REAÇÕES EM VESÍCULAS DE ANFIFÍLICOS SINTÉTICOS

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INTRODUÇÃO

O trabalho que venho desenvolvendo no Instituto de Química, desde o início da minha pós-graduação, tem como objetivo o estudo de reatividade química em agregados de anfifílicos utilizando dois sistemas: micelas e vesículas.

Apesar de cada sistema possuir características próprias, os fenômenos básicos de alteração de reatividade e compartimentalização são similares. As particularidades de cada sistema, entretanto, permitem a exploração de aspectos distintos que são mais pronunciados em cada tipo de estrutura.

Nesta Tese analisarei apenas os trabalhos realizados com vesículas por ser o sistema no qual a minha contribuição, dentro dos trabalhos realizados no grupo, é mais intensa. Foram incluídos os dois primeiros trabalhos com vesículas que resultaram do meu doutoramento com o intuito de facilitar o acompanhamento da evolução das idéias nessa área nos últimos anos.

Esta Introdução fornece elementos para a avaliação do trabalho desenvolvido nos últimos dez anos no contexto da produção científica na área não sendo uma revisão exaustiva sobre reatividade química em vesículas.

A vida, com as suas formas conhecidas, dificilmente poderia ter evoluído sem uma membrana que separasse, do meio externo, os componentes dos processos metabólicos. As membranas celulares, além de separar compartimentos, são sistemas funcionais onde ocorrem, entre outros, fenômenos de permeação, transporte, excitação, interações intercelulares, diferenciação morfológica e fusão (1).

De acordo com o modelo de mosaico-fluido de Singer e Nicolson (2), a matriz da membrana biológica é uma bicamada de fosfo- e glicolípídeos na qual proteínas estão incorporadas na sua superfície ou em seu interior.

As moléculas que compõem a membrana podem fluir livremente no plano lateral enquanto o movimento de difusão transversal ("flip-flop") é lento. A membrana age como uma barreira semi-permeável. Esta permeabilidade passiva depende do ordenamento da bicamada, o qual depende do comprimento da cadeia hidrocarbônica dos lipídeos, posição e número de duplas ligações, quantidade de colesterol, proteínas e temperatura (3). A transição do estado gel para o líquido cristalino ocorre a uma temperatura característica (temperatura de transição da bicamada, T_c) que depende da composição da bicamada (1). Na fase gel as ligações simples C-C das cadeias alifáticas dos fosfolipídeos estão em conformação trans dando origem a um estado de maior rigidez. Na fase líquido-cristal aumenta a probabilidade de conformações gauche e a bicamada passa para um estado mais fluído (1).

Os lipídeos constituintes das membranas biológicas possuem estruturas químicas diversas sendo mais frequentes os fosfolipídeos (ésteres de álcoois como colina, etanolamina, serina, inositol com 1,2 diacilfosfoglicerol), esteróis, cardiolipídeos, glicolipídeos, esfingolipídeos, gangliosídeos, etc. Os fosfolipídeos naturais, em excesso de água, se organizam espontaneamente em bicamadas. Na membrana, ordem e mobilidade estão relacionadas a estrutura de unidades funcionais formadas por lipídeos e proteínas e as funções de tais sistemas supramoleculares, formados por agregação, dependem de sua organização.

Um grande número de anfifílicos se organiza espontaneamente em presença de água. A natureza dos grupos polares e das cadeias dos anfifílicos determina, em parte, a geometria dos agregados (4). A natureza dos vários agregados, assim como a compactação dos monômeros, é função da concentração de anfifílico, polaridade do meio, temperatura, pH e força iônica. Exemplos de estruturas formadas por anfifílicos são os líquido-cristais, mono e multicamadas, micelas, lipossomos, vesículas, etc. (esquema 1).

A complexidade das membranas naturais levou a necessidade de se obterem modelos mais simples para a investigação, a nível molecular, dos fenômenos relacionados com membranas. Os modelos nem sempre apresentam todas as características do sistema original. Ocorre com frequência que algumas particularidades estão melhor definidas em um determinado modelo e outras são mimetizadas em sistemas aparentemente pouco relacionado com o original. Por isto

tem-se utilizado micelas, bicamadas planas, vesículas e lipossomos para investigar uma série grande e complexa de fenômenos que ocorrem a nível de membranas.

Esquema 1- Exemplos de anfifílicos naturais e sintéticos

Lipossomos são modelos bastante utilizados para mimetizar membranas biológicas. Lipossomos unilamelares (ou vesículas) são agregados onde uma bicamada de anfifílicos envolve parte do meio aquoso isolando-o da fase aquosa externa. Quando mais de uma bicamada envolve parte da fase aquosa estes agregados são chamados de lipossomos multilamelares (esquema 1) (5).

Vesículas podem ser produzidas por diferentes métodos tais como: sonicação, depleção de detergente, extrusão por filtros de poro controlado, fase reversa, injeção etanólica, superfície molde, etc. (6). O tamanho das vesículas depende do

método de preparação, condições do meio e o seu diâmetro pode variar de 25 nm a várias micras.

Um número crescente de fenômenos que incluem transporte de íons, compartimentalização de fármacos, solubilização de drogas e preparação de nanopartículas tem sido estudados utilizando-se vesículas e lipossomos preparados com fosfolípidos (7). A velocidade de reações químicas também é afetada por estes agregados (7). Exemplos disto são a inibição da hidrólise alcalina de procaína e análogos (8,9) por vesículas de fosfatidilcolina (PC). Este efeito protetor tem sido muito explorado na estabilização de produtos farmacêuticos.

A descoberta, em 1977, que sais de amônio quaternário com duas cadeias hidrocarbônicas longas formam lipossomos deu origem a síntese de um grande número de anfifílicos (10, 11, 12). Esta descoberta eliminou a necessidade da manutenção da estrutura básica da fosfatidilcolina (PC) para a formação de vesículas. Abriu-se a possibilidade de se utilizarem uma ampla gama de moléculas, de estruturas químicas diversas, para o estudo dos fatores envolvidos na formação, interação, estabilização e outras características de membranas.

Os anfifílicos sintéticos, que servem como unidades monoméricas de uma grande variedade de estruturas supramoleculares, incluem compostos com grupos polares positivos, negativos, zwitteriônicos e neutros com cadeias hidrofóbicas de estruturas diversas (5,7,13,14,15) esquema 2).

Esquema 2-Exemplos de estruturas supramoleculares formadas por anfifílicos

Alguns anfifílicos sintéticos são análogos dos detergentes, muito utilizados em estudos de modificação de reatividade química em micelas (7) (esquema 2). As vesículas possuem regiões com polaridades distintas, e, como as micelas, são utilizadas como micro-reatores para estudar compartimentalização de substratos e efeito de interfaces em reações (7).

Os primeiros trabalhos de de caracterização de vesículas de anfifílicos sintéticos foram feitos utilizando-se preparações obtidas por ultrassom (10,11,12). A presença de partículas de titânio nestas preparações acelera a oxidação de substratos ou do anfifílico no caso deste possuir grupos sensíveis em sua estrutura. Nestes casos utilizam-se vesículas preparadas por injeção etanólica (16). A presença

de etanol nestas preparações é uma das principais objeções ao método, mas, em alguns casos, é a única forma de se obter dados reprodutíveis.

Em 1983 foram descritas vesículas "gigantes" de cloreto de dimetildioctadecilamônio (DODAC) e dicetilfosfato (DCP) (17, 18) com diâmetro da ordem de 200 nm. Estas vesículas tem sido utilizadas como catalisadores de reações e como compartimentalizadores de substratos (19, 20) já que o volume interno é 10 a 70 vezes maior que o das sonicadas (14 l/Mol e 0,2 l/Mol respectivamente) (17). Além disso, são sistemas adequados para o estudo de transporte de íons e nucleófilos através da membrana (20, 21).

O primeiro estudo sobre reatividade em vesículas foi feito com a reação de hidrólise de acetato de p-nitrofenila (NPA) e palmitato de p-nitrofenila (PNP) catalisada pelo coesterilester do ácido imidazol carboxílico (22). Em micelas de brometo de trimetilhexadecilamônio (CTAB) a reação é acelerada quando os substratos são dissolvidos independentemente nas micelas e depois misturados para dar início à reação através de aumento de pH. A sonicação da mistura ou aquecimento, antes do aumento de pH, não alteram a velocidade. Este mesmo procedimento foi utilizado com vesículas de brometo de dimetildidodecilamônio (DDB) contendo os reagentes em frações distintas de vesículas. Observou-se um aumento da ordem de 200 vezes na constante de velocidade e concluiu-se que vesículas dificultam a difusão dos substratos o que não ocorre com micelas. Neste caso vesículas compartimentalizam os substratos muito melhor que as micelas.

A hidrólise catalítica de NPA e nonanoato de p-nitrofenila (NPN) por nucleófilos contendo imidazol ou hidroxamato ligados a cadeias hidrofóbicas longas ou a colesterol foi investigada em presença de sais de amônio quaternário com uma, duas e três cadeias (23). O fator de catálise foi maior para agregados cujos monômeros contêm mais de uma cadeia (quando o nucleófilo tem cadeia longa simples). Quando o nucleófilo é derivado de colesterol a reatividade é maior em vesículas de DDB.

Kunitake e col. estudaram a reação de descarboxilação do 6-nitrobenzisoxazol-3-carboxilato (NBC) em presença de (CTAB) e vesículas de brometo de dimetildioctadecilamônio (DODAB), entre outros (24). A grande sensibilidade desta reação a solvente permitiu obter informações a respeito da natureza da bicamada destes agregados. Vesículas positivas com cadeia hidrofóbica contendo 12 a 18 carbonos catalisam esta reação por fatores similares (acima da temperatura de transição, T_c). A 30°C vesículas de DDB aceleram a reação por um fator de 500 enquanto CTAB aumenta a velocidade 50 vezes. Nesta temperatura as vesículas de DODAB estão abaixo da T_c e por isto catalisam menos que o DDB. Em micelas e vesículas o NBC está localizado em uma região de polaridade menor que a fase aquosa. Porém, em vesículas, a temperaturas maiores que a T_c , ocorre uma mudança significativa na velocidade. Estes dados indicam que a constante de velocidade da reação é sensível a fluidez da bicamada.

Fendler e Hinze (25) estudaram a hidrólise alcalina do ácido 5,5'-ditiobis(2-nitrobenzóico) (DTNB) em CTAB e DODAC

concluindo que as vesículas são cerca de 100 vezes mais eficientes que as micelas. Este foi o primeiro trabalho com vesículas em que se fez uma análise quantitativa dos resultados (segundo modelos de Berezin (26) e Quina e Chaimovich (27)). Apesar do tratamento não ter sido feito da forma adequada (utilizou-se uma constante de associação para o DTNB e não constante de troca), concluiu-se que tanto em vesículas como em micelas a catálise obtida podia ser atribuída inteiramente a concentração de reagentes agregados, havendo um pequeno efeito das interfaces sobre os valores das constantes de velocidade de segunda ordem da reação.

Vesículas de cloreto de dimetildioctadecilamônio (DODAC), preparadas por injeção etanólica, catalisam a tiólise de acetato de p-nitrofenila (NPA) por n-Heptilmercaptana (HM) por um fator de $7 \cdot 10^6$. Este trabalho, realizado em nosso laboratório, fez parte de minha tese de Doutorado(1980). Fizemos uma caracterização parcial das vesículas preparadas por injeção etanólica (16) e, posteriormente, analisamos esta reação através do modelo de pseudofase Troca Iônica (27). Mostramos que a aceleração observada é devida, basicamente, aos efeitos somatórios de concentração de reagentes na vesículas, alteração de pK da HM e pequeno aumento na constante de segunda ordem da reação na vesícula (28).

Em nossos trabalhos com tiólise de octanoato de p-nitrofenila (NPO) e NPA por heptilmercaptana e hexadecilcisteinamida em vesículas de DODAC (etanólicas) observamos o maior fator de aceleração já obtido em sistemas simples (da ordem de $7 \cdot 10^6$)(29). A análise dos dados segundo

o modelo de Troca-Iônica nos levou a concluir que, a semelhança das micelas, os maiores responsáveis pela aceleração observada em ambas as reações são: concentração de reagentes na interface, variação de pK e pequena mudança na reatividade do nucleófilo (da ordem de 50 vezes para a reação de NPO e cisteinamida em DODAC).

Compostos de duas cadeias hidrofóbicas contendo grupo funcional quiral foram sintetizados e foi possível obter não apenas aceleração mas estereoespecificidade. Vesículas preparadas com monômeros opticamente ativos catalisaram preferencialmente a reação de hidrólise do isômero L de um éster derivado de p-nitrofenol (30, 31). Vesículas são capazes de alterar a relação de produtos na reação de monohalogenação de alquilfenil éteres levando a um aumento de cerca de 5 vezes na proporção de um dos produtos de reação (32). Estes dados mostram que a compartimentalização de substratos em vesículas leva à obtenção de propriedades seletivas, típicas de sistemas mais complexos como as enzimas.

Nestes trabalhos e em outros de nosso grupo não era possível saber qual o sítio de reação. Por isto, para o tratamento dos dados, admitia-se que os substratos difundiam livremente através da bicamada e que o compartimento aquoso interno era pequeno com relação ao externo e portanto desprezível.

J.Fuhrhop e J.Mathieu, em 1984, propuseram um esquema para descrever vesículas em meio aquoso no qual podiam ser delimitadas nove regiões ou compartimentos onde poderiam ocorrer reações (15): 1) compartimento aquoso externo, 2)

esfera de hidratação próxima das cabeças polares, 3) grupos polares da camada externa, 4) membrana hidrofóbica próxima as cabeças polares externas, 5) membrana hidrofóbica interna, 6) membrana hidrofóbica próxima das cabeças polares internas, 7) grupos polares da camada interna, 8) esfera de hidratação das cabeças polares internas, 9) espaço aquoso interno (esquema 3).

Esquema 3-Regiões em soluções de vesículas

Cada uma destas regiões pode ser funcionalizada seletivamente através de síntese de monômeros com as características desejadas ou pela incorporação de sondas de localização predizível ou determinável. O objetivo destes procedimentos é identificar os sítios onde determinadas reações ocorrem caracterizando cada região quanto a sua capacidade de alterar a velocidade, o mecanismo ou selecionar caminhos de reações. É possível, também, verificar se estes agregados catalisam reações específicas e se os efeitos observados são diferentes daqueles obtidos com outros modelos de compartimentalização (micelas, por exemplo).

Anfifílicos contendo cisteína (33), tiocolina (32,34,35), Imidazol (36,37,38), β -hidroxi-etilamônio (39)

foram sintetizados e as vesículas preparadas com estes nucleófilos foram utilizadas no estudo de reações de hidrólise de ésteres, reações de transaminação, hidrólise de ésteres de fosfato, etc. Em nenhum destes casos ficou claramente demonstrada uma diferença de reatividade entre a camada interna e externa das vesículas.

A preparação de vesículas contendo um substrato hidrofóbico distribuído na bicamada (nas superfícies interna e externa) ou incorporado no compartimento aquoso interno e posterior adição de um nucleófilo hidrofílico externamente deu origem as chamadas reações endo e exovesiculares.

Moss e Schreck estudaram a reação de DTNB com ditonito (40), sulfito e tiofenóxido (41) em CTAB e em vesículas preparadas com cloreto de dihexadeciltrimetilamônio (DHDAC). Quando o nucleófilo é misturado às vesículas vazias e, posteriormente, adiciona-se o DTNB, observa-se uma cinética monofásica. Quando a ordem de adição é invertida, a cinética é bifásica e a porcentagem de fase rápida é dependente do tempo de incubação do DTNB com as vesículas. Atribuiu-se a fase rápida a reação na superfície externa da vesícula entre o DTNB e o nucleófilo. Haveria uma entrada rápida do DTNB no compartimento interno das vesículas e a fase lenta seria devida a reação ocorrendo na superfície interna limitada pela entrada de ditonito e sulfito. No caso do tiofenol a permeação é rápida e observa-se apenas uma transiente na reação. As vesículas de DHDAC não são capazes, entretanto, de manter gradientes de pH ou compartimentalizar substratos com a mesma eficiência das vesículas de fosfolípidos naturais.

Este problema foi resolvido com a descoberta de que vesículas de DODAC são capazes de encapsular substratos por um tempo muito maior que o DHDAC (20).

Moss, e col. mostraram que vesículas de DODAC retinham o ácido 5-tio-2-nitrobenzótico (TNB) e o-iodosobenzoato durante várias horas, ao contrário do DHDAC. Demonstraram também que o dititionito atravessa lentamente apenas quando a bicamada da vesícula é feita com anfifílicos de 18 carbonos (20).

Moss e Swarup (42) conseguiram incorporar DTNB em vesículas de DODAB e diferenciar as reações na superfície interna e externa com vários tióis. Foi possível demonstrar que o DTNB reage rapidamente na superfície externa da vesícula com diferentes mercaptanais e que a reação na superfície interna de DODAC é mais lenta por ser limitada pela passagem das mercaptanas através da bicamada. A velocidade de permeação depende do número de carbonos do anfifílico, estrutura dos substratos e temperatura (42).

Iniciamos em nosso laboratório uma série de trabalhos nos quais tentamos diferenciar os diversos compartimentos de vesículas de DODAC através do uso de reações específicas.

O desenvolvimento de um método sensível para a determinação da concentração de DODAC foi fundamental para a realização destes trabalhos. Após a eliminação de substratos na fase aquosa externa é necessário determinar a concentração do anfifílico (43). Os métodos existentes (44) utilizavam grande quantidade de amostra e por isto tivemos necessidade de desenvolver um método simples e sensível para esta determinação. Para isto utilizamos um corante (Orange G) que

forma um complexo estável e de estequiometria definida com DODAC. Em presença de micelas de Brij-35, um detergente neutro, o complexo é solubilizado mudando o coeficiente de absorção, o que permite determinar a concentração do anfifílico.

A incorporação de N-metil-4-cianopiridíneo em vesículas de DODAC permitiu a demonstração de que hidróxido atravessa lentamente a membrana de DODAC e que a reatividade do mesmo no compartimento aquoso interno é idêntica a reatividade em água. Demonstramos também que a velocidade de entrada de hidróxido é pouco dependente de sua concentração no compartimento aquoso externo e que, provavelmente, depende da entrada do cátion para compensar o potencial criado pela entrada do hidróxido (21).

Incorporando DTNB no compartimento aquoso interno de vesículas gigantes de DODAC demonstramos também que é possível manter um gradiente de prótons por várias horas e que a reatividade do hidróxido na superfície interna das vesículas de DODAC é idêntica a da superfície externa. Além disso, mudando a natureza das espécies iônicas no meio aquoso externo (por exemplo, substituindo cloreto por brometo), alteramos a reatividade nas interfaces fazendo com que a interface interna, que está na presença de cloreto, tenha a capacidade de acelerar a hidrólise de DTNB por um fator maior que a superfície externa onde o OH^- compete com brometo (19). O efeito dos diferentes íons se deve a sua capacidade de se ligar as interfaces deslocando o íon hidróxido, que é o nucleófilo da reação. Portanto, nesse sistema simples foi

possível compartimentalizar e modular a velocidade de reações mudando condições de força iônica. Estes dois trabalhos estabeleceram claramente que vesículas DODAC e DODAB são pouco permeáveis a hidróxido e que é possível compartimentalizar reações dependentes de pH em sistemas simples.

A permeação de ions como iodeto e brometo também foi estudada medindo a supressão de fluorescência do ácido 1-pirennonanóico por iodeto. Verificamos que o iodeto atravessa lentamente a bicamada de DODAC e determinamos as constantes de troca I/Br e I/Cl em DODAC e DODAB (45). Estes valores são essenciais na análise de reações em vesículas já que, em geral, se usam os parâmetros obtidos em micelas no ajuste teórico de equações aos dados experimentais.

Sistemas mais complexos foram utilizados tentando compartimentalizar e controlar o fluxo de ions e reagentes em vesículas.

A síntese de 1,2-dipalmitoil-3-gliceril p-nitrofenilfosfato permitiu a preparação de vesículas negativas onde o monômero é o substrato (46). A adição de hidróxido no compartimento aquoso externo leva a hidrólise do éster da superfície externa que fica com mais carga negativa do que a superfície interna. Cerca de 50% do monômero é hidrolizado em uma hora e o restante após 24 horas o que demonstra que o hidróxido atravessa lentamente a bicamada destas vesículas. A adição de micelas a este sistema leva a hidrólise de 100% do monômero em 90 minutos. Fica evidente, portanto, a capacidade das vesículas, quando comparadas com as micelas, de compartimentalizarem reações.

A síntese do brometo de 1,2-bis (palmitoiloxi)-3-propil) dimetil (β - (p-nitrofenil-carbonato) etil) amônio permitiu a preparação de vesículas positivas funcionalizada com substrato. A hidrólise alcalina dos monômeros da superfície externa produz uma vesícula assimétrica (neutra por fora e positiva dentro) já que na interface interna, a reação é lenta por ser limitada pela passagem de hidróxido (47).

O problema deste sistema é que as vesículas se transformam durante a reação. Em trabalhos posteriores estes monômeros de di-n-octadecilamônio funcionalizados com p-nitrofenilcarbonato ou p-nitrofenilbenzoato foram incorporados em vesículas preparadas com DODAC ou DHDAC. A adição de hidróxido ou glutatona na fase aquosa externa leva a uma cinética bifásica no caso de vesículas de DODAC e monofásica com vesículas de DHAC (48).

