, 1854 (1956)

14. S. Ghosh, I. Kobal, D. Zanette and W.F. Reed, Macromolecules, **26**, 4685 (1993)

fig. 1

FINKCIB.PSD

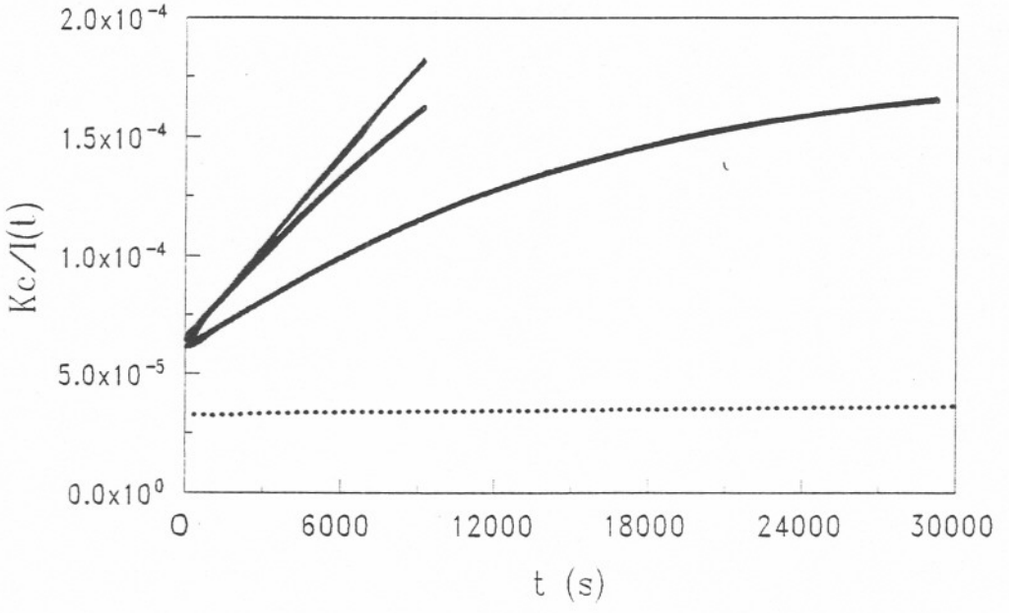


fig2a

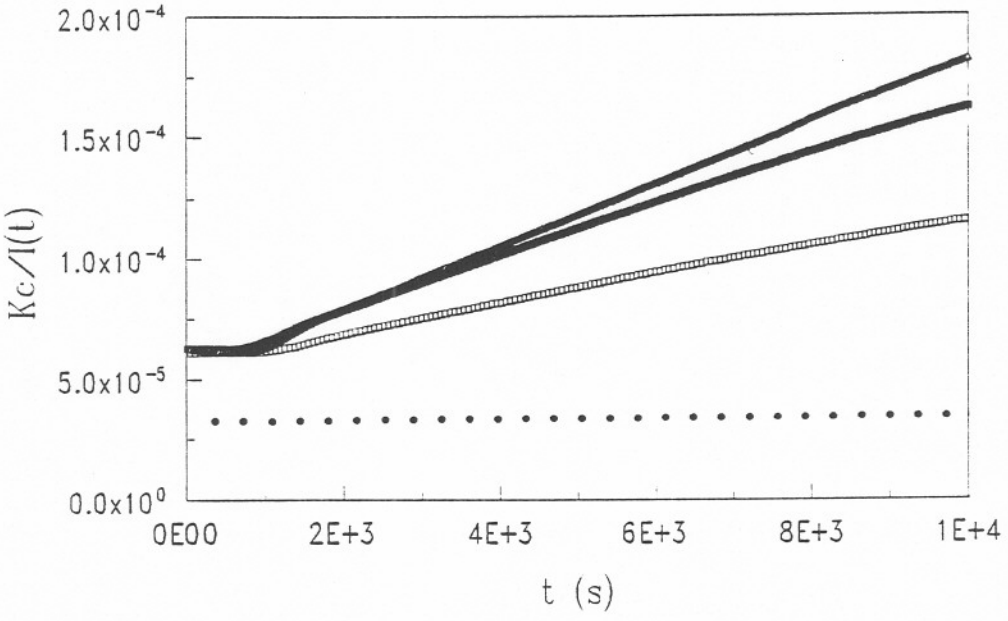
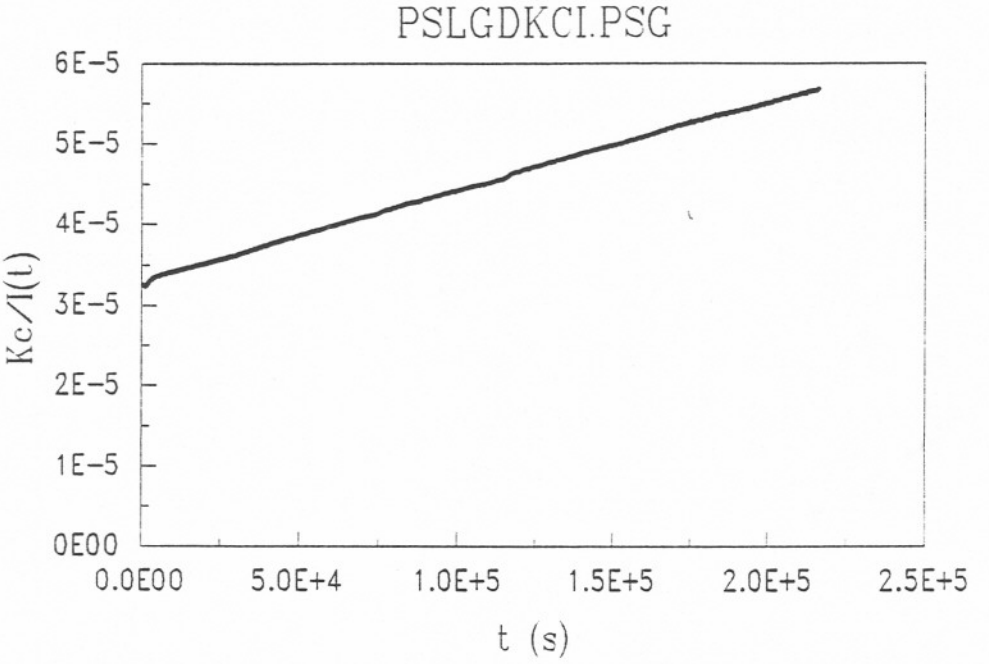

2.0x10⁻⁴
1.5x10⁻⁴
1.0x10⁻⁴
5.0x10⁻⁵
0.0x10⁰
Kc/I(t)
0E00
2E+3
4E+3
6E+3
8E+3
1E+4
t (s)

Fig. 2b


PSLGDKCI.PSG
6E-5
5E-5
4E-5
3E-5
2E-5
1E-5
0E00
Kc/I(t)
0.0E00
5.0E+4
1.0E+5
1.5E+5
2.0E+5
2.5E+5
t (s)