A demonstração de "flip-flop" também foi possível neste sistema. Após a reação externa com cisteína haver terminado acidificou-se o meio, as vesículas foram incubadas acima da T_c e, após resfriamento, o pH foi elevado novamente. Neste caso verificou-se de novo uma reação rápida e outra lenta sendo a porcentagem de cada uma das fases dependente do tempo de incubação. Este efeito foi atribuído ao "flip-flop" dos monômeros e sua velocidade depende do comprimento da cadeia do surfactante sendo maior em DHDAC do que em DODAC (48, 49, 50).

Utilizando os mesmos substratos Moss e col. demonstraram que a inserção de insaturações na cadeia hidrofóbica de monômeros das vesículas (derivados de diacilglicerol) aumenta a permeabilidade da bicamada a H^+/OH^- e a velocidade de

Flip-Flop (51).

Os diferentes tamanhos das vesículas de DODAC haviam, até aqui, sido explorados com o objetivo de obter uma maior incorporação de substratos. As propriedades destas bicamadas, entretanto são muito diferentes. Demonstramos que além do tamanho, existem diferenças no grau de dissociação externo dessas vesículas sendo que quanto maior o diâmetro, menor o grau de dissociação. Este último parâmetro, ao contrário das micelas, é bastante afetado por força iônica (52). Isto sugere que o grau de compactação da bicamada é muito diferente nas diversas preparações.

Outra questão a ser resolvida é se existe um efeito do grau de "compactação" dos monômeros na velocidade de reações em vesículas. Em alguns sistemas havia evidências claras deste fenômeno. Em sistemas mistos compostos por CTAB e um anfifílico capaz de formar vesículas, o brometo de dimetilditetradecilamônio (DTAB) a reação de um peptídeo contendo histidina com um éster de p-nitrofenila de cadeia longa é acelerada diferentemente dependendo da relação CTAB/DTAB observando-se um máximo de aceleração quando a relação é 2:3. Nesta relação CTAB/DTAB obtém-se também enantioseletividade $L \gg D$. A natureza dessa mistura particular de anfifílicos é incerta pois a turbidez das amostras não permitiu o uso de métodos clássicos de medida (53).

As reações de hidrólise de p-nitrofenilésteres de organo fosfinatos por ácido o-iodosobenzóico foram estudadas em líquido-cristais preparados com misturas de brometo de miristoiltrimetilamônio, decanol, brometo de amônio e água.

Na transição da fase discóide (Nl) para a fase cilíndrica (Nc) observou-se uma mudança na velocidade da reação de três ordens de magnitude enquanto na fase esférica (I), a velocidade é cerca de 3 vezes maior que na fase Nc. Atribuíram-se estas diferenças a solubilização dos substratos numa região próxima a interface nos agregados cilíndricos e esféricos enquanto que nos agregados lamelares os substratos estão localizados na região hidrocarbônica e, portanto, menos acessíveis ao nucleófilo (54).

A foto-isomerização unimolecular da merocianina a Indolinospirano também é afetada pela transição de fase Nl -Nc e I obtidas por variação de temperatura ou concentração de detergente (55). Na reação de bromação do trans-estilbeno as constantes de velocidade da reação também é afetada por transições de fase I-Nc-Nl (56). Estes efeitos foram atribuídos a diferentes localizações dos substratos nos agregados.

Esta mesma reação foi estudada com trans-estilbenos com cadeia hidrocarbônica longa em presença de micelas de SDS, vesículas de dihexadecil fosfato e dipalmitoilfosfatidilcolina (DPPC) e observou-se dependência entre a constante de velocidade e estrutura do agregado (57). Concluiu-se que vesículas solubilizam os substratos com estruturas diferentes em sítios diferentes enquanto em micelas todos ocupam o mesmo sítio (57).

Talvez o exemplo mais claro do efeito da compactação dos monômeros dos anfifílicos na velocidade de uma reação seja o da reação de lactonização, em meio ácido, de ~~monocamadas~~ de

ácido γ -hidroxiesteárico (58). A velocidade desta reação é constante até a pressão do filme chegar a 8 dinas/cm e, acima desta pressão, decresce por um fator de 3 ao chegar em 16 dinas/cm.

A compreensão, a nível molecular, de como a interface altera a velocidade de reações depende fundamentalmente do conhecimento das propriedades da bicamada de anfifílicos. O estudo de reações em vesículas preparadas por diversos métodos levou a ampliação desses conhecimentos.

Estudamos as reações de hidrólise alcalina e tiólise de NPA e NPO por n-heptilmercaptana com vesículas de DODAC de diversos tamanhos. Observamos uma grande diferença entre as diversas preparações não só em termos de fatores de aceleração das reações como nas constantes de associação dos substratos, alterações de pK e reatividade intrínseca dos nucleófilos. Em suma, as vesículas gigantes são pouco permeáveis a entrada de substratos e, por isso, as constantes de ligação dos substratos com estas vesículas são menores do que com as sonicadas ou etanólicas. Isto leva também a um efeito menor no pK da mercaptana já que esta também se incorpora menos (59).

O trabalho realizado nos últimos anos em nosso laboratório contribuiu em vários aspectos para a compreensão do efeito de vesículas em reatividade química:

- 1) Vesículas podem aumentar a velocidade de algumas reações bimoleculares por fatores da ordem de 10^6 .
- 2) Sistemas complexos como vesículas podem ser tratados segundo modelos similares aos utilizados com micelas.

3) Parâmetros físicos como tamanho, grau de dissociação, constantes de troca de íons foram determinados para vesículas de DODAC, DODAB e DCP .

4) Vesículas com diferentes graus de compactação da bicamada podem ser discriminadas por reações adequadas .

5) Vesículas de DODAC e DODAB podem compartimentalizar substratos e serem eficientes barreiras para a difusão passiva de íons.

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EFFECT OF DIALKYLDIMETHYLAMMONIUM VESICLES ON THE THIOLYSIS OF
p-NITROPHENYL ACETATE

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SUMMARY: Cationic surfactant vesicles, prepared by an alcohol injection technique and characterized by gel filtration, are extremely effective at increasing the rate of ester thiolysis at near neutral pH.

We and others have recently demonstrated that synthetic long-chain dialkyl amphiphiles preferentially form positively¹ and negatively² charged vesicles upon sonification in aqueous media. Since, unlike natural phospholipids³, these vesicles are chemically stable, they are unique models for the study of chemical reactivity in membranes. In our initial attempts to study the thiolysis of p-nitrophenyl acetate (NPA) by n-heptylmercaptan (C₇SH) in the presence of dialkyldimethylammonium (Herquat)⁴ vesicles, we found that vesicles preparations obtained by sonication contained probe-derived contaminants capable of catalyzing the oxidation of C₇SH. We therefore investigated the possibility of obtaining these vesicles by the alcohol injection method⁵. Vesicles are indeed obtained from Herquat⁴ by this technique⁵. When prepared in the presence of boric acid-borax buffer (9×10^{-3} M), the vesicle preparations is stable for at least 24 hrs. The existence of vesicles was confirmed by gel filtration on Sephadex G-25 (fine) using three independent criteria: (1) The gel filtration data in Fig.1A show that our Herquat vesicles are capable of entrapping (¹⁴C)-glucose to the extent of 8.8%, a value which compares favorably with that reported for analogous¹ vesicles. The stability of the vesicles under these conditions is indicated by the retention of the incorporated glucose upon refiltration of the vesicle preparation (Fig.1B). (2) Gel filtration of a vesicle preparation labelled with the water-insoluble fluorescent probe methyl 4-(1-pyrene)-butyrate, which should be totally incorporated in the bilayer phase⁶ resulted in a very sharp fluorescence peak in the void volume of the column (Fig.1C). (3) Preliminary kinetic studies, with unfiltered vesicle dispersions, demonstrated that the thiolysis of NPA by (excess) C₇SH is pseudo first-order under all conditions

and that the rate increases with Herquat concentration up to ca. $0.4 \times 10^{-3} \text{ M}$. That the observed rate acceleration is indeed attributable exclusively to vesicles is patent from the agreement between the observed rate acceleration profile for the gel filtration aliquots (Fig.1D) and the profiles for both entrapped (^{14}C)-glucose and the fluorescent pyrene derivative.

In contrast to these three criteria for the vesicle elution profile, the chloride ion profile was found to be significantly broader on the trailing edge (Fig.1E). The sensitivity of this broadening to the ionic content of the medium leads us to conclude that this tailing is related to ion exchange⁷ at the vesicle surface(s).

Fig.1 - Gel filtration of Herquat vesicles prepared by injection ($9 \times 10^{-3} \text{ M}$ borate buffer, pH 7.6). (A) Separation of vesicle-entrapped from free (^{14}C)-glucose; (B) Refiltration of an aliquot of peak I of A; (C) Relative fluorescence intensity of vesicle-incorporated methyl-4-(1-pyrene)-butyrate; (D) Effect of column aliquots on the relative rate of thiolysis of NPA by n-heptanethiol; (E) Chloride ion profile.

Having thus shown that vesicles are present and are the only source of the observed rate increase, we examined the amphiphile dependence of the thiolysis of NPA by C_7SH (Fig.2). There is a distinct rate maximum at $0.5 \times 10^{-3} \text{ M}$ Herquat with a smooth fall off in rate at higher amphiphile concentrations, the maximum rate increase of 7×10^4 in the presence of Herquat vesicles at pH 7.6 being truly remarkable. In contrast, the corresponding maximum rate increase in the presence of CTAB ($2 \times 10^{-3} \text{ M}$) is only 4.0×10^3 under the same conditions⁸.

This remarkable rate enhancement may reflect the mutual interplay of interfacial effects on the apparent pK of C_7SH , the binding of the resultant mercaptide and NPA to the vesicle and (possibly) intrinsic effects on nucleophilic reactivity at the vesicular interface(s). Indeed Herquat ($5 \times 10^{-4} M$) vesicles have been found to decrease the pK_{ap} of C_7SH from 10.7⁹ to 9.45. Quantitative studies of this system, envisaging the dissection of the relative roles of the above factors, are in progress.

Fig.2 - Effect of Herquat vesicles on the rate of thiolysis of p-nitrophenyl acetate. An appropriate aliquot of Herquat vesicles (prepared by injection of ca. 1 mL of a 0.2 M ethanol solution of Herquat into 19 mL of $1 \times 10^{-2} M$ boric acid-borax buffer pH 7.6) diluted to 2.5 mL with the same buffer ($1 \times 10^{-2} M$, pH 7.6) and temperature equilibrated in a Beckman 25 spectrophotometer. 15 μL of a solution of n-heptanethiol ($8 \times 10^{-3} M$ in ethanol) were added and the reaction started by addition of 10 μL of a CH_3CN solution of NPA ($1.25 \times 10^{-3} M$). Reaction was followed at 405 nm and apparent first-order rate constants were calculated as described¹⁰. k_v for the uncatalyzed thiolysis of NPA by C_7SH under the same conditions is $2.4 \times 10^{-6} s^{-1}$.

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A REMARKABLE ENHANCEMENT OF THE RATE OF ESTER THIOLYSIS BY SYNTHETIC AMPHIPHILE VESICLES

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Abstract—Cationic surfactant vesicles accelerate the rate of thiolysis of *p*-nitrophenyl octanoate by *n*-heptyl-mercaptan by several million fold in the pH range from 4 to 6, providing an efficient system for ester thiolysis in aqueous solution that is functional even at pH 4, i.e. more than 6 pH units below the pK_a of the SH group. Analysis of the data in terms of an ion exchange formalism implies that this rate acceleration is due primarily to concentration of the reagents in the dimensionally-restricted environment provided by the vesicle, coupled with small contributions from enhanced dissociation and reactivity of the nucleophile at the vesicle surface(s).

Recently, there has been an increasing interest in the solution properties of synthetic amphiphiles having a hydrophobic-hydrophilic balance similar to that of natural lipids.^{1,2} Although the polar groups of these synthetic amphiphiles bear little³ or no⁴ structural similarity to those of natural lipids, they have been shown to form a variety of lipid-like structures¹ in aqueous dispersions, including both single^{5,6} and multilayer^{6,7} vesicles and other types of bilayers.⁸ The fact that the chemical nature of these amphiphiles can be readily varied provides a unique opportunity to investigate the interrelationships between the structure of the monomer and the properties and architecture of the resultant aggregates. These features also make synthetic-amphiphile aggregates an extremely attractive model system⁹⁻¹¹ for the study of chemical reactivity in well-defined, dimensionally-restricted environments such as those prevailing at the active sites of enzymes and in biological membranes.

In this work, we report that the thiolysis of *p*-nitrophenyl octanoate (NPO) by *n*-heptyl mercaptan (HM) at pH 4 is accelerated by more than 10^6 fold in the presence of vesicles formed from dimethyl di-(*n*-alkyl) ammonium chloride. Analysis of this marked rate acceleration in terms of an ion-exchange formalism¹² permits estimation of the relative contributions of local concentration, local pH effects, and changes in intrinsic reactivity to the overall rate enhancement.

MATERIALS AND METHODS

NPO was kindly furnished by Dr. O. A. El Seoud.[†] Dimethyldi-(*n*-alkyl) ammonium chloride (DODAC)[‡] was purified and analyzed as described previously.¹⁰ HM[§] was distilled *in vacuo* (b.p. 37–38°C, 1 mm Hg) and stored at –18° under argon. All other reagents were analytical grade or superior and solutions were prepared in deionized water, doubly distilled in glass, which had been freshly boiled and allowed to cool under an argon atmosphere.

Kinetic measurements were carried out at 30.0° with a Beckman M25 spectrophotometer. The thiolysis of NPO by HM was followed by monitoring the appearance of the *p*-nitrophenoxide

ion at 405 nm (or of *p*-nitrophenol at 330 nm at the lower pHs). Rate constants were calculated as previously described¹⁰ from $\log(A_\infty - A_t)$ vs time plots, which were linear for at least three half-lives in all cases. pH was measured with a Mettler Herisau E 388 compensator equipped with a semimicro combination electrode (Beckman Inc.) and calibrated against standard reference buffers (Beckman Inc.). DODAC vesicles were prepared as previously described¹⁰ by injection of 1.0 ml of an 0.2 M ethanolic soln of DODAC into 19.0 ml of the appropriate buffer thermostatted at 50°. The soln was stirred vigorously throughout the injection procedure, which required ca. 2 min.

Gel filtration of DODAC vesicles (0.50 ml aliquot) was performed on Sephadex G-25 (Pharmacia Fine Chemicals, AB) columns (0.8 × 47 cm) equilibrated with appropriate buffers and eluted with the equilibrating buffer at 4.8 ml/hr; 1.0 ml aliquots were collected. Chloride ion concentrations were determined by titration¹³ using a Beckman Spinco 153 Microtitrator and vesicle concentration was estimated by measuring the apparent turbidity at 240 nm. The apparent pK (pK_{app}) of HM was calculated from the relationship $pK_{app} = pH + \log [(HM^-)/(HM_T - HM^-)]$ where HM_T is the total concentration of added HM. The concentration of mercaptide ion (HM^-) was determined at 230 nm¹⁴ as a function of DODAC concentration at constant pH^{12,14} and HM_T was determined by titration with 5,5'-dithiobis (*p*-nitrobenzoic acid).¹⁵

RESULTS

The integrity of DODAC vesicles, prepared by direct injection of concentrated (0.2 M) ethanolic solutions of the DODAC into the appropriate aqueous buffers, was verified by Sephadex G-25 gel filtration.¹⁰ Filtration of DODAC vesicles prepared and filtered in borate buffers results in incomplete borate/chloride ion exchange, most of the chloride eluting at or near V_0 .¹⁰ In contrast, when DODAC vesicles are prepared and filtered in acetate buffers, all of the amphiphile-derived chloride elutes at the internal (included) volume of the Sephadex G-25 column, indicating complete displacement of chloride due to ion exchange during the preparation-filtration sequence (Fig. 1).

The influence of unfiltered DODAC vesicles on the pK_{app} of HM is shown in Fig. 2. The effects of unfiltered DODAC vesicles, prepared in acetate buffers, on the apparent first order rate constant (k_{app}) for thiolysis of NPO by HM are shown in Fig. 3.

The maximum increase in the apparent first order rate constant in the presence of vesicles was calculated from

[†]The Instituto de Química da Universidade de São Paulo.

[‡]Herquat 2HT; 85% *n*-C₁₈ and 15% *n*-C₁₆, Herga Ind. Quim., Brasil.

[§]The Connecticut Hard Rubber Co. Chemical Division.

Table 1. Effect of DODAC vesicles on the rate of thiolysis of *p*-nitrophenyl octanoate by *n*-heptylmercaptan

pH	DODAC (M) $\times 10^5$	$k_V^{\max}$ (s ⁻¹) $\times 10^3$	k_V^0 (s ⁻¹) $\times 10^3$	$R_{\max}$ ($k_V^{\max}/k_V^0$)
4.00	2.3	2.3	0.30	7.7×10^6
4.35	6.0	3.9	0.67	5.8×10^6
4.60	6.4	4.7	1.20	3.9×10^6
5.00	4.7	8.0	3.00	2.7×10^6
5.35	8.3	18.4	6.70	2.7×10^6
5.60	10.0	27.0	11.90	2.3×10^6

Fig. 1. Gel filtration of DODAC vesicles prepared in 0.005 M Na⁺-Acetate buffer, pH 5.30; (○) chloride; (●) Abs₂₄₀. Fig. 2. Effect of DODAC vesicles on the apparent pKa (pKap) of *n*-heptylmercaptan (HM). The pKap of HM (5×10^{-5} M) was determined in the same buffer used to prepare the vesicles (see Materials and Methods). Vesicles were prepared in: A. (●) Tris-HCl 0.02 M, pH 9.1, and (○) Borate (Na⁺) 0.01 M, pH 9.3; B. (□) Tris-Acetate 0.02 M, pH 9.05, and (■) Tris-Acetate 0.04 M, pH 9.05. All curves are calculated (see Discussion). Fig. 3. Effect of DODAC vesicles on the thiolysis of *p*-nitrophenyloctanoate (NPO) by *n*-heptylmercaptan (HM). Rate constants were measured (see Materials and Methods) using 5×10^{-6} M NPO and 5×10^{-5} M HM in Na⁺ Acetate buffers. (Δ) pH 4.0; (■) pH 4.60; (□) pH 5.0; (●) pH 5.35; (○) pH 5.60. All curves are calculated (see Discussion).

the relationship:

$$R_{\max} = k_V^{\max}/k_V^0 \quad (1)$$

where $k_V^{\max}$ is the maximum value of k_V at each pH (Fig. 3). The corresponding apparent first order rate constants in the absence of vesicles (k_V^0) were calculated from

$$k_V^0 = k_2^0 \times HM_T \times \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (2)$$

using the measured values of $k_2^0 = 30 \text{ M}^{-1} \text{ s}^{-1}$ for the second order rate constant of thiolysis in the aqueous phase, 10.70 for the pKa of HM^{14,16} and the total concentration of HM (HM_T). The relevant data are summarized in Table 1.

DISCUSSION

The mere demonstration of a million fold increase in the apparent constants in a non-enzymatic model system sheds little light on either the origin of the rate increase or its relationship to the rates observed in enzyme-catalysis. In the present case, the overall rate acceleration is so large relative to those observed in other model systems that it becomes essential to understand the various factors that contribute to this rate enhancement.

The existence of a minimum and the effect of added acetate on the pKap vs DODAC profiles (Fig. 2) are completely analogous to the effects of cationic micelles on the dissociation of HM and other weak acids.^{12,14} In the case of micelles, the pKap profiles can be analyzed quantitatively using an ion-exchange formalism which describes this complex pattern in terms of the distribution of HM between the aqueous and micellar phases and local pH effects (ion-exchange) at the micellar surface.^{12,14} The applicability of these concepts of ion exchange in the DODAC system follows directly from the gel filtration data, which provide unequivocal evidence for Cl⁻/borate and Cl⁻/acetate ion exchange at the DODAC vesicle surface(s). Moreover, the observation of complete displacement of chloride by acetate (Fig. 1) implies that, in this case, ion exchange occurs at both the inner and outer surfaces of the vesicle. Thus, by analogy to the micellar system, the effect of unfiltered DODAC vesicles on the pKap of HM should be described¹⁴ by the relationship:

$$K_{ap} = K_a \frac{1 + K_{HM-Cl} \frac{Cl_b}{Cl_f}}{1 + K_{HM} [DODAC]} \quad (3)$$

where K_a is the acid dissociation constant of HM in water, K_{HM} is the distribution constant of HM between the water and vesicle phases, and K_{HM-Cl} is the weighted selectivity coefficient for mercaptide ion/chloride ion exchange at the outer and inner vesicle surfaces. In the presence of an "ideal" buffer such as Tris-HCl,¹⁷ the analytical concentrations of bound and free chloride ions, Cl_b and Cl_f respectively, can be calculated from expressions derived previously.^{12,20}

$$Cl_b = (1 - \alpha) [DODAC] - OH_b \quad (4)$$

$$Cl_f = \alpha [DODAC] + Cl_{AD} + OH_b \quad (5)$$

where Cl_{AD} is the concentration of added chloride and OH_b the concentration of bound hydroxide ion. The value for the selectivity coefficient for hydroxide/chloride exchange ($K_{OH/Cl} = 0.13$) was taken to be that derived for hexadecyltrimethylammonium micelles.^{14,17} Using a ratio of K_{HM-Cl}/K_{HM} of 0.03, a value close to that obtained in micellar systems¹⁴ and a K_{HM} of 3700,¹⁴ the pKap profile obtained in Tris-HCl (Fig. 2a) could be fitted with a constant value of 0.16 for the parameter α , which reflects the apparent degree of counterion dissociation at both the inner and outer vesicle surfaces. The pKap vs DODAC profiles depicted in Fig. 2 could all be reproduced by simply taking into account the effects of borate/Cl and acetate/Cl exchange on Cl_b/Cl_f using the corresponding ion exchange selectivity coefficients determined for cationic micelles.^{14,18,19}

Although the absolute values of the parameters

employed in the curve fitting procedure represent only estimates, the fact remains that the experimental results for the effects of vesicles on pKap can be fitted remarkably well with a micelle-type ion exchange formalism. Hence, we are led to conclude that the vesicle surface(s) are micelle-like with respect to counterion exchange, the "small" effect of DODAC vesicles on the dissociation of HM being attributable to distribution of HM between the aqueous and vesicle "phases" and ion-exchange effects on the local pH at the vesicle surface(s).

The overall reaction rate for the thiolysis of NPO by HM in the presence of DODAC vesicles is the sum of the rates of reaction in the aqueous phase (governed by the rate constant in the aqueous phase and the analytical concentrations of the free reagents) and in the vesicle "phase" (governed by the rate constant and the local concentrations of reagents in this phase). Since the volume fraction occupied by the vesicles is very small¹¹ and both NPO and HM can be safely assumed to partition in favor of the vesicle bilayer¹⁴ the local reagent concentration in the vesicles can be several orders of magnitude higher than those in the bulk aqueous phase. For example, taking the partial molar volume ($\bar{V}$) of DODAC to be 0.44 L/M,¹¹ the volume fraction occupied by the vesicles (excluding the internal aqueous compartment) at 10^{-4} M DODAC would be 4.4×10^{-5} .

Thus, for a bimolecular reaction between two totally vesicle-incorporated substrates at 10^{-4} M DODAC, one would expect a 5×10^8 fold increase in reaction velocity (or a 5×10^4 fold increase in k_p) on the basis of local substrate concentration effects alone.

Considering, in addition to substrate distribution, the effects of local pH on the dissociation of HM, it can be shown^{12,14} that:

$$k_p = (HM)_T K_a \frac{(k_2^*/\bar{V}) K_S K_{HM-Cl} \frac{Cl_b}{Cl_f} + k_2^0}{(1 + K_{HM} [DODAC]) (H^+ + K_{ap}) (1 + K_S [DODAC])} \quad (6)$$

Using the same parameter values derived from the analysis of the effect of DODAC vesicles on pKap and a value of K_S (4×10^4 M⁻¹), approximately equal to that for NPO in CTAB,¹⁴ the best fit value for $k_2^*/\bar{V}$ was found to be 1095 s⁻¹ at all the pH's studied. The increase in external pH produces an increase in the local concentration of bound hydroxide ion at the vesicle interface, and a corresponding increase in k_p and k_p^{app} due to enhanced dissociation of HM (Fig. 3, Table 1). However, at constant buffer concentration, the increase in pH is accompanied by an increase in the acetate concentration enhancing the relative displacement of bound counterions (including ⁻OH) by acetate from the vesicle surface(s). The decrease in R_{max} with pH (Table 1) and the non-unity slope (0.75 at 1×10^{-4} M DODAC) of a log k_p vs pH function (data from Fig. 3) are thus nicely accounted for by Eq. 6, via the Cl_b/Cl_f term, on the basis of local pH effects¹⁷ (i.e. ion-exchange at the vesicle interface(s)).

An absolute value for the apparent intrinsic reactivity¹⁷ of the mercaptide ion for the thiolysis reaction in the vesicles (k_2^*) can be calculated from an estimate of $\bar{V}$ and the value of $k_2^*/\bar{V}$. Thus, depending on the value

selected for $\bar{V}$ (ranging from 0.37 to 0.44 L M⁻¹),^{10,11} k_2^* varies from 405 s⁻¹ M⁻¹ to 481 s⁻¹ M⁻¹. Compared to the value of $k_2^* = 30$ s⁻¹ M⁻¹, these k_2^* values are indicative of only a moderate increase in the intrinsic reactivity (ca 15 fold) of the mercaptide ion in the DODAC vesicle implying that the main acceleration factor is indeed a local concentration effect.

Although the relationship between rate accelerations obtained in amphiphile aggregates and kinetic data for enzymes is not straightforward,²⁰ the results of the present work bear directly on the role of entropic factors in enzyme catalysis.²⁰ Thus, the analysis of the kinetic data for the present model system demonstrates that, even in the case of relatively non-specific substrate binding, quite large rate enhancements can arise as a result of concentration of the components of a bimolecular reaction in a dimensionally restricted environment.

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²⁰See ref. 20 for a cogent description of the difficulties involved.

ANALYSIS OF THE EFFECT OF MICELLES AND VESICLES ON THE REACTIVITY
OF NUCLEOPHILES DERIVED FROM THE DISSOCIATION OF WEAK ACIDS

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Micellar solutions of hexadecyltrimethylammonium bromide(CTAB) accelerate the thiolysis of p-nitrophenyl octanoate(NPO) and acetate(NPA) by n-heptyl mercaptan(HM) by several orders of magnitude. The rate of thiolysis of NPO by HM increases two hundred fold in the presence of the zwitterionic detergent dodecyltrimethylammonium propane sulfonate(DDAPS) while sodium dodecyl sulfate (SDS) produces a seventy fold rate decrease. The detergent-induced rate effects were analyzed within the framework of a pseudophase ion-exchange formalism, the requisite parameters being determined independently of the kinetic results. Excellent stimulations were obtained using ratios of second order rate constants in the aqueous and micellar phases close to one. CTAB and vesicles derived from dimethyldioctadecylammonium chloride accelerate the thiolysis of esters by cysteinylhexadecyl amide by more than a million fold. This rate enhancement was also analyzed using pseudophase ion-exchange formalism. The maximum rate effects, produced by the addition of amphiphile aggregates on the thiolysis of esters, extend over a range of one hundred million. Although changes of the interphase affect the reactivity of the nucleophile, this factor does not determine the extent of catalysis or inhibition. The main kinetic effect of micelles and vesicles is due to changes in the local concentration of the reactants in the restricted environment of the organized pseudophase.

INTRODUCTION

Pseudophase models, with explicit consideration of ion exchange, successfully describe micellar effects on reaction kinetics and equilibria involving both neutral and ionic reagents.¹⁻⁶ Application of such models shows that nucleophilic reactivities, assessed by the values of calculated rate constants, are of comparable magnitude in the micellar pseudophase and in the intermicellar aqueous phase. Similar reactivities have been found for reactions such as hydroxide ion attack on esters,^{5,7} ester thiolysis,^{7,8} acid catalyzed benzidine rearrangement,⁹ acid catalyzed hydration of pyridinium ions,¹⁰ nucleophilic substitution on halobenzenes,¹¹ cyanide addition on nicotinamides¹² and ester oximolysis.^{6,13} The use of pseudophase models has recently been extended to the analysis of reactions in synthetic amphiphile vesicles.^{14,15}

Our aim in this work was to examine the limits of the applicability of the ion exchange formalism, and of the concept of similar reactivities in amphiphile aggregates and in the aqueous phase. For that purpose we analysed the effect of positive, negative and zwitterionic detergents on the rate of thiolysis of esters. In addition we compared the effect of micelles and synthetic amphiphile vesicles on the same reaction. The maximum rate effects ranged from seventy fold inhibition by negatively charged micelles to ten million fold acceleration by positively charged vesicles. Pseudophase ion-exchange formalism was shown to apply in all cases. The analysis of such wide range of kinetic effects demonstrated that local reagent concentration in the main contributor to the observed "catalysis" or "inhibition" by these amphiphile aggregates.

EXPERIMENTAL SECTION

Materials. Hexadecyltrimethylammonium bromide (CTAB) (Merck, Darmstadt, Germany) and dioctadecyldimethylammonium chloride (DODAC) (Herga Ind. Quim. Rio de Janeiro, Brasil) were purified and analyzed as previously described.^{8,16} Sodium dodecylsulfate (SDS) (Specially pure grade, BDH, Poole, England) gave no minimum in surface tension plots, was used as received. Heptyl mercaptan (HM) (Aldrich) was distilled (37 - 38°C, 1 mmHg) and maintained at -18°C under Ar. p-Nitrophenyl acetate (NPA) was purchased from Sigma Chem. Co.. Dodecyldimethylammoniumpropanesulfonate (DDAPS) and p-nitrophenyl octanoate (NPO) were synthesized by Dr. O.A. El Seoud of this Institute. Cysteinylhexadecylamide (HCys) was synthesized as shown in Scheme 1, while compounds I and II were synthesized according to the literature.¹⁷ p-Nitrophenylester of L-4-carboxy-3-formyl-2,2-dimethylthiazolidine (III): 25 g of dicyclohexylcarbodiimide (DCC) was added to a stirred suspension of II (18.9 g) in chloroform (300ml) containing p-nitrophenol (16 g) at

SCHEME I

15°. The mixture was stirred for 4 hrs at room temperature (22°). The copious precipitate of dicyclohexylurea was filtered and the solution evaporated to dryness. The residue was crystallized from absolute ethanol (m.p. 113-114, nmr, ir.).

L-3-Formyl-2,2-dimethylthiazolidine-4-(hexadecyl)carboxamide (IV): 21.7 g of III and 14.5 g of hexadecylamine, dissolved in chloroform, were left overnight. The solvent was removed and residue (orange-red liquid) was added to a dilute solution of sodium carbonate (100 ml, 10%) to remove p-nitrophenol. The aqueous layer was decanted and the residue was washed with ethanol. Cysteinyl hexadecylamide (HCys): Crude IV was dissolved in a mixture of 10 ml of concentrated HCl and 120 ml of methanol and refluxed on a steam bath for 30 min and then left at room temperature for 48 hrs. The solvent was evaporated and the residue recrystallized from MeOH-ether. Elemental analysis: Required for $\text{C}_{19}\text{H}_{41}\text{ClN}_2\text{OS}$: C, 59.88; H, 10.84; N, 7.35. Found: C, 58.91; H, 10.43; N, 7.05. NMR, IR. and SH analysis are in agreement with those required by structure.

METHODS

DODAC vesicles were prepared by alcohol injection as described previously.¹⁶ DODAC and CTAB concentrations were determined by halide titration with $\text{Hg}(\text{NO}_3)_2$.¹⁶ Stock solutions (ca. 10^{-2} M) of HM (in CH_3CN) or HCys (in CH_3OH) were maintained at -18°C under Ar. SH concentration was determined by reaction with 5,5' dithiobis (2-nitrobenzoic acid).¹⁸ Stock solutions of NPA and NPO (ca. 10^{-3} M) were prepared in CH_3CN . Pseudo first order rate constants k_V (excess mercaptan) for the thiolysis of p-nitrophenyl esters ($30.0 \pm 0.1^\circ\text{C}$) were obtained as described previously.^{8,15}

The distribution constant for the partitioning of HM between the micellar pseudo-phase and the intermicellar aqueous phase (K_{HM}) can be described by Equation (1):⁶

$$K_{\text{HM}} = \frac{(\text{HM}_b)}{(\text{HM}_f)C_D} \quad (1)$$

where (HM_b) and (HM_f) are the analytical concentration of HM bound to the micelles and free in the aqueous phase, respectively. The concentration of micellized detergent, C_D , is equal to the total detergent concentration (C_T) minus the critical micelle concentration (CMC). CMC's in the presence of HM were determined by measuring surface tension as a function of detergent concentration. In all other cases the CMC's were corrected for salt and/or the increase in detergent concentration by numerical iteration of Equation 2:^{19a}

$$\log \text{CMC} = A - b \log (\text{salt} + \text{CMC} + \alpha C_D) \quad (2)$$

The constants A and b for CTAB and SDS were taken from the literature.^{19a,b} The degree of counterion dissociation of the micelle (α) was taken as 0.2 for CTAB and 0.218 for SDS.²⁰ Equation (1) can be expressed as

$$(\text{HM}_O) = (\text{HM}_f) + (\text{HM}_f) K_{\text{HM}} C_D \quad (3)$$

where HM_O is the total analytical concentration of HM at a particular detergent concentration. In practice, 0.10 ml of HM were added to Teflon-stoppered Ar-flushed tubes containing 2.00 ml of 0.01 M HCl. The solutions were equilibrated for 2 days at 30°C with gentle agitation. After aspiration of the HM layer, HM_O was determined in an aliquot of the aqueous layer. K_{HM} was calculated from the slope of the linear plot relating HM_O with C_D (Equation 3).

The apparent acid dissociation constant (K_{ap}) of a weak acid, such as HM or HCys, in the presence of an amphiphilic

aggregate can be defined⁶ as:

$$K_{ap} = H_f \frac{(M_b) + (M_f)}{(HM_b) + (HM_f)} = H_f \frac{(M_T)}{(HM_T)} \quad (4)$$

where H_f represents the intermicellar concentration of hydrogen ions. HM and M are the undissociated and dissociated forms of the weak acid. The subscripts b, f and T represent the bound, free and total analytical concentrations of each species.

The absorptivities of the protonated (E_{HM}) and anionic (E_M) forms of the mercaptans, determined in 0.01 M HCl and 1.0 M NaOH, were found to be insensitive to the presence of detergent. For n-heptylmercaptan E_M and E_{HM} were 5650 and 115 $M^{-1} cm^{-1}$ and for cysteinylhexadecylamide the corresponding values were 5495 and 153 $M^{-1} cm^{-1}$. The absorbance (240 nm) of a solution of HM (or HCys) can be described by

$$Abs = E_{\psi} HM_0 = E_{HM} HM_T + E_M M_T \quad (5)$$

where E_{ψ} is the observed absorptivity of the added mercaptan. From Equations (4) and (5) it can be shown that

$$pK_{ap} = pH + \log \left| \frac{E_M - E_{\psi}}{E_{\psi} - E_{HM}} \right| \quad (6)$$

The variation of pK_{ap} with C_D was determined (Equation 6) at a single, fixed pH using a fixed mercaptan concentration (ca. $5 \times 10^{-5} M$).

RESULTS

Cationic detergents, such as CTAB, increase the rate of thiolysis of esters.^{6,8,21} The effect of CTAB on the rate of thiolysis of NPA and NPO by HM was determined as a function of detergent concentration at several pH's (Figure 1). The shape of the curves relating k_{ψ} with CTAB is characterized by a maximum at low detergent concentration, followed by a smooth decrease upon increasing [CTAB]. The concentration of detergent at which the maximum rate is attained (k_{ψ}^{max}), and the values of k_{ψ}^{max} are pH dependent (Figure 1). For comparison, we calculated the pseudo-first order rate constant (k_{ψ}^0) that would be observed for the thiolysis of NPO and NPA on the absence of micelles under comparable conditions. The second order rate constant for reaction of NPA with HM in the aqueous phase (k_2^0) was found to be 30 $M^{-1} s^{-1}$.²² The relationship $k_{\psi}^{max}/k_{\psi}^0$ gives the maximum rate acceleration in each case (Table I). The value of $k_{\psi}^{max}/k_{\psi}^0$ increased with pH in the case of NPO as a reflection of the decrease in the concentration of bromide needed to maintain the pH with Tris buffer. For NPA the small variations of the values of $k_{\psi}^{max}/k_{\psi}^0$ were attributed to a change in the ionic composition of the borate and borate-borax buffers in this pH region and to the corresponding differences in the affinity of these ionic species to the CTAB micelle.

Figure 1. Effect of CTAB on the rate of thiolysis of NPA (A) and NPO (B) by n-heptyl mercaptan. A. (NPA) = 5×10^{-6} M, (HM) = 5×10^{-5} M, Borate buffer (0.02 M) pH's 8.9 (○), 8.6 (●) and 8.3 (□). Borax/borate buffer (0.02 M) pH's 8.0 (■) and 7.7 (Δ). Inset shows the variation of pK_{ap} a function of (CTAB), borate buffer (0.02 M) pH 10.10. B. (NPO) = 5×10^{-6} M, (HM) = 5×10^{-5} M, all reactions on 0.02 M Tris/HBr pH's 7.7 (■), 8.0 (□), 8.3 (●), 8.55 (○) and 8.90 (Inset).

Table 1. Effect of CTAB on the rate of thiolysis of NPO and NPA by n-heptyl mercaptan.

Ester	pH ^a	$k_{\psi}^{\max}/k_{\psi}^o$ ($\times 10^3$)	k_2^m/k_2^o
NPA	7.7	6.09	3.3
	8.0	6.35	3.6
	8.3	6.94	4.2
	8.6	6.13	4.8
	8.9	5.55	4.6
NPO	7.7	3.05	1.0
	8.0	5.02	1.2
	8.3	5.06	1.0
	8.55	5.80	1.0
	8.9	15.80	1.2

a. Buffer composition is given in the legend of Figure 1. b. CTAB concentration at $k_{\psi}^{\max}$ was 2×10^{-3} M for NPA and 1×10^{-3} M for NPO

The quantitative analysis of a reaction of a nucleophile derived from the dissociation of a weak acid in a micelle solution starts by considering the effect of the detergent on the acid dissociation. The relationship between K_{ap} and C_D for a neutral weak acid such as HM in the presence of a positively charged detergent (CTAB) can be expressed⁶ as:

$$K_{ap} = K_a \frac{1 + K_T \frac{(Br_b)}{(Br_f)}}{1 + K_{HM} C_D} \quad (7)$$

where K_a is the acid dissociation constant of HM. The analytical concentration of bromide bound to the micelle (Br_b) can be described by^{4,6}

$$(Br_b) = (1 - \alpha) C_D - OH_b - M_b \quad (8)$$

where OH_b represents the analytical concentration of hydroxide ion bound to the micelle. The analytical concentration of bromide in the intermicellar aqueous phase (Br_f) can be expressed as

$$(Br_f) = \alpha C_D + OH_b + M_b + Br_{AD} \quad (9)$$

where Br_{AD} is the concentration of added bromide. In the presence of a buffer which contains ions other than bromide the binding of all ionic species have to be considered in Equations (8) and (9).^{7a} The selectivity constant for the mercaptide/bromide ion exchange (K_T) was calculated by fitting the data for the variation of pK_{ap} with C_D (inset Figure 1A) with Equation (7) using K_T as a variable parameter and a value of 3700 M^{-1} for K_{HM} (see Methods). In this particular case K_T can also be calculated from the data (inset Figure 1A) at the point where $pK_{ap} = pK_a = 10.7$.²³ Under such condition Equation (6) reduces to

$$K_T = K_{HA} C_D \frac{(Br_f)}{(Br_b)} \quad (10)$$

Both procedures yield a value of 75 for K_T .

The kinetic data for the thiolysis of NPA and NPO by HM (Figure 1) were analyzed quantitatively using an ion-exchange model.⁶ This formalism takes into account: a) the partitioning of the neutral form of the mercaptan (HM) and of the neutral esters (NPA and NPO) between the micellar pseudophase and the aqueous phase b) selective ion exchange of all ionic species, including the dissociated form of the mercaptan, with bromide counterions and c) independent reactivities in the micellar and aqueous phases. We have demonstrated⁶ that this system can be described by Equation (11).

$$k_y = K_{HM} K_a \frac{k_2^0 + (k_2^m / \bar{V}) K_S K_T (Br_b / Br_f)}{H_f (1 + K_{HM} C_D) + K_a (1 + K_T (Br_b / Br_f)) (1 + K_S C_D)} \quad (11)$$

k_2^m and $\bar{V}$ represent the second order rate constant in the micellar phase and partial molar volume of the micellized detergent, respectively. H_f and maintained constant by the use of an appropriate buffer^{7a} and the distribution constants for NPA and NPO (K_S) were taken as 54 and 1.5×10^4 respectively. The values of K_T and K_{HM} were those determined previously (vide supra). The data in Figure 1 were fitted to Equation (11) by iteration varying exclusively the value of $(k_2^m / \bar{V})$. The correspondence between the data and the k_y vs. CTAB functions generated at several pH's by the ion exchange model was excellent (Figure 1).

Calculation of second order rate constants in the micelle pseudophase in conventional units is possible after selection of a volume element for the reaction. We^{7a,15} and others^{1,6} have used the partial molar volume of the micellized detergent as such volume element. The k_2^m 's for the thiolysis of NPO and NPA were calculated from the respective best fit values of $(k_2^m / \bar{V})$ taking $\bar{V} = 0.37 \text{ LM}^{-1}$ (Table I). For the thiolysis of NPO by HM the values of k_2^m/k_2^0 are identical within experimental error. The values of k_2^m/k_2^0 for the NPA series exhibit a definite increase upon changing the buffer from borax/borate to borate.

Since the early work of Hartley²⁵ it is known that negatively charged detergents increase the apparent acid dissociation constant of neutral weak acids. In fact, the addition of SDS caused an increase in the pK_a of HM (Inset Figure 2). The dissociated form of the mercaptan (M), bearing a negative charge, will distribute as a pseudo monomer of the detergent²⁶. With these consideration and the usual assumptions of ion exchange^{4,20} it can be shown that the function relating the variation of K_a of HM with SDS concentration is

$$K_{ap} = K_a \frac{1 + (K_b \cdot K_{HM} \cdot C_D / K_{H/Na} \cdot K_a) ((Na_f) / (Na_b))_l}{(1 + K_{HM} C_D)} \quad (12)$$

The acid dissociation constant of HM in the micellar phase (K_b) was defined⁶ as:

$$K_b = \frac{(M_b)_l (H_b)_l}{(HM_b)_l} \quad (13)$$

where the subscript l represents the local concentration in the micellar pseudophase. The value of the sodium/hydrogen selectivity coefficient ($K_{H/Na}$) for ion exchange was taken as 1.27^b (Na_f) and $(Na_b)_l$ represent the analytical concentrations of free sodium

Figure 2. Effect of SDS on the rate of thiolysis of NPO by HM. $[HM] = 2 \times 10^{-4}$ M, $[NPO] = 4 \times 10^{-6}$ M, $[NaOH] = 2.0 \times 10^{-3}$ M (pH = 11.13, 30°C). Inset shows the variation of pK_{ap} with SDS. Sodium phosphate (0.02 M) buffer pH 11.30, HM = 7×10^{-5} M.

ions and the local concentration of bound sodium ions respectively. The mathematical expressions relating Na_f and $(Na_b)_i$ with detergent concentration, salt and pH have been published.^{7b,12} Equation (12) is equivalent to Equation (14)

$$K_{ap} = K_a \frac{1 + (K_T^M/m_f) C_D}{1 + K_{HM} C_D} \quad (14)$$

The distribution constant of the pseudo monomer M (K_T^M) can be described by Equation (15)

$$K_T^M = \frac{M_b m_f}{M_f C_D} \quad (15)$$

where m_f represents the analytical concentration of free monomers in the intermicellar aqueous phase. The solid line in the inset of Figure 2 was obtained by iteration to best fit (Equation 12) using K_b as the adjustable parameter. The calculated value for K_b was 1×10^{-12} .

SDS strongly inhibits the thiolysis of NPO by HM (Figure 2), the maximum inhibitory effect, at the plateau region, being ca. 70 fold (Table II). The kinetic data presented in Figure (2) were analyzed using Equation (16). This equation can be derived from a ion exchange formalism^{4,6,20} taking into account all the factors described above for the variation of pKap with [SDS] and the distribution of NPO

$$k_{\psi} = HM_0 \frac{k_2^0 K_a K_{H/Na} + (k_2^m/\bar{V}) K_b K_{HM} K_S C_D \frac{(Na_f)}{(Na_b)_l}}{(1+K_S C_D) \left[K_a K_{H/Na} + K_{H/Na} H_f (1+K_{HM} C_D) + K_b K_{HM} C_D \frac{(Na_f)}{(Na_b)_l} \right]} \quad (16)$$

The values of K_a , $K_{H/Na}$, K_{HM} , K_b and the calculation of C_D , (Na_f) and $(Na_b)_l$ were those described in the analysis of the variation of pKap with [SDS] and K_S for NPO was taken as $1 \times 10^4 M^{-1}$. Equation (16) was fitted to the experimental results by varying $(k_2^m/\bar{V})$. Taking $\bar{V}$ as 0.25²⁴ the best fit value of k_2^m is lower than k_2^0 by a factors of ca. 3 (Table II).

The effect of a zwitterionic detergent (DDAPS) on the pKap of HM is similar to that observed for SDS (Insets Figures (2) and (3)). In this case the pKap vs. DDAPS profile can be described by Equation 17⁶ employing a (constant) value of 0.002 M for the cmc of DDAPS.

$$Kap = K_a \frac{1 + K_M C_D}{1 + K_{HM} C_D} \quad (17)$$

The partitioning of $M(K_M)$ and of $HM(K_{HM})$ were represented as distribution constants between the micellar pseudophase and the aqueous phase. The value of the distribution constant for neutral,

Table II. Effect of SDS and DDAPS on the rate of thiolysis of NPO by HM.

Detergent	pH ^a	$k_{\psi}^{max}/k_{\psi}^{ob}$ ^b	k_2^m/k_2^0
SDS	11.30	0.09	0.27
DDAPS	10.15	266	1.0

a. The buffer compositions are given in the legends of Figures (2) and (3). b. For SDS this ratio refers to the k_{ψ} observed at 0.10 M, for DDAPS the concentration of detergent at k_{ψ}^{max} was 0.004 M.

long chain reagents between the micellar pseudophase and the aqueous phase depends mostly on the length of the alkyl chain of the surfactant.²⁷ Thus K_{HM} in DDAPS was taken as equal to the value in SDS. Equation (17) was fitted to the experimental data by varying K_M and the best fit value was found to be 100 M^{-1} .

Although both DDAPS and SDS cause an increase in the pK_a of HM, the zwitterionic detergent produces an increase in the rate of thiolysis of NPO (Figure 3), the maximum rate enhancement (k_y^{max}/k_0) being ca. 250 fold (Table II). The effect of the variation of [DDAPS] on the pseudo first order rate constant for the thiolysis of NPO by HM can be described by Equation (18).

$$k_y = k_0 + \frac{(k_2^{\text{H}}/\bar{V}) K_M K_S C_D}{(1 + K_M C_D) (1 + K_S C_D) (1 + H_f/K_a)} \quad (18)$$

Equation (18) was fitted to the experimental data (Figure 3) using $(k_2^{\text{H}}/\bar{V})$ as a variable parameter. Taking $\bar{V}$ as 0.25 LM^{-1} , the second order rate constant in the micellar pseudophase is equal to that in the aqueous phase²⁸ (Table II).

Figure 3. Effect of DDAPS on the rate of thiolysis of NPO by HM. $[HM] = 5.7 \times 10^{-5} \text{ M}$, $[NPO] = 4 \times 10^{-6} \text{ M}$ in borate buffer $0.02 \text{ pH } 10.15$. Inset shows the variation of pK_a with DDAPS under the same conditions.

We have also employed a functional detergent derived from cysteine (HCys) as a model to study SH reactivity in micellar and vesicular systems. CTAB produced an effect on the pK_{ap} of HCys entirely analogous to that observed with HM (compare Figure 1 with Figure 4).

For a single dissociation step

the effect of CTAB on the pK_{ap} the SH group of HCys can be described by Equation (19)

$$\text{K}_{\text{ap}} = \frac{\text{K}_a (1 + \text{K}_{\text{HC}} \text{C}_D)}{\text{K}'_{\text{W}} \text{K}_a \text{K}_{\text{HC}} \text{C}_D ((\text{Br}_f)/(\text{Br}_b)_L)} \quad (19)$$

$$1 + \frac{\text{K}_b \text{K}_{\text{OH/Br}}}{\text{K}'_{\text{W}} \text{K}_a \text{K}_{\text{HC}} \text{C}_D ((\text{Br}_f)/(\text{Br}_b)_L)}$$

Figure 4. Effect of CTAB on the pK_{ap} of cysteinyl hexadecylamide [HCys] = 4×10^{-5} M. A. Borate buffer 0.01 M pH 8.5 (O) B. Tris.HBr 0.01 M pH 8.55 (O), Tris.Acetate 0.01 M pH 8.60 (●).

where K_b is the corresponding dissociation in the micelle, K_{HC} the distribution constant of the zwitterionic form of HCys between the aqueous phase and micellar pseudo phase and K'_w the ratio between the ionic product of water in the micellar pseudophase and in the aqueous phase, respectively. The value of K_{HC} was taken as $1 \times 10^4 \text{ M}^{-1}$, a value which is reasonable in view of the hydrophobicity of HCys. In fact, the pK_{ap} vs $[CTAB]$ function can be simulated by Equation (18) with any value of K_{HC} exceeding $1 \times 10^4 \text{ M}^{-1}$. pK_a for HCys was taken as 8.50². Equation (19) was fitted to the experimental data using K'_w/K_b as a adjustable parameter. In the case of borate and tris.HBr buffers the fit was reasonable when the exchange constants employed were those used previously ($K_{OH}/Br = 0.08$; $K_{OH}/Borate = 2.25$). The best fit value for K'_w/K_b was found to be 8.3×10^9 for borate and 9.1×10^9 for Tris.HBr. Study of the variation of pK_{ap} as a function of CTAB in tris.acetate furnished a value of 7.1×10^9 for the parameter K'_w/K_b . In the latter case, the hydroxide/acetate selectivity constant was used as a varying parameter in order to obtain the fit shown in Figure 4B. For this system $K_{OH}/Acetate$ was calculated as 0.1.

The addition of CTAB causes a remarkable increase in the rate of thiolysis of NPO by HCys. The reaction can be conveniently followed down to pH 4.0, the maximum acceleration being ca. 10^6 fold (Figure 5, Table III).

Figure 5. Effect of CTAB on the rate of thiolysis of NPO by HCys. $[HCys] = 4 \times 10^{-5}$, $[NPO] = 4 \times 10^{-6}$ M. Na acetate buffer 0.01 M pH 5.5 (O), 5.05 (●), 4.55 (□), 4.05 (■).

Table III. Effect of CTAB on the rate of thiolysis of NPO by hexadecylcysteinamide.

pH ^a	$k_y^{\max}/k_y^0$ ($\times 10^{-6}$) ^b	$k_2^{\max}/k_2^0$
4.05	1	13
4.55	0.39	17
5.05	0.14	20
5.50	0.12	23

a. In 0.01 M acetate buffer b. The value of k_2^0 was taken as $6.6 \text{ M}^{-1} \text{ s}^{-1}$,³⁰

The variation of k_y with $[\text{CTAB}]$ (Figure 5) for the reaction of HCys with NPO was analyzed using Equation (20),

$$k_y = \text{HCys} \frac{k_2^0 + (k_2^{\max}/\sqrt{V}) K_S K_{\text{HC}} C_D}{(1 + K_S C_D) \left[(1 + K_{\text{HC}} C_D) + \frac{K_f}{K_a} \left(1 + \frac{K_{\text{HC}} K' K_a C_D}{K_{\text{OH/Br}} K_b} \frac{(\text{Br}_f)}{(\text{Br}_b)_f} \right) \right]} \quad (20)$$

The values of the parameters were those obtained by the analysis of the variation of pK_{ap} with $[\text{CTAB}]$, except for $(k_2^{\max}/\sqrt{V})$ that was varied to fit Equation (20) with the data in Figure (5).

The effect of a vesicle prepared with a synthetic amphiphile (DODAC) on the same reaction was also investigated. The rate enhancement produced by DODAC vesicles is almost one order of magnitude larger than that observed in the CTAB, and is dependent upon the nature and concentration of ions present in the solution. (Figure 6, Table IV). The kinetic data were analyzed using Equation (20), and the following parameters $K_{\text{HC}} = 8 \times 10^4 \text{ M}^{-1}$, $K_S = 3 \times 10^4$, $\alpha = 0.15$, $(K'_a/K_b) = 7.1 \times 10^9$, $K_{\text{OH/Cl}} = 0.14$, and varying $(k_2^{\max}/\sqrt{V})$ to best fit. Taking the value of $\sqrt{V}$ as 0.37 LM^{-1} , the second order rate constant in the vesicle is larger than the value of k_2^0 by a factor of 60^{28} (Table IV). The maximum rate effects obtained are summarized in Table V. The most remarkable feature of these results lies in the range of the kinetic effect observed for a single reaction type. It is important to note that within this 10^6 fold rate difference, and over all conditions (surfactant type and concentration, thiol, pH, salt), the pseudophase ion exchange model accounts successfully for the effects of amphiphile aggregates on the rate of thiolysis. Although partial failures of the pseudo phase model have been reported,^{31a,b} under conditions in which the micelle coverage by the reacting nucleophile is small, the pseudophase model is widely applicable. The comparison between the maximum rate effects and the ratio of second order rate

Figure 6. Effect of dioctadecyldimethylammonium vesicles on the rate of thiolysis of NPO by HCys. $[HCys] = 4.0 \times 10^{-5}$, $[NPO] = 4 \times 10^{-6}$ M. A. HCl = 2.65 mM, pH 2.65 (O), 5 mM, pH 2.35 (●) B. Na Acetate buffer 0.005 M pH 4.50 (O), 3.75 (●).

Table IV. Effect of DODAC vesicles on the rate of thiolysis of NPO by HCys.

pH	k_t^{max}/k_t^0 ^c ($\times 10^{-6}$)	k_2^B/k_2^0
2.35 ^a	3.2	60.6
2.65 ^b	5.2	60.6
3.75 ^b	8.8	53
4.15 ^b	5.8	60.6

a. HCl b. Acetate buffer 0.005 M c. All k_t^{max} values were obtained at 1×10^{-5} M DODAC.

Table V. Maximum rate effects and calculated second order rate constants for the thiolysis of p-nitrophenol octanoate in the presence of amphiphile aggregates.

Thiol	Detergent (structure)	$k_y^{\max}/k_y^{\circ}$	$k_2^{\max}/k_2^{\circ}$
NH	BDS (micelle)	0.09	0.27
NH	DDAPS (micelle)	266	1.0
NH	CTAB (micelle)	1.6×10^3	1.2
HCys	CTAB (micelle)	1×10^6	15
HCys	DODAC (vesicle)	9×10^6	50

constants (Table V) indicates unequivocally that the main contributing factor for the rate enhancements is concentration of the reagents in the amphiphile pseudophase (s). The quantitative analysis of the effect of vesicles on the thiolysis (Tables IV and V, see also Ref. 15) suggests that in this case there may be a significant difference in the reactivity of the thiol between the aqueous and vesicular phases. It should be pointed out, however that there are major problems with the analysis of the data in the vesicular systems. Firstly it is not clear whether the reaction is taking place in the outer or inner surfaces of the vesicles³². Moreover, although vesicles do exchange ions¹⁵ absolute values for the extent of ion dissociations from the surface (s) are presently lacking. Thus the quantitative analysis must be considered tentative at this stage.

CONCLUSIONS

We have presented here the initial part of a systematic study of the effect of variations of the structure and charge of amphiphile aggregates on the thiolysis of esters. The main features of the results are: (i) the kinetic effects range from a tenfold inhibition by negatively charged micelles to 10^7 fold rate enhancement by positively charged vesicles, (ii) all these kinetic effects can be analyzed quantitatively using a pseudophase model with explicit consideration of ion exchange, and (iii) the

analysis demonstrates that the main factor determining the effect of the amphiphile aggregates on the thiolysis is reagent concentration in the dimensionally restricted environment of the micellar or vesicular pseudophase.

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 28. A reaction volume element within micelles or vesicles must be chosen to convert the calculated rate constants (k_2^0/V) to conventional units for comparison with rate constants for the same reaction in water. In this work the values of V for micellar DOAPS and vesicular DODAC were taken as 0.25^{24} and 0.32^8 LM⁻¹ respectively in order to facilitate comparisons with reactions in SDS and CTAB. The partial molar volume of aggregated DDAPS and DODAC were 0.31 and 0.81 LM⁻¹ respectively (Cuccovia, I.M., unpublished). The choice of a reaction volume element is in fact arbitrary and has been usually taken as either the partial molar volume of the aggregated detergent^{8,24} or the calculated volume of the Stern Layer¹. For a cogent analysis of the importance of the choice and significance of the reaction volume element in the analysis of bimolecular reactions in micelles the reader should consult the article by L.S. Romsted in these proceedings.
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Quantitative Determination of Alkylammonium Amphiphiles Using Neutral Detergents

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Orange G forms stable complexes with dioctadecyldimethylammonium and hexadecyltrimethylammonium ions which can be solubilized in aqueous solutions containing Brij 35. The changes in absorbance, due to complex formation in the presence of Brij 35, were used to develop a fast, reproducible, and sensitive method for the quantitative analysis of long-chain alkylammonium amphiphiles. © 1987 Academic Press, Inc.

INTRODUCTION

In view of the wide range of uses and applications of positively charged amphiphiles, their quantitative determination is relevant. Several of the methods (1) used for the analysis of these amphiphiles are based upon the formation of water-insoluble salts which can be weighed (1), titrated (2), or extracted with an organic solvent (3).

Solubilization of water-insoluble complexes in micelles is a convenient alternative to solvent extraction (4). In addition to cost, time, sensitivity, and hazard considerations, the use of micelles allows direct analysis of small samples of low concentration (5).

In this paper we describe a method for the determination of hexadecyltrimethylammonium bromide (CTAB) and dioctadecyldimethylammonium chloride (DODAC) based on the solubilization of a dye-amphiphile complex in solutions of the neutral detergent Brij 35. This method is applicable to amphiphiles that form micelles or vesicles.

MATERIALS AND METHODS

CTAB (Merck, Darmstadt, Germany, analytical grade) was recrystallized (3×) in ace-

tone/ethanol. DODAC (Herga, Ind. Quim. S.A., R.J., Brazil) was purified and analyzed as described previously (6). Orange G (QEEL, SP, Brazil) was recrystallized from water/methanol and Brij 35 (Calbiochem, research grade) was used as received. All other reagents were analytical grade and deionized, glass-bi-distilled water was used.

The concentrations of CTAB and DODAC in standard solutions were determined by titration of the halide counterion with (standard) $\text{Hg}(\text{NO}_3)_2$ (7). Enough ethanol was added to ensure a transparent solution during the titration. DODAC vesicles were obtained by sonication as previously described (8).

Analytical Procedure

The standard curve for DODAC (or CTAB) monomer was obtained as follows. A solution (2.0 ml) containing Orange G ($6.0 \times 10^{-5} M$), Brij 35 ($6.0 \times 10^{-5} M$), and NaCl (0.1 M) (Solution I) was temperature equilibrated (30°C) in the sample compartment of a Beckman-25 spectrophotometer and the initial absorbance (A_b) was recorded using NaCl (0.1 M) as reference. After each addition of standard amphiphile (5 to 50 μl of a $5 \times 10^{-3} M$ solution), in the same cuvette, the absorbances were registered (A_b). For samples containing

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FIG. 1. (A) Visible spectra of Orange G ($6.1 \times 10^{-5} M$) in an aqueous solution of NaCl (0.1 M) (—), Brij 35 ($6.1 \times 10^{-5} M$) and NaCl (0.1 M) (---), and in ethanol (· · ·). (B) Effect of CTAB on the spectrum of a solution of Orange G ($6.1 \times 10^{-5} M$). The solution contained NaCl (0.1 M) and Brij 35 ($6.1 \times 10^{-5} M$). CTAB concentrations ($M \times 10^5$) were a, 0; b, 2.2; c, 4.4; d, 6.7; e, 8.4; f, 13.3; g, 17.8; and h, 22.2.

DODAC vesicles it is convenient, after each addition, to heat the cuvette to 45°C for at least 1 min to ensure destruction of the vesicles. After temperature reequilibration A_b 's were recorded. All absorbances were corrected for dilution. The standard curves were obtained plotting $A_b - A_f(\Delta A_b)$ against CTAB or DODAC concentrations.

Samples (5 to 50 μl) containing unknown amounts of amphiphile were added to a cuvette containing 2.0 ml of Solution I (see above) and the ΔA_b 's, obtained as above, were compared with the standard curve to obtain the amphiphile concentrations.

RESULTS AND DISCUSSION

The visible spectrum of Orange G exhibits a broad absorption band with an absorptivity

(E_1) of $17,000 M^{-1} \text{cm}^{-1}$ at 480 nm in aqueous solution. The spectrum was altered in ethanol, the absorptivity decreasing between 430 and 500 nm (Fig. 1A). The addition of Brij 35 to an aqueous solution of Orange G did not alter the spectrum significantly (Fig. 1A). The solvent sensitivity of the spectrum and the high water solubility of the dye suggest that, under our conditions, Orange G was not solubilized by the Brij 35 micelles. While CTAB caused precipitation of Orange G from aqueous solution (not shown) the addition of the cationic detergent to a Brij 35 solution of the dye produced spectral changes which, at high CTAB, are comparable to the spectra of the free dye in ethanol. The spectral change depended on [CTAB] up to a certain detergent concentration (Fig. 1B). The absence of visible precipitate and the presence of spectral shifts are strong indications of the formation of a CTAB–Orange G complex solubilized in the pseudophase of the mixed Brij 35 aggregates.

The absorbance of Orange G–Brij solutions decreased with CTAB (or DODAC) reaching a plateau with increasing positive amphiphile concentration (Fig. 2). For both DODAC and CTAB the value of the observed absorbance depended on the total Brij added (Fig. 2). However, at a fixed Orange G and amphiphile concentration the increase in absorbance as a function of [Brij] was less pronounced for DODAC than for CTAB (insets Fig. 2).

These results can be analyzed qualitatively on the basis of the following minimal scheme for this system:

SCHEME I.

In this scheme D represents Orange G, A represents the (mono)positively charged detergent, and the equilibrium constants K_1 and

FIG. 2. Effect of amphiphile concentration $[A]$ on the absorbance of a solution of Orange G ($6.1 \times 10^{-5} M$). Brij 35 concentrations ($M \times 10^3$) were 5.5 (O) and 38 (●). (A) DODAC. (B) CTAB. Insets show the variation of Ab as a function of $[Brij\ 35]$ at $CTAB = 9.95 \times 10^{-3} M$ and $DODAC = 7.7 \times 10^{-3} M$.

K_3 correspond to the formation of a neutral complex between the dye and DODAC (or CTAB) in the aqueous phase and in the Brij aggregate, respectively. K_2 and K_4 represent the distribution constant of the positively charged detergent and the complex between the aqueous phase and the Brij aggregates, respectively. The binding of the dye to Brij was not considered for the reasons discussed above. We have not attempted to fit our experimental data to this model since it is clear that a mixed detergent micelle, formed from Brij and CTAB (or DODAC) will result in an exceedingly complicated system in which the adjustable parameters will be larger than the experimental data obtained in this work. However, it is reasonable to expect that for both CTAB and DODAC an increase in the concentration of Brij will produce a decrease in the concentration of free A. Thus the concentration of DA_2 and DA_2 . Brij will be determined by the relative values of both the formation constants and the distribution constants. The higher the value of K_1 relative to K_2 the less sensitivity the system will exhibit to dilution of the free positive amphiphile. This seems to be the case when one compares CTAB with DODAC (Fig.

2). For a more hydrophobic amphiphile (DODAC) it is expected that the association constant with the dye will be high (9) and thus the variation of the concentration of complex with Brij will be correspondingly lower than that in the case of CTAB.

Extrapolation of the linear parts of the absorbance/concentration functions defines an intercept which can be interpreted as an apparent stoichiometry for complex formation (Fig. 2). This result is expected on the basis of the scheme if the values for K_1 are high and the relative values of K_1 and K_3 are higher than K_2 . The calculated stoichiometry for DODAC/Orange G is 2.0 and varies little with Brij concentration. This value corresponds to the expected formation of a neutral complex between the positively charged amphiphile and the (di)negatively charged dye. As expected from the Brij dependence of the absorption the apparent stoichiometry for CTAB corresponds to 2.0 only at low $[Brij]$.

From the data described above it was clear that the decrease in absorbance due to complex formation in Brij could be used to determine the concentration of DODAC and CTAB under a specified range of conditions. If one assumes the formation of a major complex with a stoichiometry of 1:2 (dye:CTAB or DODAC) and that this complex is solubilized in mixed aggregates containing the neutral detergent Brij 35, the following set of equations describe the concentration dependence of the absorbance,

$$Ab_i = E_1 \cdot D_i, \quad [1]$$

where E_1 is the absorptivity coefficient of the free Orange G and D_i is its initial concentration. For any CTAB (or DODAC) concentration the absorbance will be given by

$$Ab_j = E_1 D_j + E_2 Y_j, \quad [2]$$

where E_2 is the extinction coefficient of the complex, Y_j and D_j are the concentrations of the complex and free dye respectively at CTAB (or DODAC) concentration A . Defining ΔAb as the absorbance difference between Ab_i and Ab_j at any A_j , from Eqs. [1] and [2] and the

FIG. 3. Effect of CTAB (A) and DODAC (B) on the absorbance of a solution of Orange G. Standard curves relating ΔA_b (see text) with CTAB (C) and DODAC (D). Concentrations of Brij 35 and NaCl were $6.1 \times 10^{-3} M$ and $0.1 M$, respectively. Orange G concentrations were ($M \times 10^5$): (○) 10.7, (●) 9.5, (◐) 7.9, (△) 5.9, (▲) 5.1, (□) 4.0, and (■) 2.8.

mass conservation equations for the system we can show that

$$\begin{aligned}\Delta A_b &= E_1 \cdot (D_1 - D_2) - E_2 \cdot Y_j \\ &= (E_1 - E_2) \cdot Y_j.\end{aligned}\quad [3]$$

Thus the difference in absorbance between the sample with no added CTAB (or DODAC) and that containing the positively charged detergent will be linearly related to the concentration of the complex and consequently with A .

In Figs. 3A and 3B we present data for the variation of absorbance as a function of total concentration for CTAB and DODAC, respectively, obtained at several concentrations of Orange G. The absorbance varied with concentration until reaching the expected stoichiometry (Figs. 3A and 3B). The concentration of the dye has to be higher than the concentration of the detergent for a sample with an unknown concentration. However, this condition is general for any method depending on the formation of a complex. The relationship between the total concentration of CTAB (or DODAC) and ΔA_b is presented in Figs. 3C and 3D for several concentrations of Orange G. As expected from Eq. [3] these functions were linear with a correlation coefficient of 0.995. Thus Beer's law for ΔA_b is obeyed

strictly. From Figs. 3C and 3D one can calculate the absorptivity coefficients of the complexes which, for the conditions shown, were $11,600$ and $12,050 M^{-1} cm^{-1}$ for CTAB and DODAC, respectively. From calibration curves such as those shown in Figs. 3C and 3D we have routinely determined the concentration of CTAB and DODAC as described under Materials and Methods. The precision of the determination was higher than that of other methods tested in our laboratory. The most useful range of concentrations determined with this method is 1×10^{-5} – $2 \times 10^{-4} M$ of CTAB or DODAC.

Amphiphile solutions may contain variable amounts of different salts and it is well established that the solubilization of a complex in a micelle may be affected by the medium (9–11). It was thus important to test the salt dependence of the absorbances measured in this system. For both DODAC and CTAB the absorbances of the complexes with Orange G in Brij 35 micelles are insensitive to salt concentration (Fig. 4). As could be anticipated (Scheme I) large organic cations (such as tet-

FIG. 4. Salt effect on the formation of the amphiphile/Orange G complex in Brij 35. The solutions contained $5.9 \times 10^{-5} M$ Orange G, $6.3 \times 10^{-3} M$ Brij 35, and the following salts (concentration): $CaCl_2$ ($0.39 M$) (●), NaCl ($0.097 M$) (○), tetramethylammonium bromide ($0.09 M$) (□), tetramethylammonium chloride ($0.097 M$) (▲), and $MgCl_2$ ($0.037 M$) (△). (A) DODAC. (B) CTAB.

ramethylammonium) capable of forming a complex with the dye cause absorbance changes, presumably due to competition with the dye for complex formation. This analytical method is applicable from pH 2.0 to 10.0. Above pH 10 the dye becomes further dissociated ($pK = 11.3$) and we decided not to extend the measurements to this region.

CONCLUSION

The formation of water-insoluble complexes between Orange G and positively charged amphiphiles in micellar solutions of neutral detergents can be used for the quantitative determination of the amphiphiles. The large amount of data concerning complex formation between dyes and amphiphiles (11) and the judicious selection of detergents for solubilization of the complex make this method a rapid and convenient alternative for the quantitative determination of any ionic amphiphile.

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periments allowed correlations between site-specific proton assignments³ and ¹³C chemical shifts to be made. These site-specific ¹³C shifts were then compared to the 1D chemical shifts, and the 1D chemical shifts were assigned accordingly. These assignments are marked with superscript italic c in Table I. All of the others are degenerate or within 0.1 ppm of the other, and are correctly assigned according to carbon class.¹⁹

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Supplementary Material Available: Figures S1 and S2 containing details of the same spectrum as Figure 1 etc., level 2.5 lower than Figure 1, and cross peaks A8(H8-C5), G3(H8-C5), and G5(H8-C5) and G5(H8-C4) and G3(H8-C4), respectively (2 pages). Ordering information is given on any current masthead page.

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Control of Reaction Rates in Vesicular Systems

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Vesicles prepared with synthetic amphiphiles catalyze numerous reactions with high efficiency.¹⁻⁵ Several studies have attempted to separate the kinetic and mechanistic effects of outer and inner surfaces of such vesicles on various chemical reactions.⁵ We here report that a negatively charged substrate (5,5'-dithiobis(2-nitrobenzoic acid)) (DTNB) can be selectively incorporated in the inner and/or outer surfaces of vesicles prepared with a positively charged amphiphile (dioctadecyldimethylammonium chloride) (DODAC). Selective incorporation permitted the demonstration of similar interfacial catalysis of the alkaline hydrolysis of DTNB³ by both surfaces. We also demonstrate here, for the first time, that the ratio of the rates of reaction at the inner and outer surfaces can be modulated by changing the composition of the medium.

DTNB (Sigma Chem. Co.) was incorporated in large unilamellar DODAC (Herga Ind. Quim. Brasil) vesicles by injecting a CHCl₃ solution of DODAC (20 mM) into an aqueous solution (70 °C)⁶ containing 5 mM NaCl, 90 mM erythritol, and 0.15 mM DTNB, pH 5.3. This preparation was annealed,⁷ and aliquots were filtered through two columns of Dowex 21K (Cl) (Serva Fine Chemicals)⁸ and eluted with 5.0 mM NaCl/90 mM erythritol. This procedure led to vesicles in which DTNB was adsorbed only

Figure 1. A. Chloride and bromide effects on the endo- and exovesicular hydrolysis of DTNB. [DODAC] = 5.3×10^{-3} M, [DTNB] (endovesicular, analytical) = 2×10^{-6} M. The arrow indicates the addition of external DTNB 2×10^{-6} M final concentration: (—) NaOH 1 mM, NaCl 4 mM, pH 10.9; (---) NaOH 1 mM, NaBr 4 mM, pH 10.8. B. pH effects on the rate of the endo- and exovesicular hydrolysis of DTNB: [DTNB] (endovesicular, analytical) = [DTNB] (exovesicular) = 2×10^{-6} M; [DODAC] = 4×10^{-3} M. NaCl was added to maintain the total anionic concentration at 5 mM. Full points indicate exovesicular, and open points refer to the endovesicular reactions, respectively: (●, ○), borate buffer, 5 mM; (■, □), triethylamine-HCl buffer, 5 mM at pH 9.7, 3.3 mM at pH 10.5, and 10 mM at pH 10.9; (▲, △), NaOH 1 mM. C. Effect of DODAC concentration on the rate of endo- and exovesicular hydrolysis of DTNB. [DTNB] (endovesicular, analytical) = [DTNB] (exovesicular) = 2×10^{-6} M. NaCl was added to maintain the total ionic concentration at 5 mM. Full points indicate exovesicular, and open points refer to endovesicular reactions, respectively: (●, ○) triethylamine-HCl buffer, 3.3 mM, pH 10.5; (■, □) NaOH 1 mM, pH 10.91.

to the internal surface.⁹ DTNB hydrolysis (30 °C) was followed (437 nm)³ in a Beckmann DU7 spectrophotometer.

The endovesicular reaction was started by adding aliquots of the (column-eluted) vesicle pool (0.6 mM DODAC¹⁰ and 0.03 mM DTNB) to a solution at the desired pH with osmolarity and ionic strength controlled with erythritol and NaCl, respectively. After the complete reaction 2 μ L of a 1 mM aqueous solution of DTNB was added to the same cuvette. The ensuing absorbance increase was attributed to reaction at the external vesicular surface since (a) the rate constant¹¹ for this exovesicular process was similar to one obtained previously³ and (b) DTNB does not penetrate the bilayer under our conditions.⁹ Figure 1A shows a typical endo- and exovesicular hydrolysis of DTNB. Using the same procedure, kinetic data were obtained under several conditions (Figure 1B, Table I). In the absence of buffer and at high pH, the exovesicular reaction was 100 times faster than the endo process (Figure 1 (parts B and C), Table I). However, with borate or triethylamine buffers, the endo- and exovesicular rate constants were of the same order of magnitude (Table I, Figure 1 (parts B and C)). The maximum rate enhancement in the presence of vesicles, with respect to the uncatalyzed reaction, was 200-fold (Table I). The (low) rate observed for endovesicular

(9) Twenty hours after vesicle preparation the rate constant for the endovesicular reaction was identical with that obtained immediately after annealing indicating that DTNB did not leak out of the vesicles.

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Table 1. Effect of Buffer Composition on the Endo- and Exovesicular Rates of Hydrolysis of DTNB^a

buffer composition ^a	pH	DODAC (M) × 10 ³	k_1^b (s ⁻¹) × 10 ³	k_2^b (s ⁻¹) × 10 ³	k_2/k_1
borate 5 mM, NaCl	9.2	5.3	2.0	5.6	2.8
2.5 mM					
borate 5 mM, NaBr	9.2	5.3	1.8	3.7	2.1
2.5 mM					
NaOH 1 mM, NaCl	10.9	5.3	2.0	180	90
4 mM					
NaOH 1 mM, NaBr	10.8	5.3	3.3	44	13.3
4 mM					
triethylamine-HCl	9.7	5.3	7.8	13	1.7
5 mM					
triethylamine-HBr	9.7	5.3	6.2	3.9	0.6
5 mM					
triethylamine-HCl	10.8	13	72	160	2.2
10 mM					
triethylamine-HBr	10.9	13	98	63	0.6
10 mM					

^a All buffer, vesicle, and salt solutions contained erythritol (90 mM). ^b Observed pseudo-first-order rate constants for the endovesicular (k_1) and exovesicular (k_2) reactions, respectively. ^c DTNB concentration was 2.0×10^{-6} M for both endo- and exovesicular reactions. The second-order rate constant for the alkaline hydrolysis of DTNB, calculated from ref 3, was $0.818 \text{ M}^{-1} \text{ s}^{-1}$ (30 °C).

hydrolysis at high pH in the absence of buffer may be attributed to rate-limiting ⁻OH permeation. Endovesicular rates limited by ⁻OH diffusion have been reported.^{3c} Buffers containing neutral species can accelerate the rate of ⁺H/⁻OH permeation across bilayers.^{5b,12} The similarity of endo and exovesicular rates in this system can be understood in terms of similar reactivities at the inner and outer surfaces and of faster permeation rates for ⁻OH with buffers.

Addition of bromide produced a decrease in the rate of the exovesicular reaction, not affecting the endovesicular process (Figure 1A, Table I). The ratio between the exo- and endovesicular rate constants (k_2/k_1) can even be reversed by the addition of external salts (Table I). The inhibitory effect at the outer surface was attributed to a ⁻OH/⁻Br exchange.¹³ The permeability of halide ions is (at least) 1000 times less than ⁺H/⁻OH for the same bilayer.¹⁴ Therefore a bromide inhibitory effect on the endovesicular reaction was not to be expected since the (externally) added ion did not penetrate during reaction.

The DODAC concentration-dependence of the exovesicular reaction was identical with that previously reported³ (Figure 1C). The endovesicular rate constant decreased slightly with [DODAC] irrespective of the buffer (Figure 1C). These results were rationalized by using the pseudophase ion-exchange formalism.¹³ Thus, an increase in [DODAC] leads exclusively to a displacement of the externally bound ⁻OH since the concentration of free ⁻Cl increases with [DODAC] only in the external aqueous compartment. Since the concentration of free ⁻Cl in the internal aqueous compartment does not change with [DODAC] the local concentration of (internally) bound ⁻OH was not a sharp function of [DODAC]. The small decrease of the exovesicular rate constant with [DODAC] can be attributed to the lower local concentration of externally bound ⁻OH and thus a lower ⁻OH penetration rate.

In conclusion, we have demonstrated that the effects of the internal and external surfaces of DODAC vesicles on the alkaline hydrolysis of DTNB are similar. Moreover these results show that the rate of the endo- and exovesicular reactions can be controlled by the rate of reagent permeation and also by the nature (and concentration) of the ions present in both inner and outer aqueous compartments. Since vesicles prepared with synthetic

amphiphiles are extremely efficient catalysts,¹⁻⁴ the control of both site and rate of reaction opens new perspectives in the understanding and use of these and other microreactors.

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Double Alkylation of α,β -Unsaturated Acetals. An Inverse Polarity Approach

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Regiochemistry of enolate formation-alkylation sequence (double alkylation) can often be controlled very effectively via Michael additions.¹ Noyori's prostaglandin synthesis immediately comes to mind.² Reported herein is an umpolung³ version of the process, and the general scheme is illustrated below.

We have found that an α,β -unsaturated acetal undergoes rapid metalation upon treatment with allylzinc in the presence of nickel catalyst. Allylzinc reagent, as a nucleophile, attacks at α carbon of the acetal 1, and then the resulting carbanion at β -position reacts with a variety of electrophiles to afford α,β -dialkylated acetal effectively, eq 1.

Copper-⁴ or nickel-⁵catalyzed reaction of the Grignard reagent with α,β -unsaturated acetals was reported to produce only the corresponding Michael-type addition (β -alkylation) products in moderate yields. In some cases, the more reactive allylic Grignard reagent reacts with nonactivated double bonds.⁶ Allylic zinc reagents, in contrast, are relatively unreactive toward alkenic bonds.^{6b,7}

Treatment of 1 equiv of α,β -unsaturated acetal with a solution of allylzinc bromide (3.5 equiv) in CH_2Cl_2 ⁸ under the influence

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ALKALINE HYDROLYSIS OF N-METHYL-4-CYANOPYRIDINIUM ION IN THE INTERNAL AQUEOUS COMPARTMENT OF LARGE VESICLES AND OH^-/H^+ PERMEATION ACROSS THE BILAYER

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RESUMEN

Cloruro de N-metil-4-cianopiridinio (MCP) fue atrapado en el compartimento acuoso interno de vesículas de bromuro de dioctadecildimetilamonio o de lecitina de huevo. A pH's mayores que 10,54 la velocidad de la hidrólisis alcalina del MCP intravesicular fue menor que la del MCP libre. La menor velocidad de reacción del MCP intravesicular es explicada en términos de la permeación del OH^- a través de la bicapa como etapa limitante.

SUMMARY

N-Methyl-4-cyanopyridinium (MCP) chloride was entrapped in the internal aqueous compartment of dioctadecyldimethylammonium bromide or egg lecithin vesicles. The rate of alkaline hydrolysis of intravesicular MCP was lower than that of free MCP at pH's higher than 10.54. Rate limiting OH^- permeation across the bilayer was responsible for the slower intravesicular reaction rate.

Vesicles of synthetic amphiphiles can increase reaction rates by factors reaching a million fold (1). The internal aqueous compartment, bilayer, internal and external interfaces constitute potential reaction sites (2). In view of the importance of these microreactors it is essential to understand the mechanism (s) leading to the rate enhancements and, in particular, the contribution of each reaction site to the observed effects.

The kinetics of vesicular effects on some chemical reactions has been analyzed quantitatively (3,4). In these studies it is likely that the reactions were taking place at the outer surface of the small sonicated vesicles (3,4) and that the major contribution to the rate enhancement was reagent concentration in the vesicular pseudophase (5).

Here we demonstrate that the reaction rate of OH^- with a positively charged substrate confined to the internal aqueous compartment of large unilamellar dioctadecyldimethylammonium bromide (DODAB) and egg lecithin vesicles is identical to that of the same reaction in the bulk water phase.

In order to probe exclusively the internal aqueous compartment of the positively charged vesicles prepared with DODAB, we selected a positively charged hydrophilic probe: N-methyl-4-cyanopyridinium (MCP). MCP does not associate with positively charged micelles over a large range of detergent

concentration (6a,b). MCP should therefore exhibit no tendency to associate with the inner interface of DODAB vesicles and thus be confined to the inner water compartment.

DODAB vesicles containing MCP were added to solutions of the desired pH (9) and the rate of MCP hydrolysis measured at 265 nm⁶ in a Beckman M-25 spectrophotometer (30.0°C). The absorbance data were analyzed as a first order process (11). At external pH = 10.2, the observed rate constant for MCP hydrolysis (k_v) in the aqueous phase was identical to that in the internal aqueous compartment (Fig. 1A). As the pH increased, there was an increasing divergence of the observed rate constants in water and DODAB vesicles (Fig. 1A). The data could be rationalized on the basis of: 1) a rate limiting OH⁻ permeation across the vesicle bilayer as the pH increased, 2) a change in OH⁻ reactivity and/or 3) a change in substrate distribution with increasing pH.

FIG. 1. Effect of external acidity on intravesicular pH and MCP hydrolysis. A. Effect of pH on the hydrolysis of free and DODAB-entrapped MCP. DODAB vesicles were prepared by chloroformic injection (7) in a solution containing 5 mM MCP and 0.095 M erythritol. Untrapped MCP was removed by Sephadex 6-25 filtration (8,9). MCP-containing vesicles were added to a cuvette at the desired pH (pH 10.20 borate buffer (5 mM) (9), all other NaOH (9)). Hydrolysis in water (○) and in the internal aqueous compartment of DODAB vesicles (●), respectively. (DODAB) = 3.2×10^{-5} M, (MCP) = 5×10^{-6} M. B. Effect of pH on the absorbance of intravesicular PAB. DODAB vesicles were prepared as above in 5 mM PAB and untrapped PAB eliminated by Sephadex filtration. An aliquot (50 μ l) of PAB-containing vesicle-suspension

was added to (1.5 ml) 0.095 M erythritol, pH 11.59 and the absorbance (340 nm) followed with time. Final concentration of DODAB and PAB were 1.9×10^{-5} and 1.5×10^{-6} M, respectively. After ten half lives HCl was added (arrow) to obtain pH 2.70. After 5 min NaOH was added to yield a final pH = 11.7. C. Effect of pH on the hydrolysis of free and lecithin-entrapped MCP. Large lecithin vesicles (pure lecithin from egg yolk (19) with 10 mol percent dicetyl phosphate) were prepared in solutions containing 5 mM MCP and 0.16 M KCl. Untrapped MCP separated by Sephadex 6-25 filtration. Vesicles containing MCP were added to buffer solutions to give 5.7×10^{-5} M lecithin and 5.3×10^{-6} M (analytical) MCP. MCP hydrolysis was measured as described above. In one set of experiments (three runs) the MCP-containing vesicle suspension was preincubated (15 min) with valinomycin (4×10^{-5} M) before sample addition to the NaOH solution (pH 11.4). In a separate experiment the MCP-containing vesicle suspension was preincubated for 90 min with valinomycin

and refiltered through Sephadex G-25. No free MCP was detected and an aliquot of the refiltered suspension was added for the kinetics. (o) no vesicles, (e) intravesicular hydrolysis in lecithin, (Δ) valinomycin-containing lecithin and (Δ) refiltered-valinomycin treated lecithin, respectively.

The rate of OH⁻ permeation in DODAB vesicles was estimated using a pH-sensitive probe: 2-hydroxy-5- (2-trimethyl-ammonium) acetyl benzoate (PAB). PAB (pK_a = 10.5) was entrapped to DODAB vesicles and a time-dependent absorbance (340 nm) change was observed after a change in the external pH (Fig. 1B). From these experiments we also demonstrated that, at equilibrium, the pH in the internal aqueous compartment was identical to that in the external phase (13). After reaching equilibrium, the pH in the external phase was lowered by addition of HCl (Fig. 1B). The rate of internal pH decrease, as estimated by the absorbance of PAB, was faster than our time resolution (14). However upon realkalinization, another pH change was detected (Fig. 1B). The reversibility of our system constitutes firm evidence that this particular vesicle system is not leaky. Since the pK of the probe does not change upon entrapment (13) it was possible to express the absorbance data for internal alkalinization as OH⁻ permeation rates using the Henderson Hasselbach equation. From the kinetic data ([OH⁻] versus time) (not shown) we calculated an apparent first order rate constant of $1.7 \times 10^{-3} \text{ s}^{-1}$ for the permeation of OH⁻ into DODAB vesicles. The value of this rate constant is within the range obtained for MCP hydrolysis in the internal aqueous compartment of DODAB vesicles at high pH (Fig. 1A). Thus permeation could indeed be rate limiting in the intravesicular hydrolysis of MCP.

Definitive proof for the identity of rate constants for MCP hydrolysis in water and in the internal aqueous compartment was obtained analyzing a system of large unilamellar vesicles prepared with egg lecithin containing MCP. Large egg lecithin vesicles were obtained by reversed phase evaporation (16) and MCP incorporated in the internal aqueous compartment. The pH profile for MCP hydrolysis in the internal compartment and in the aqueous phase is presented in Figure 1C. The profile is very similar to that obtained in DODAB vesicles. Valinomycin is known to accelerate OH⁻/H⁺ equilibration across lipid bilayers, probably by increasing the rate of permeation of K⁺ (17,18). When valinomycin was added to the lecithin vesicles containing MCP, substrate leakage was not detected by Sephadex G-25 filtration. The rate of MCP hydrolysis in the internal aqueous compartment of valinomycin-containing vesicles was identical to the rate in water (Figure 1C). Since valinomycin increases the rate of OH⁻/H⁺ equilibration without promoting substrate leakage these data demonstrate unequivocally that the difference in the intravesicular versus bulk rates observed were due to rate limited OH⁻ diffusion across the vesicular bilayer.

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9. For DODAB vesicles the total ionic strength was maintained at 5×10^{-3} M (with NaCl) throughout and 0.095 M erythritol was present in all buffers, column elutions or reactions in order to maintain constant osmolarity. Small changes in osmolarity can produce light scattering effects (10a) which can obscure absorbance changes. Vesicles were annealed (10b) at 50 °C for 1 hr and slowly cooled. This procedure was essential for reproducibility of the data.
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11. In fact the rate of alkaline hydrolysis of a substrate entrapped in the inner aqueous compartment of a vesicle is a complex result of, at least, two consecutive processes, i.e., the rate of OH⁻ permeation and the rate of reaction. A full kinetic analysis will be published elsewhere. For comparative purposes, however, it is reasonable to use the apparent first order rate constant obtained from the first two half lives of the reaction. Moreover, from kinetics in the presence of buffers, the neutral form (of the buffer) may contribute to increase the rate of approaching equilibrium.
12. PAB was furnished by Prof. D. Zanette, Department of Chemistry, Universidade Federal de Santa Catarina, Brasil.
13. The deprotonated form of PAB absorbs at 340 nm ($\epsilon = 19091 \text{ M}^{-1} \text{ cm}^{-1}$), the protonated form has negligible absorbance at this wavelength ($\epsilon = 140 \text{ M}^{-1} \text{ cm}^{-1}$). The absorbance of the solutions of vesicles containing PAB, at different external pH's and infinite time was taken as being due to the deprotonated probe. Plotting this absorbance vs. pH, a titration curve with a pK of 10.50 was obtained. This value is identical to the pK of PAB in bulk aqueous solution.
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This paper is dedicated to the memory of Professor Luis
Sepulveda, respected scientist, kind human being, dedicated
friend, collaborator. We will miss him.

Selectivity Coefficients for Iodide/Bromide and Iodide/Chloride Counterion Exchanges at the Surfaces of Dioctadecyldimethylammonium Vesicles

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Selectivity coefficients for the I^-/Cl^- and I^-/Br^- ion exchanges at the surfaces of dioctadecyldimethylammonium (large) vesicles were obtained from fluorescence-quenching experiments. The fluorescence of 1-pyrenenonanoic acid incorporated into dioctadecyldimethylammonium chloride and dioctadecyldimethylammonium bromide vesicles is quenched by iodide addition to the intervesicular media. Below the phase transition temperature of the bilayers, temporal changes in probe fluorescence intensity after iodide addition can be interpreted in terms of fast ion exchange at the outer surface, followed by a slow iodide permeation until a final state is achieved in which ion exchange takes place at both surfaces. From data obtained immediately after the iodide addition to DODAB vesicles, it is learned that $K(I^-/Br^-)$ at the outer surface is 8.0 ± 1.5 . From quenching data after total counterion equilibration, values of $K(I^-/Br^-) = 6.6 \pm 1$ and $K(I^-/Cl^-) = 31 \pm 5$ are obtained.

Introduction

Fluorescence-quenching techniques are particularly well suited for the study of ion exchange and permeation in vesicles.¹⁻⁵ In a previous work,¹ we have used these techniques to determine the selectivity coefficient for the bromide/chloride counterion exchange at the surfaces of dioctadecyldimethylammonium vesicles and have shown that bromide ions slowly permeate the vesicle, even below the phase transition temperature of the bilayer.^{1,6} In the previous work,¹ and due to the low efficiency of the bromide counterions to quench the fluorescence of the probes employed (naphthalene derivatives), the Br^-/Cl^- exchange was only evaluated at large bromide surface coverages. Similarly, the bromide permeation could only be monitored in situations where there was a significant difference in the counterion composition between the inner and the outer surfaces of the vesicle, conditions under which the bilayer cannot be considered a stable system. The easy permeation of the bromide ions under these conditions, in contrast with the behavior observed for other ions,⁷ could then be promoted by the stress generated in the membrane as a consequence of the asymmetrical counterion distribution.¹

In the present work, we have taken advantage of the high efficiency of iodide ions as quenchers of the fluorescence of pyrene derivatives. Using this system, we were able to evaluate I^-/Cl^- and I^-/Br^- exchanges in dioctadecyldimethylammonium (DODA) vesicles at very low quencher coverage of the vesicular surface(s). Selectivity coefficients can then be obtained for the ion exchanges taking place

either at the outer surface or at both surfaces of the vesicle. Moreover, iodide permeation across the bilayer under conditions of minimal difference in the counterion composition at both vesicle surfaces can also be revealed from the time dependence of the probe fluorescence intensity after addition of iodide to the intervesicular medium.

Experimental Section

Dioctadecyldimethylammonium chloride (DODAC) (Herga Industria Quimica Brasil) was purified as previously described.⁸ Dioctadecyldimethylammonium bromide (DODAB) was purified by repeated recrystallizations from acetone. Sodium chloride (Merck), sodium bromide (Baker), and sodium iodide (Fluka) were analytical grade. 1-Pyrenenonanoic acid (PN) (Molecular Probes) was employed as a fluorescent probe. All solutions were prepared in deionized doubly distilled water.

Vesicles (large) were prepared from DODAC or DODAB following the injection with simultaneous vaporization of the solvent (chloroform) method.⁹ The fluorescent probe was added to the chloroformic solution of the surfactants prior to its injection. Probe to surfactant mole ratios employed were below 10^{-4} . All experiments were performed in the presence of added common salt. The working solutions were prepared by injection of the chloroformic solution of the surfactant containing the fluorescent probe into either sodium chloride (for DODAC) or sodium bromide (for DODAB) aqueous solutions at a given concentration (typically 2 and 5 mM concentrations of the common salt added were used). The surfactant concentrations employed were variable below 5 mM. Fresh (prepared daily) solutions were used. Iodide was introduced to the already prepared vesicle solutions by addition of small aliquots of a concentrated stock solution (prepared at the same ionic strength as that corresponding to the vesicle solution to be employed). Addition of ca. 0.1% of sodium thiosulfate to the iodide solution did not significantly modify the results obtained.

Fluorescence intensities were measured on a Perkin Elmer LS5 luminescence spectrometer (the samples were excited at 337 nm and the emission registered at 380 nm; under the conditions employed, no significant light from scattering was ob-

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Figure 1. Time dependence of the relative fluorescence intensity of vesicle-incorporated PN after addition of iodide to a solution of DODAC (1 mM) in NaCl 5 mM. Iodide added: 0.066 mM (●) and 0.17 mM (▲).

Figure 2. Effect of iodide addition on the fluorescence intensity of PN from a solution of DODAB (2 mM) at [NaBr] = 2 mM. (▲) Measured at 18 °C immediately after (ca. 15 s of elapsed time) quencher addition. Each iodide concentration was achieved by iodide addition to a fresh, iodide-free vesicular solution. (●) Measured after a heating (47 °C)–cooling (18 °C) cycle.

served). Lifetime measurements were performed by employing a Nitromite LN 100 nitrogen laser as a light source coupled to a Tektronix 7633 oscilloscope. Experiments were carried out at room temperature (18 °C) in air-equilibrated solutions.

Results and Discussion

The fluorescence of the probe, both in DODAC and DODAB vesicles, is efficiently quenched by addition of iodide at very low concentrations (10^{-6} – 10^{-4} M range), either at temperatures below (18 °C) or above (47 °C) the phase transition temperature (T_c) of the bilayer.^{6,9} Addition of iodide above the T_c produces a quenching of the fluorescence that remains invariant with the time elapsed after the quencher addition. In contrast, the effect observed at 18 °C increases with time (Figure 1). This change is faster if the samples, after iodide addition at 18 °C, are heated at 47 °C. Heating at this temperature for ca. 5 min considerably decreases the fluorescence intensity (registered after cooling the sample at 18 °C). The fluorescence intensity measured after this treatment is not modified by further heating (47 °C)–cooling (18 °C) cycles. This

Figure 3. Fluorescence intensity data measured immediately after iodide addition plotted according to eq 1. (●) PN in a 1 mM DODAB solution at [NaBr] = 2 mM. (▲) PN in a 0.52 mM DODAB solution at [NaBr] = 2 mM.

result is indicative of a fast iodide diffusion across the bilayer in the liquid crystalline (fluid) state. If the value obtained for the fluorescence intensity after the heating (47 °C)–cooling (18 °C) cycle is considered as that corresponding to the equilibrium distribution of the iodide between the inner and outer surfaces of the vesicles (time infinite), the data in Figure 1 indicate that half of the fluorescence decrease occurs in ca. 20 min. This value, although related to the rate of iodide ion permeation through the bilayer, cannot be taken as a direct measure of the rate of the process since the fluorescence intensity is not a straightforward measure of the distribution of iodide ions in the vesicle (see below).

The effect of iodide addition on the pyrene moiety fluorescence intensity is shown in Figure 2. These data show that, while the values of I^0/I (I^0 , emission intensity in the absence of iodide; I , emission intensity in the presence of iodide) measured immediately after the iodide addition show a noticeable downward curvature at low I^0/I values, those values measured after the heating–cooling cycle are almost linear. The downward curvature can then be attributed to selective quenching of the probes located at the outer layer of the vesicle. The quenching behavior under these conditions can be described by

$$I^0/(I^0 - I) = (f_e)^{-1} + ((f_e)K'_{SV}[I])^{-1} \quad (1)$$

where f_e is the fraction of the light emitted from the probe at the outer surface and K'_{SV} is the Stern–Volmer constant (defined in terms of the analytical concentration of iodide). Analyzing the effect of added iodide ion on the fluorescence of vesicle-incorporated PN at 18 °C immediately after the quencher addition yields a good linear correlation (Figure 3). The extrapolated f_e is compatible with half of the emission arising from the probe at the outer surface. This result is in accord with what can be expected¹ for a large vesicle and demonstrates that the iodide ions, under these conditions, do not penetrate the bilayer.

At fixed salt concentration, the quenching of PN fluorescence by iodide decreases when the surfactant concentration increases (Figure 4). Furthermore, and particularly at low surfactant concentrations, the iodide-quenching efficiency slightly decreases when the concentration of the added common salt increases (data not shown). These effects are consistent with ion competition (exchange) between I^- and the surfactant counterions at the vesicular surfaces.¹⁰

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Figure 4. Effect of surfactant concentration on the fluorescence quenching of vesicle incorporated PN by iodide. Data shown correspond to measurements taken after the heating-cooling cycle in the presence of added NaBr at 2 mM. DODAB concentrations: 0.24 mM (a); 0.52 mM (b); 1 mM (c); 2 mM (d).

Figure 5. Experimental data (from Figure 4) plotted according to eq 3. $[I^-]_T$ values taken for F/F_0 equal to 1.3 (●) and 1.5 (▲). Iodide concentration given in mM.

Since the free surfactant concentration and the dissociation degree^{10,12} of vesicles are extremely low, and the amounts of iodide added are much smaller than the concentration of added common salt, these systems are particularly suitable to obtain counterion exchange constants following the procedure originally proposed by Encinas and Lissi¹² for neutral molecules and adapted by Abuin and Lissi¹³ for counterions in micelles. For a family of quenching curves obtained at a fixed concentration of added common salt but different surfactant concentrations (such as those shown in Figure 4), a given value of F/F_0 corresponds to equal fractional coverage of the vesicle surfaces by iodide, θ_I (and hence equal fractional coverage by the vesicle's counterion, θ_X) for all the surfactant concentrations.

Since under the present conditions $[X^-]_{free} \approx [\text{added common salt}]$ and taking into account that

$$K(I^-/X^-) = \frac{\theta_I [X^-]_{free}}{\theta_X [I^-]_{free}} \quad (2)$$

the free iodide concentration can also be considered as constant. Hence, the analytical concentration of $[I^-]_T$

Table I. Summary of Experimental Results

$[X^-]$, mM	vesicle surface	F/F_0	intercept, ^a mM	slope ^b	$K(I^-/X^-)^b$
bromide	both	2	1.3	0.007	7.5
			1.5	0.013	6.0
		5	1.3	0.014	6.5
			1.5	0.025	6.6
	outer	2	1.1	0.006	8.0
		5	1.1	0.017	6.3
chloride	both		1.2	0.017	9.4
			1.2	0.040	8.0
		2	1.8	0.002	28
		5		0.004	35

^a Values of F/F_0 at which the set of data producing the results given in columns four and five was taken. ^b Values given are averages of two or three independent determinations.

necessary to produce a given F/F_0 value must be simply related to the total surfactant concentration by

$$[I^-]_T = [I^-]_{free} + \theta_I [\text{surfactant}] \quad (3)$$

If the total iodide concentration needed to reach a given value of F/F_0 is plotted against the surfactant concentration, a straight line must be obtained with the free iodide concentration as intercept and, as slope, the amount of iodide bound to the vesicles per mole of surfactant (Figure 5). The values obtained are summarized in Table I. These values show that, for a given F/F_0 , the iodide bound per surfactant is almost independent of the added salt, while the free iodide significantly increases when the concentration of added common salt increases. These results are those expected if the pseudophase counterion exchange formalism applies, to the outer surface or to both surfaces of the vesicle.

Furthermore, since

$$K(I^-/X^-) = \frac{[I^-]_b [X^-]_{free}}{[X^-]_b [I^-]_{free}} \quad (4)$$

where b stands for bound, and

$$[X^-]_b = \beta [\text{DODAX}] - [I^-]_b \quad (5)$$

where $\beta = 0.5$ if only the external surface is considered or $\beta = 1$ if the exchange takes place at both surfaces, the exchange constant can be expressed

$$K(I^-/X^-) = \frac{\text{slope} [\text{added salt}]}{(\beta - \text{slope}) \text{intercept}} \quad (6)$$

where slope and intercept are evaluated by plotting the data according to eq 3. The values of $K(I^-/X^-)$ so obtained are collected in Table I. These data give, for the exchange constants involving both surfaces, $K(I^-/Br^-) = 6.6 \pm 1$ and $K(I^-/Cl^-) = 31 \pm 5$, while a value of 8.5 ± 1.5 is obtained for the I^-/Br^- exchange constant when only the outer surface is considered. If we accept the counterion exchange formalism,

$$K(Br^-/Cl^-) = K(I^-/Cl^-)/K(I^-/Br^-) = 4.7$$

This value is in very good agreement with that (4 ± 1) reported previously by Lissi et al.¹ Furthermore, for the I^-/Br^- exchange in cetyltrimethylammonium micelles, a value of $K(I^-/Br^-) = 13 \pm 3$ has been reported.¹⁰ This value, although larger than that found in the present work, is also compatible with a considerably higher affinity of iodide both in the micelle and the vesicles, a result expected do to the larger polarizability of this anion.

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Figure 6. Plot of the experimental data according to eqs 7 and 8. (●): According to eq 7 ($\alpha = 1$). (▲): According to eq 8 ($\alpha = 0.5$).

The data collected in Table I allow an evaluation of the Stern-Volmer constant, K_{SV} , in terms of θ_I . If a linear Stern-Volmer behavior is assumed, the data obtained after equilibration can be represented by

$$I^0/I = 1 + K_{SV}\theta_I \quad (7)$$

while the values obtained when the exchange takes place only at the outer surface must follow

$$\frac{0.5(I^0/I)}{1 - 0.5(I^0/I)} = 1 + (K_{SV})_{outer}\theta_I \quad (8)$$

The data, plotted according to these equations, are shown in Figure 6. This figure shows that $(K_{SV})_{outer}$ appears to be considerably smaller than K_{SV} . The probe lifetime can be taken as 75 ± 3 ns at both surfaces, as indicated by the linearity of the emission decay in the absence of iodide. Furthermore, addition of iodide (0.033 mM) to a 2 mM DODAB solution leads (at short times after the quencher addition) to a decay with a long component of 72 ± 3 ns, which can be assigned to the probe emitting from the inner side of the bilayer. From these data, the quenching rate

constants k_Q can be derived. The values of k_Q obtained were 0.11×10^9 and 0.2×10^9 s $^{-1}$ when the iodides are located only at the outer surface and equally distributed at both vesicle sides, respectively, a result that could indicate a closer approach of the iodides to the probe when the vesicle is totally equilibrated (i.e., with equal counterion composition at both sides). This could result from a different probe location and/or a closer approach of the counterions under these last conditions. Similar conclusions were obtained in a study of the fluorescence quenching of naphthalene derivatives by bromide in the same vesicle system.¹

The data obtained for DODAC lead to $K_{SV} = 28.6$ (in terms of θ_I). The probe lifetime in DODAC solutions is 135 ± 5 ns, leading to $k_Q = 0.21 \times 10^9$ s $^{-1}$. This value is very close to that obtained in DODAB vesicles for the quenching taking place in the equilibrated state, suggesting that the accessibility of the probe to iodide counterions under these conditions is very similar in DODAC and DODAB vesicles.

The change in the probe fluorescence intensity with time after addition of iodide at 18 °C to the intervesicular medium (see Figure 1) implies that iodide ions permeate through the bilayer, even below its T_c . This change could then be used to calculate the rate of iodide permeation through the vesicle bilayer. Nevertheless, the different efficiencies observed for the quenching of the probe fluorescence by iodide, when located at the outer surface or equally distributed at both surfaces, preclude, with the present data, a quantitative relation between the effect observed and the kinetics of iodide crossing through the bilayer.

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Registry No. DODAC, 107-64-2; DODAB, 3700-67-2; PN, 72165-42-5; I $^-$, 20461-54-5; Cl $^-$, 16887-00-6; Br $^-$, 24959-67-9.

Size, Electrophoretic Mobility, and Ion Dissociation of Vesicles Prepared with Synthetic Amphiphiles

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Vesicles prepared with synthetic amphiphiles (dioctadecyldimethylammonium bromide and chloride, dihexadecyl phosphate and its sodium salt) were obtained by sonication, ethanol injections, and chloroform injections. The hydrodynamic diameter of vesicles (D_h), estimated from the diffusivity measured by quasielastic light scattering, ranged from 230 to 3000 Å. The electrophoretic mobility (U_m) was measured by free-flow electrophoresis. The zeta potential (ζ) and the degree of counterion dissociation (α) of the vesicles were calculated from U_m and conductivity data. α decreased with increasing D_h of the vesicles, probably due to the decreasing headgroup area and the increasing counterion association needed to relax the surface electrostatic potential. The electrophoretic mobility was also calculated (U_c) according to an impenetrable, nonconducting sphere model with a spherically symmetric charge distribution approximation. Within the limits of the experimental error(s) of the (different) methods employed and the assumptions made in the calculations, the fact that the U_m/U_c ratio ranged from 1.3 to 7.5 was considered to be a good agreement between the calculated and the experimental values.

Introduction

Vesicles prepared with both natural and synthetic amphiphiles are convenient systems for the study of structural and functional properties of bilayers as well as carriers of drugs and genetic information into cells.¹ Particularly, synthetic amphiphile vesicles are extremely efficient catalysts for both ground- and excited-state reactions.¹ The rate of vesicle-modified reactions can be controlled by substrate compartmentalization and changes in the composition of the reaction medium.^{2,3} The size of vesicles can be varied from 250 to 5000 Å in diameter by use of different preparation methods.⁴ Several features, such as solubilization and permeability, are strictly related to vesicle size. Not only the association constants but also the relative positions of bound substrates vary with vesicle size.⁵ These data led us to investigate the degree of counterion dissociation (α) as a function of vesicle size. This parameter is essential for the quantitative analysis of kinetic data using models such as the pseudophase ion exchange.⁶ Moreover, the determination of the parameters needed for the calculation of α of synthetic amphiphile vesicles of different sizes (e.g., hydrodynamic radius, electrophoretic mobility, zeta potential) may be of relevance for the understanding of the (several) differences between lipid and synthetic amphiphile bilayers.

Here we have used free-flow electrophoresis to determine, for the first time, the degree of ion dissociation from vesicles as a function of their size (measured by quasielastic light scattering). The experimental values of electrophoretic mobility of vesicles were lower than those calculated according to theory,⁷ and this discrepancy was analyzed. For both positively and negatively charged amphiphiles the degree of ion dissociation decreased with increasing size of the vesicle.

Materials and Methods

Dioctadecyldimethylammonium chloride (DODAC) (Herga Industrias Químicas, Rio de Janeiro, Brazil) and dioctadecyldimethylammonium bromide (DODAB) (Eastman Kodak) were purified by recrystallization.⁸ Dihexadecyl phosphate (DHPH) (Sigma Chemical Co.) was used as received. The sodium salt of DHPH (DHPNa) was prepared by neutralization of a hexane solution of DHPH with NaOMe in MeOH. After solvent stripping the solid was recrystallized from ethanol:acetone. 1-

[(2-methylphenyl)azo]-2-naphthol (Orange OT) was obtained from *o*-toluidine and 2-naphthol (Aldrich) according to standard procedures.⁹ All other reagents were of analytical grade. Deionized, twice-distilled water was used throughout.

Vesicle preparation. (1) Sonication: Enough amphiphile to give a final concentration of ca. 10 mM was weighed in a sonication tube, water (or aqueous NaCl) was added, and the tube was maintained for 10 min above the phase transition temperature (T_c) of the (corresponding) bilayer. The mixture was then sonicated at the T_c for 1 h (DODAC) or 1.5 h (DODAB) in a bath sonicator (Branson do Brazil, São Paulo, Brazil, MSX-10). Sonication was also performed with a tip sonifier (Braunsonic 1510) for 20 min (above the T_c), and mixtures were later centrifuged at 10000g, for 30 min. (2) Chloroform injection: Large DODAC and DODAB vesicles were prepared by injection of chloroform solutions of DODAX ($X = Cl^-$ or Br^-) into water or aqueous salt as previously described.¹⁰ (3) Ethanol injection: 1.0 mL of an ethanolic solution of DODAC (0.2 M) was slowly injected into 19.0 mL of either water or aqueous NaCl maintained at 50 °C.

Conductivity was determined (30 °C) in a digital apparatus (Digimed, São Paulo, Brazil) with a cell constant of 1.00. Absorbance was measured in a Beckman M25 spectrophotometer at 495 nm. To minimize light scattering errors, vesicles were destroyed before absorbance measurements by addition of detergent and incubation at 60 °C for 5 min. For DODAC or DODAB we used hexadecyltrimethylammonium bromide (CTAB), and for DHPH or DHPNa we used sodium dodecyl sulfate (SDS). DODAB and DODAC concentrations were measured by halide titration,¹¹ and DHPH was determined, after

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TABLE I

methods and conditions ^a	D_p , Å	$(dA/dr)/(r \times 10^3)$	Q
DODAC			
EtOH injection ^b			
no salt (0.92)	230	4.47	0.29
no salt (8.0)	230	4.47	0.35
8 mM NaCl (9.0)	250	2.83	0.23
sonication			
no salt (5.0)	330 (484 ^c)	3.32	0.21
no salt (1.0)	330	3.32	0.22
0.93 mM NaCl (5.0)		2.90	0.20
2.42 mM NaCl (5.0)		2.35	0.17
4.76 mM NaCl (5.0)	380	1.73	0.13
9.27 mM NaCl (5.0)	400	1.40	0.11
CHCl ₃ injection			
no salt (0.3)	2850 (5000, ^d 3000 ^e)	0.61	0.044
4.4 mM NaCl (0.3)	3500		
9.0 mM NaCl (0.3)	3500		
DODAB			
sonication			
no salt (5.0)	650	1.64	0.13
CHCl ₃ injection (0.3)	2750	0.58	0.043
DHPNa			
sonication (5.0)	673 (508 ^f)	2.50	0.22
DHPH			
sonication (4.0)	1162 (500, ^g 900 ^h)	2.92	0.068
CTAB			
NaBr 0.025 M, 30 °C	61 (64.4 ⁱ)	2.23	0.203
SDS			
25 °C	51 ^j	2.52	0.26
30 °C		2.69	0.24

^a Amphiphile concentrations (mM) in parentheses. ^b 5% (v/v) EtOH. ^c Reference 28. ^d Reference 10. ^e Reference 19. ^f Reference 29. ^g Reference 30. ^h Reference 31. ⁱ Reference 32.

hydrolysis, by measuring inorganic phosphate.¹²

Electrophoretic mobility (U_m) was determined according to the procedure described by Hoyer et al.¹³ as modified by Sepulveda and Cortes.¹⁴ The agar (2%) bridge contained 0.2 M NaCl. Labelling was done by adding 20 μ L of an acetonitrile solution of Orange OT (0.025 M) to vesicle preparations to yield 5×10^{-5} M final dye concentration. Current was measured with a Keithley 197 multimeter. The applied voltage (300 V) was maintained with a Shandon SAE 2761 source. The silver electrodes (tapes $1 \times 10 \times 0.1$ cm, generously supplied by Degussa S. A. Brasil) were immersed in NaCl (0.2 M). During electrophoresis the temperature was maintained at 30.0 ± 0.02 °C with a thermostating-circulating pump (Quimis Brasil) in a 50 L water bath. U_m was calculated from the equation¹³

$$U_m = \frac{\lambda(A_i - A_f)c}{\kappa A_p} \quad (1)$$

where A_i and A_f are the initial and final absorbances, respectively, of the Orange OT labeled vesicle solution contained in a capillary of volume (v) = 3.71 cm³; t is the time (in seconds) during which the current (i , amperes) was measured; λ is the conductivity of the sample (mho per centimeter).

Dynamic light scattering measurements were made at 25 °C in an apparatus equipped with a Coherent Innova 90-S argon ion laser operating at 488 nm and a Thorn EMI 9863Q-35 phototube with an APED-II amplifier for single photon detection, connected to a Brookhaven PI-2030 digital correlator. Vesicle solutions were passed through nylon filters before quasielastic light scattering (QELS); for sonicated and ethanolic vesicles the filter cutoff was 2200 Å and for chloroformic vesicles it was 8000 Å.

Results and Discussion

Results are presented in Tables I and II. Vesicles of various sizes have been prepared with lipids (for a review see ref 4), and some of the methods used for lipids have been adapted for synthetic amphiphiles.^{6,10} In particular, dialkyldimethylammonium (DODA) amphiphiles form aggregates of different architecture (e.g., micelles or vesicles) depending not only on the preparation method but also on the alkyl chain length and counterion type.¹⁵ Size determinations using QELS depend on several assumptions, including corrections for particle-particle interactions.¹⁶ The reported hydrodynamic diameters (D_h) were estimated from the diffusivity measured by QELS using the Stokes-Einstein relation.¹⁷ We note that for all reported D_h 's the polydispersity index, Q ,¹⁸ ranged from 0.1 to 0.2. In the case of micelles, extrapolation to the critical micelle concentration (cmc) generally affords consistent sizes from QELS data.¹⁶ The apparent sizes of sonicated vesicles varied little with concentration up to 10 mM amphiphile (not shown). Hence, for the purpose of this work, we elected to report all D_h 's at a single amphiphile concentration (Table I). The micellar D_h of CTAB was extrapolated to the cmc and agrees with published data.¹⁶

Ethanol injection (DODAC) produced aggregates with an D_h of 230 Å with or without added salt. Sonicated DODAC vesicles exhibited a D_h of 520 Å when prepared in water. Sonication of DODAC in salt ($(5-10) \times 10^{-3}$ M) produced smaller vesicles (D_h = 380–400 Å). For vesicles obtained by chloroform injection the presence of salt in the preparation medium led to an increase in D_h from 2850 Å (no salt) to 3500 Å ($(4-9) \times 10^{-3}$ M NaCl). Comparison of the values obtained here for D_h with published data (presented in Table I) show good agreement for sonicated vesicles. In the case of large (chloroformic) vesicles the present values are smaller than those obtained by electron microscopy¹⁹ but similar to those obtained by QELS.¹⁶ Since these vesicles are salt-sensitive,²⁰ it is probable that the difference in the sizes reflects the effect of the staining salt used for contrast in electron microscopy. A limited study of the size of DHPH vesicles was also undertaken (Table I). It should be noted that the size of DHPH vesicles was extremely sensitive to the nature of the counterion. Sonicated DHPH vesicles exhibited a D_h of 1162 Å while for DHPNa D_h was 673 Å. This difference is probably related to the formation of intrabilayer hydrogen bonds²¹ in the DHPH bilayer which could prevent the formation of small vesicles.

Measured mobilities are sensitive functions of both amphiphile and salt concentrations.²¹ Without added salt the values of U_m of large vesicles were 30–50% lower than those of small (sonicated) vesicles irrespective of the nature of the counterion. The mobility of positively charged vesicles was similar to those of DHPNa and DHPH vesicles and to micelles (Table II). In spite of the remarkable size difference between the amphiphilic structures, ranging from 40 to 5000 Å in diameter, the maximum difference in U_m was ca. 2-fold. The data in Table I suggest that there is no relationship between particle size and electrophoretic mobility. This lack of relationship could be expected for particles in which the size increases were accompanied by a decrease in ion dissociation, e.g., a decrease in effective surface potential (vide infra).

From the electrophoretic mobility (U_m) we estimated the streaming potential (ξ) of the particles using a simple Smoluchowski model. The ξ value calculated by using the (uncorrected) Smoluchowski expression is known to yield a minimum estimate of the streaming potential.²² There are serious doubts that ξ values can be calculated from U_m data even using more rigorous data-

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TABLE II

methods and conditions ^a	$U_m (\times 10^3)$, cm ² /(V s)	$U_c (\times 10^3)$, cm ² /(V s)	U_c/U_m	U_c/U_m^b	ξ , mV
DODAC					
EtOH injection ^d					
no salt (0.92)	8.2	4.3	5.2	2.7	96.4
no salt (8.04)	4.5	3.4	7.5	6.6	52.9
5 mM NaCl (9.42)	4.0	1.4	3.4	3.0	47.0
sonication					
no salt (1.0)	7.2				85.0
no salt (5.0)	7.8	4.1	5.3	3.3	91.5
0.93 mM NaCl (5.0)	6.2				72.6
2.42 mM NaCl (5.0)	5.9				70.1
4.76 mM NaCl (5.0)	4.9	0.92	1.3	1.1	57.9
9.27 mM NaCl (5.0)	4.6	0.86	1.9	1.6	54.1
CHCl ₃ injection					
no salt (5.0)	5.4	2.8	5.2	4.2	63.6
DODAB					
sonication					
no salt (5.0)	6.7	3.0	4.5	3.2	78.4
CHCl ₃ injection					
no salt (5.0)	5.1	2.4	4.5	3.8	60.4
DHPNa					
sonication (4–10)	6.6	4.6	5.8		77.9 (77–127) ^e
DHPH					
sonication (4.0)	8.3	2.9	3.6	1.8	97.3
CTAB					
25 mM NaBr (5.0)	5.0 (2.45/ 4.75) ^f	0.94	1.9	1.6	58.9
SDS					
25 °C (16)	4.8 (4.5) ^g	0.8	1.7	1.4	56.2
30 °C (16)	5.6	0.8	1.5	1.1	66.2

^a Amphiphile concentrations (mM) in parentheses. ^b Corrected U_c values; see text. ^c Calculated from U_m ; see text. ^d 5% v/v EtOH. ^e Reference 33. ^f Reference 14a. ^g Reference 14b. ^h Reference 21.

analysis models. Nevertheless, the calculations were considered to be illustrative for comparative purposes. ξ was calculated by using the expression²²

$$\xi = \frac{\eta U_m}{\epsilon_r \epsilon_0} \quad (2)$$

where η is the water viscosity (7.975×10^{-3} dyn s/cm²), ϵ_0 the permittivity of free space (8.85717×10^{-7} dyn/V²), and ϵ_r the dielectric constant of water (76.55) at 30 °C. The calculated ξ values (Table II) ranged from 50 to 90 mV, well within the range published for similar supramolecular aggregates of this type at low salt concentration.²²

From U_m and conductance data we calculated the relative counterion dissociation of the supramolecular aggregate (α)²³ using the relationship

$$\alpha = \frac{10^3 d\lambda/dc}{F(U_m + U_s)f} \quad (3)$$

where the Faraday constant (F) is equal to 96 500, U_s is the mobility of the counterion, and $d\lambda/dc$ is the slope of the conductance vs total amphiphile concentration plot. For micelles $d\lambda/dc$ was taken above the cmc.¹⁴ In the case of vesicles $d\lambda/dc$ was corrected for the fraction of amphiphiles residing in the external surface (f). The dissociation of ions from the internal surface should not contribute to the measured conductance.²⁴ Hence f is 1.0 for micelles and was taken as 0.7, 0.6, and 0.5 for vesicle diameters up to 250 Å, from 250 to 700 Å, and greater than 700 Å, respectively.¹⁰ The conductances of solutions of large vesicles were significantly lower than those containing smaller structures. In some cases a deviation from linearity in the λ vs

c plots was observed. The values of $d\lambda/dc$ are presented in Table I. Vesicles of DHPNa and DHPH exhibited similar $d\lambda/dc$. However, since the specific conductance of H⁺ is larger than that of Na⁺, the calculated value of α for DHPH was much lower than that of DHPNa. Function-dependent changes due to replacement of H⁺ in DHPH monolayers have been described for intra- and interlayer forces.²⁰

As previously observed for micelles,¹⁴ the degree of counterion dissociation (α) from small DODAX vesicles was larger with Cl⁻ ion than with Br⁻ ion. The difference in α , however, is negligible when Cl⁻ ion and Br⁻ ion are compared in large DODAX vesicles (Table I). Ion dissociation from the large vesicles was very small, and the detection of minor differences in α (between DODAC and DODAB) in large vesicles would be outside the sensitivity of the present method. The salt dependence of α , especially in the small DODAC vesicles, suggests that ion dissociation in vesicles is affected by ionic strength more strongly than in micelles. It is attractive to speculate that the effective size of the ion pair at the surface¹⁵ affects both ion dissociation and vesicle size. The size dependence of α is probably partially due to the difference in headgroup packing in the bilayer as a function of changing radius of curvature. Abuin and Lissi⁵ have observed marked changes in the partition coefficients of neutral substrates as a function of vesicle size and interpreted their data in terms of a lesser monomer packing in the small vesicles. Lesser packing should reduce the electrostatic repulsion at the surface and permit a larger extent of ion dissociation as observed. The values of α presented here were utilized to analyze quantitatively vesicle size dependent catalytic effects on chemical reactions and resulted in excellent fits.²⁵ The large differences in α , varying by as much as a factor of 8 (Table I), cannot be wholly explained by changes in headgroup area. The latter is not expected to exceed a factor of about 2 in going from a vesicle diameter of 220 Å to 2850 Å.²⁶

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The surface potential (ψ) of the vesicle should be roughly proportional to

$$\frac{\alpha R}{(1 + \kappa R)}$$

where κ is the Debye-Hückel inverse screening length and R is taken as half of D_h . At very low salt concentration the interaction energy between an ion and the surface would increase with increasing vesicle size to levels well above kT unless some counterions condense back to the headgroups; i.e., α must decrease with increasing vesicle size in order to maintain the thermal distribution of bound and free counterions.

Vesicles, because they can be prepared with a number of different monomers in varied sizes and charge distributions, are interesting systems to test theories for particle movement as well as particle-particle interactions in solution. Although a completely satisfactory model for the relationship between electrophoretic mobility, polyion parameters, and solution parameters does not exist, the impermeable sphere model should be a better approximation for vesicles and micelles than for polymeric polyelectrolytes. In the approximation of an impenetrable, nonconducting sphere with a spherically symmetric charge distribution, the leading term for the expression of electrophoretic mobility is given by⁷

$$U_c = z\epsilon X(kR) / [(6\pi\eta R)(1 + kR)] \quad (4)$$

Here k is the Debye-Hückel parameter, R is the radius of the sphere, and $X(kR)$ is a sigmoidal function which varies from 1.0 to 1.5 over the range of kR from zero to infinity. The hydrodynamic radii were used for R in the above equation.

In order to estimate the mobility according to eq 4, it was necessary to make approximations for the values of z and k in the cases of no added electrolyte. Two assumptions were used to estimate z : First, the headgroup area per monomer in all the vesicles was taken as 75 Å². Literature values for the surface areas of monomers in bilayers range from 40 to 100 Å² for amphiphiles as different as DODAC to (a number of) naturally occurring lipids.²⁷ Thus, while headgroup area is expected to decrease with increasing radius, the (constant) value chosen should not affect z by more than a factor of 2 in any of the cases. Second, the value of α was used to compute the net charge via

$$z = \alpha(\pi D_h^2) / 75 \quad (5)$$

where D_h is measured in angstroms. This equation implicitly assumes that only the dissociated headgroups on the external surface contribute to the vesicles' net charge and mobility.

In the cases of added NaCl the usual prescription for the inverse Debye-Hückel screening length was used:

$$k = (I)^{1/2} / (3.04) \quad (6)$$

where I is the ionic strength in terms of moles and k has the units of inverse angstroms.

In the case where no added salt was present, the value of k was estimated by assuming the ionic strength to be due to the concentration of (external) unassociated counterion, e.g., to $\alpha/2$ [amphiphile]/2. No attempt is made to justify this procedure since in the absence of added salt the applicability of the assumptions of Debye-Hückel theory is uncertain. It is important to note that by this estimation even low effective free counterion concentrations, on the order of 0.1 mM lead to ionic atmosphere radii on the order of the vesicles' radii and hence to appreciable values for kR in eq 4.

The shortest interparticle distance (800 Å) was calculated for ethanol-injected vesicles (8 mM DODAC, Table I), and even in this case, the screening length (100 Å) was lower than the intervesicle distance. As the size of the particle increases, at the amphiphile concentrations used, the interparticle distances increase. Thus, for the large DODAC vesicles (0.3 mM DODAC, Table I) the intervesicle distance was ca. 1.36×10^4 Å and the screening length was 409 Å. It is therefore clear that the assumption of no interparticle repulsions, in the calculation of α , was adequate.

Table II shows the values for the calculated mobilities (U_c) and the ratio U_c/U_m . The latter values range from 1.3 to 7.5. Even this level of agreement is surprising given the number of approximations. In the case of all vesicles the U_c values are larger than U_m values. Booth⁷ carried out the expansion in terms of zeta potential to third order for the electrophoretic mobility of impenetrable, uniformly charged, nonconducting spheres in symmetric electrolytes and found that for potentials above about 40 mV the mobility begins to deviate downward from the linear relation of eq 4. For a potential of 100 mV the linear fit overestimates the mobility by a factor of 2. In light of this correction, all of the U_c values in Table II can be revised downward. Table II shows the values of U_c/U_m with U_c corrected to third order using Booth's graphical summary.⁷ Since Booth's theory also only applies in the case of added symmetric electrolyte, it is most interesting to see the effect of the correction on the case of 5 mM salt for sonicated DODAC in the Table II, using the associated estimate for the zeta potential. The revised value was within 10% of the measured value (Table II). The good agreement between measured and calculated values for the micelles is probably due to the fact that the error inherent in the approximation of k has a relatively small effect on the value of kR , since the micellar radius is quite small. The larger deviations for the ethanolic vesicle preparations may have to do with differences in dielectric constant around the vesicles due to the (unknown) distribution of the ethanol between the bulk and vesicular pseudophases.

Given the good agreement between experiment and theory in the case of the sonicated DODAC vesicles and added NaCl, it would be interesting to conduct more extensive tests on vesicles or liposomes that are stable at high ionic strengths.

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Evidence That the Effects of Synthetic Amphiphilic Vesicles on Reaction Rates Depend on Vesicle Size

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Small and large vesicles were prepared with dioctadecyldimethylammonium chloride and bromide. The hydrodynamic radius of the small vesicles ranges from 11 to 30 nm, while that of the large vesicles is ca. 140 nm. Both small and large vesicles increased the rate of alkaline hydrolysis and thiolysis (heptyl mercaptan, HM) of *p*-nitrophenyl octanoate (NPO). The rate/amphiphile patterns were different and the small vesicles were 2–5-fold more effective as catalysts. From the quantitative analysis of the kinetic results, using a pseudophase model with ion exchange, we concluded that the size dependence is due to differences in ion dissociation, substrate binding constants, and intrinsic reactivities. The effect of vesicle size on substrate distribution was confirmed by measuring binding constants with a fluorescence quenching method. These data show that the rate differences arise mainly from a variation in the capacity of the bilayer to solubilize substrates and dissociate counterions. This work also shows that even small differences in bilayer packing, with no changes in medium or aggregate composition, can modulate the rate of supramolecular-modified chemical reactions.

Introduction

Amphiphile aggregates such as micelles, vesicles, microemulsions, and liquid-crystalline phases modify the rates and/or the mechanisms of a variety of reactions.¹ The aggregate structure, as well as the composition of the reaction medium, can lead to modulation of reaction rates by factors reaching several million fold.^{1–3} Subtle differences in aggregate structure can sometimes produce significant effects. Thus, the photochemical isomerization of merocyanine changes abruptly when (liquid crystalline) potassium laurate passes from cylindric (N_c) to disklike (N_d) or

micellar (L) phases.⁴ Similar effects were observed for the bromination of *trans*-stilbene in the N_c to N_d transition in sodium decyl sulfate/water/1-decanol.⁵ The same reaction exhibits structure-dependent changes in sodium dodecyl sulfate (SDS) micelles, dihexadecyl phosphate and dipalmitoylphosphatidylcholine vesicles.⁶ Stereoselectivity, in the peptide-catalyzed

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TABLE I: Alkaline Hydrolysis of *p*-Nitrophenyl Octanoate^a

amphiphile (method, pH)	α^b	$D_h, \text{\AA}$	$10^{-3}K_{\text{app}}^d$ M^{-1}	$k_{\text{app}}^{\text{max}}/k_{\text{app}}^{\text{min}}$ ([C], mM)	$k_{\text{app}}/k_{\text{app}}^{\text{min}}$ ^c
DODAC (EtOH, 9.47)	0.23	220	5.0	61 (0.50)	0.32
DODAC (sonic, 9.47)	0.21	520	5.2	39 (0.40)	0.20
DODAC (CHCl ₃ , 9.41)	0.044	2850	1.7	33 (1.00)	0.17
DODAB (sonic, 9.30)	0.13	650	18.0	42 (0.20)	0.15
DODAB (CHCl ₃ , 9.20)	0.043	2750	1.2	28 (1.3)	0.21

^a Borate buffer (0.01 M), parameters used (see text) were $K_{\text{OH}/\text{NPO}} = 0.08$, $K_{\text{OH}/\text{Cl}} = 0.16$, $K_{\text{OH}/\text{NPO}} = 2.25$, $k_{\text{app}}^{\text{max}} = 9.69 \text{ M}^{-1} \text{ s}^{-1}$, $P = 0.58$ (DODAC), 0.62 (DODAB) (L/mol). ^b Degree of ion dissociation. ^c Hydrodynamic diameter. ^d NPO-vesicle binding constant (see text). ^e Ratio of the maximum observed rate constant/rate constant in the absence of amphiphile at the same pH. ^f Ratio of vesicular/aqueous-phase second-order rate constants.

hydrolysis of a hydrophobic ester, is dramatically affected by the size of aggregates composed of hexadecyltrimethylammonium and ditetradecyldimethylammonium bromide.⁷ Differences in the local polarity of the reaction sites in the aggregates are a possible source of the structure-dependent rate modulation.⁴ Substrate distribution, localization, orientation, and local order can also be determining factors for the rate and reaction mechanism differences observed in the distinct aggregates.¹⁻⁷

The structure-function relationships involved in rate (or mechanism) control by the variation of the structure of the aggregate are far from clear. The physical properties of bilayers depend on headgroup packing, which in turn is a function of the aggregate diameter.⁸ Controlled variation of the vesicle diameter allows structural changes of the bilayer without changes in the molecular structure of the monomer and/or medium composition.

Here we studied the effect of vesicle size on the alkaline hydrolysis and thiolysis of *p*-nitrophenyl octanoate (Scheme 1).

SCHEME 1

Vesicles of dioctadecyldimethylammonium chloride (DODAC) and bromide (DODAB), of diameters ranging from 200 to 3000 Å, were obtained by using different preparation methods,⁹ and their effects on the rate of both reactions were determined. The kinetic data were analyzed quantitatively by using the pseudophase model with ion exchange (PPIE).^{2,10,11} The rate changes were due mainly to size-dependent differences in the substrate-vesicle binding constants, variations in surface ion dissociation, and differences in intrinsic reactivity.

Materials and Methods

p-Nitrophenyl octanoate (NPO, kindly provided by Prof. O. A. El Scoud from this Institute) and *n*-heptyl mercaptan (HM, Aldrich) were used without purification. Stock solutions of NPO and HM were prepared in CH₃CN. Dioctadecyldimethylammonium chloride (DODAC, Herga Ind. Chim., Brazil) was purified as described.¹¹ Dioctadecyldimethylammonium bromide (DODAB, Eastman Kodak) was recrystallized from methanol/acetone (1:3 v/v).

Spectra were obtained with a Beckman spectrophotometer (M-25 or DU-7) at $30.0 \pm 0.1^\circ\text{C}$. HM concentration was determined by titration.¹² The concentrations of DODAC and DODAB were determined by monomer titration.¹³ Monomer concentration of the chloroform-injected vesicles cannot be directly obtained by using our previously described procedure¹³ (applicable

to sonicated and ethanol-injected vesicles) since chloroform-injected vesicles were not totally destroyed by addition of Brij-35 even upon heating. We found that dilution of these vesicles with ethanol (50%, v/v) before the addition to orange-G gave excellent results. Probably ethanol addition led to destruction of the bilayer, solubilization of the DODAC (or DODAB) monomers and thus quantitative complexation with the dye. The rate of hydrolysis or thiolysis of NPO was followed at 405 (pH > 7) or 330 (pH < 7) nm, respectively. Apparent first-order rate constants (k_{app}), obtained from plots that were linear for (at least) 4 half-lives, are the average of three different experiments with deviations no greater than 5%.

Sonicated vesicles were prepared by treating a 0.01 M suspension of the amphiphile for 1 h at 50°C in a Branson (MSX-10) bath sonicator. The preparation of ethanol-¹ and chloroform-injected¹⁴ vesicles has been described. Vesicles were prepared in the presence of the buffers indicated in the Results section.

The apparent $\text{p}K_{\text{app}}$ ($\text{p}K_{\text{app}} = \log(1/K_{\text{app}})$) of HM in the presence of vesicles was measured by determining the absorbance of the mercaptide ion at 240 nm by using the following relationship:¹¹

$$\text{p}K_{\text{app}} = \text{pH} - \log \frac{A_{\text{v}} - A_{\text{HM}}}{A_{\text{M}} - A_{\text{v}}} \quad (\text{I})$$

A_{v} is the absorbance of the HM solution measured at a pH in the vicinity of the $\text{p}K_{\text{a}}$ (between 9 and 10), A_{HM} the absorbance of the protonated HM (determined in 0.005 M HCl, pH 2.3), and A_{M} the absorbance of the mercaptide ion (determined at pH 12). The values of A_{M} and A_{HM} did not change upon addition of vesicles. $\text{p}K_{\text{app}}$ was determined at a single pH, and the absorbance measured as a function of added vesicles at constant [HM].

The kinetic results were fitted to the PPIE model (see Results) by using one or two adjustable parameters. The input data consisted of $k_{\text{app}}/[\text{amphiphile}]$ data pairs, and the program allowed, after introduction of the proper equations and fixed parameter values, a multiparametric fit of the data to the corresponding equations. A PC-XT-compatible computer was used for all calculations.

The binding constants of NPO and HM to DODAC vesicles were determined using a fluorescence quenching method previously described.¹⁵ Pyrene (Aldrich) was purified by recrystallization from ethanol and incorporated after vesicle preparation. All spectra and emission intensities were registered in a Perkin-Elmer LS5 luminescence spectrometer at 27°C . Samples were excited at 337 nm, and emissions measured at 400 nm. The pyrene/DODAC mole ratio was maintained below 10^{-4} . All experiments were done in air-equilibrated solutions. Small aliquots of stock solutions of NPO and HM in CH₃CN were added to previously prepared vesicles. The total added volume of CH₃CN never exceeded 1% (v/v) of the volume of the aqueous solution.

Results

The effects of sonicated and ethanol-injected DODAC vesicles on the rate of alkaline hydrolysis of NPO were similar: a sharp

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Figure 1. Effect of vesicles on the rate constant for alkaline hydrolysis of *p*-nitrophenyl octanoate. Borate buffer 0.01 M; [NPO] = 1×10^{-3} M. (A) DODAC (pH = 9.45): (O) ethanolic; (●) sonicated; (▲) large vesicles. (B) DODAB: (O) sonicated (pH = 9.3); (●) large vesicles (pH = 9.2).

increase of k_2 followed by a plateau and a decrease at higher amphiphile concentrations (Figure 1A). This pattern is comparable to several observations of the effect of sonicated vesicles and micelles on bimolecular reactions.¹⁻³ The effect of chloroformic vesicles (large vesicles, Table I) was similar, but the maximum observed rate constant (k_2^{max}) was lower and the rate decrease less pronounced (Figure 1A). The ratio between k_2^{max} and the k_2 obtained in the absence of added amphiphile at the same pH (k_2^0) for large vesicles was approximately half that obtained with sonicated vesicles (Table I). The differences between the effects of sonicated (small, Table I) and large vesicles on the alkaline hydrolysis of NPO was independent of the nature of the counterion, a very similar phenomena being observed for DODAB (Figure 1B, Table I).

The results shown in Figure 1 were analyzed by using eq 2,¹⁶

$$k_2 = \frac{[\text{OH}^-](k_{2m}/V)K_s K_{\text{OH}/V}(Y_b/Y_f) + k_2^0}{1 + K_s C} \quad (2)$$

where k_{2m} and k_2^0 ($9.69 \text{ M}^{-1} \text{ s}^{-1}$)¹⁶ are the second-order rate constants in the vesicle and in the aqueous phase, respectively. K_s and $K_{\text{OH}/V}$ are the NPO-vesicle binding constant and the ion-exchange constant ($\text{OH}^-/\text{halide}$), respectively. $[\text{OH}^-]$ is the hydroxide ion concentration in the intervesicular aqueous phase, C the analytical concentration of amphiphile, and V the partial molar volume of the amphiphile. Y_b and Y_f the analytical concentrations of bound and free vesicular counterions (Cl⁻ ion in the case of DODAC and Br⁻ ion with DODAB), were calculated by using eq 3 and 4, where α is the degree of ion dissociation of

$$Y_b = (1 - \alpha)C - [\text{OH}^-] - [\text{B}_2] \quad (3)$$

$$Y_f = \alpha C + [\text{OH}^-] + [\text{B}_2] \quad (4)$$

the vesicle and C the total concentration of the amphiphile (expressed as moles/liter of monomer).³ $[\text{B}_2]$ and $[\text{OH}^-]$ are the concentrations of the anionic form of the buffer and OH^- ion

TABLE II: Binding Constants of *p*-Nitrophenyl Octanoate (K_s) and *p*-Nitrophenyl Octanoate (K_2) to DODAC Vesicles^a

vesicle prep method	K_s	$10^{-3} K_2, \text{M}^{-1}$	
		K_2	K_2^0
CHCl ₃ injection	0.5 ± 0.02	1.0 ± 0.1	2.8 ± 0.4
sonicated	1.2 ± 0.1	4.0 ± 0.2	3.9 ± 0.2
ethanol injection	2.8 ± 0.2	3.9 ± 0.2	3.3 ± 0.4

^a Vesicles prepared in 0.002 M HCl; values are the averages of at least three independent experiments $\pm$ the standard deviation. Experiments were done with 0.5–3 mM DODAC.^{12b} ^b NPO-vesicle binding constants measured in the presence of HM maintaining a constant value of 0.03 for the mole ratios of incorporated HM/DODAC.

bound to the vesicle, respectively.¹⁶ The values of $K_{\text{OH}/V}$, $K_{\text{OH}/V}$, and $K_{\text{OH}/V}$ used to calculate $[\text{OH}^-]$ and $[\text{B}_2]$ were 0.08, 0.16, and 2.25, respectively.^{16,17} The values of α were those determined previously¹⁷ and are presented in Table I. We have assumed that α is identical in the external and internal surfaces. V was calculated by multiplying the partial specific volume determined for lecithin (0.9848 mL/g)¹⁸ by the molecular weight of the amphiphiles.¹⁹ The values of V were 0.58 and 0.62 L/mol for DODAC or DODAB, respectively. The computer fit (Materials and Methods) of the data presented in Figure 1 gave the values of K_s and k_{2m} presented in Table I. The (best fit) value for K_s of NPO in sonicated vesicles was significantly higher than that observed for the large vesicles of the same amphiphile. The k_{2m}/k_2^0 ratio, a measure of the effect of the vesicles on intrinsic reactivity, was not particularly sensitive to vesicle size (Table I).

In addition to the assumptions underlying the PPIE model, the use of this formalism in vesicular systems implies the use of a single k_{2m} (corresponding to intra- and extraventricular reaction)¹⁹ and fast equilibration of NPO between the reaction sites. The assumption of a single rate constant is reasonable because we have shown previously that OH^- reactivity in the external and internal interfaces of large vesicles is comparable.¹⁹ The rate of transmembrane equilibration of relatively low molecular weight hydrophobic compounds (such as NPO and HM) is fast compared with the time scale of the reactions described here.²⁰ Evidence for fast inner-outer equilibration was obtained demonstrating that the quenching of vesicle-incorporated pyrene by NPO was the same before and after a heating (50 °C)–cooling (27 °C) cycle (not shown). Thus, NPO was rapidly and uniformly distributed in this time scale when added externally, even below the phase transition temperature of the DODAC bilayer.^{14,21}

The distributions of NPO and HM between vesicles and bulk solution were measured by using a method based on the analysis of the changes in fluorescence intensity of a vesicle-incorporated probe upon substrate addition.¹⁵ The values of the NPO and HM binding constants (K_s) to DODAC vesicles, as well as the effect of HM incorporation in the vesicles on the K_s of NPO, obtained in 0.002 M HCl, are given in Table II. The values of K_s of NPO obtained by fluorescence are very similar to those calculated from the analysis of kinetic data (compare Tables I and II). As observed previously for the incorporation of alcohols,¹⁹ HM and NPO partitioned more effectively into smaller vesicles. Incorporation of HM, particularly in large vesicles, increased the K_s of NPO (Table II).

The association of HM to the vesicles was also evaluated determining the effect of the amphiphile on the apparent pK_a ($pK_{a, \text{app}}$) of HM. The data shown in Figure 2 were obtained at different pH's to facilitate the spectrophotometric measurements. $pK_{a, \text{app}}$ decreased to a minimum upon vesicle addition (Figure 2). In all these experiments the absorbance at 240 nm (see methods) was

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TABLE III: Thiolysis of *p*-Nitrophenyl Octanoate by *n*-Heptyl Mercaptan^a

amphiphile (method, pH)	$10^{-3}K_{HM}^b$ M ⁻¹	$K_{M/Y}^c$	$10^{-3}K_1^d$ M ⁻¹	k_4^{max}/k_4^0 ([C], mM)	k_{2m}/k_2^0
DODAC ^e (EtOH, 5.35)	3.3	127	12.5	3.4×10^6 (0.11)	28
DODAC ^e (sonic, 5.35)	2.0	102	7.0	3.6×10^6 (0.16)	38
DODAC ^e (CHCl ₃ , 7.17)	0.3	5.1	2.5	2.7×10^5 (0.48)	68
DODAB ^f (sonic, 6.46)	2.5	18.1	30 (18)	7.4×10^5 (0.10)	20 (19)
DODAB ^f (CHCl ₃ , 6.55)	0.6	5.3	10 (1.2)	1.3×10^5 (0.20)	11 (15)

^a $K_{OH/B} = 0.08$, $K_{OH/sonic} = 1.14$, $k_2^0 = 30 \text{ M}^{-1} \text{ s}^{-1}$, $P = 0.58$ (DODAC), 0.62 (DODAB) (L/mol). ^b Best-fit parameter (see text). ^c Mercaptide-halide ion-exchange constant. ^d Na acetate, 5 mM. ^e Tris-acetate, 6 mM. ^f Piperazine-acetate, 6 mM. ^g Alternative values of K_1 and k_{2m}/k_2^0 used in the simulation of the data presented in Figure 3B.

Figure 2. Effect of vesicles on the apparent pK_a of heptyl mercaptan. [HM] = $(5-8) \times 10^{-3} \text{ M}$. (A) DODAC: (O) ethanolic (Tris-acetate buffer, 0.05 M, pH = 9.02); (●) sonicated (Tris-acetate buffer, 0.05 M, pH = 9.2); (Δ) large vesicles (borate buffer, 0.01 M, pH = 10.14). (B) DODAB: (O) sonicated (borate buffer, 0.01 M, pH = 9.18); (●) large vesicles (borate buffer, 0.06 M, pH = 9.86).

measured by using a reference containing the same concentration of vesicles. With large vesicles the increase in light scattering at this wavelength prevented the use of higher amphiphile concentrations. Quantitative analysis of the variation of pK_{ap} (Materials and Methods) with amphiphile concentration was done with using eq 5,^{10,16} where K_a is the acid dissociation constant of

$$K_{ap} = K_a \frac{1 + K_{M/Y}(Y_b/Y_l)}{1 + K_{HM}C} \quad (5)$$

HM in the aqueous phase ($pK_a = 10.75$),^{2,22} K_{HM} the association constant of the protonated form of HM, and $K_{M/Y}$ the ion-exchange constant for the mercaptide/halide exchange. The calculated (best fit, see Materials and Methods) values of K_{HM} and $K_{M/Y}$ are presented in Table III. Both the $K_{M/Y}$'s and the K_{HM} 's obtained in small vesicles were significantly higher than those obtained in large vesicles both for DODAC and DODAB. The best fit values of K_{HM} were very similar with those obtained by fluorescence (compare Tables II and III).

Vesicles of DODAC and DODAB markedly increased the rate of thiolysis of NPO by HM (Figure 3). The ratio between k_4^{max} and the calculated rate constant for uncatalyzed thiolysis (k_4^0) (k_4^{max}/k_4^0) reached 4×10^6 -fold (Table III). The k_4^{max}/k_4^0 ratio

Figure 3. Effect of vesicles on the thiolysis of *p*-nitrophenyl octanoate by heptyl mercaptan. [NPO] = $5 \times 10^{-6} \text{ M}$. (A) DODAC: (O) ethanolic (Tris-acetate buffer, 0.05 M, pH = 5.35, [HM] = $5 \times 10^{-3} \text{ M}$); (●) sonicated (Na⁺ acetate buffer, 0.05 M, pH = 5.35, [HM] = $5 \times 10^{-3} \text{ M}$); (---) large vesicles, pH = 5.35, calculated (see text). Inset shows large vesicles (Tris-acetate buffer, 0.006 M, pH = 7.17, [HM] = $8.2 \times 10^{-3} \text{ M}$). (B) DODAB: (O) sonicated (piperazine-acetate buffer, 0.006 M, pH = 6.46, [HM] = $8.18 \times 10^{-3} \text{ M}$); (●) large vesicles (piperazine-acetate buffer, 0.0066 M, pH = 6.57, [HM] = $8.4 \times 10^{-3} \text{ M}$). Lines were calculated (see text) by using the following values of K_1 : sonicated $3.0 \times 10^6 \text{ M}^{-1}$ (a) and $1.8 \times 10^6 \text{ M}^{-1}$ (b); large vesicles $1.0 \times 10^6 \text{ M}^{-1}$ (c) and $1.2 \times 10^5 \text{ M}^{-1}$ (d).

was larger for small vesicles with both DODAC and DODAB (Table III). A significant difference in k_4^{max} between sonicated and large vesicles is evident upon inspection of Figure 3. With sonicated DODAC vesicles the reaction rate at pH 5.35 was significantly higher than that of HM oxidation (not shown). At the same pH, however, the rate of thiolysis in the presence of large vesicles was smaller and the rate of HM oxidation becomes significant (not shown). Thus, thiolysis with large DODAC vesicles was studied at pH 7.17 (Figure 3, inset). For comparative purposes we have included a calculation of thiolysis at pH 5.23 for large vesicles using the parameters obtained at pH 7.17 (Figure 3). Quantitative analysis of thiolysis was done using eq 6,^{2,10} where

$$k_4 = [HM_T]K_s \left[\frac{(k_{2m}/P)(K_s K_{M/Y})(Y_b/Y_l) + k_2^0}{(1 + K_{HM}C)([H_l] + K_{ap}(1 + K_s C))} \right] \quad (6)$$

[HM_T] is the total concentration of HM, k_2^0 the second-order rate constant for thiolysis in aqueous solution ($k_2^0 = 30 \text{ M}^{-1} \text{ s}^{-1}$),^{2,23}

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k_{2m} the second-order rate constant in the vesicle, and $[H_1]$ the proton concentration in the aqueous solution (calculated from the measured pH). The values used for K_{HM} and $K_{M/V}$ were those calculated from the analysis of pK_{ap} variation (see above). Curve fitting with the values of K_1 previously calculated from the hydrolysis data (see above) was not possible, and K_1 was used as an adjustable parameter, together with k_{2m} . The best fit value of K_1 for DODAC was 2 times larger than that obtained from the hydrolysis data (compare Tables I and III). For DODAB we show the effect of K_1 variation upon the shape of the k_2 vs concentration profile (Figure 3B). The most discriminating region in our fitting procedure is that at low amphiphile concentration, where a large variation of k_2 with [amphiphile] occurs and the molar ratio of HM/amphiphile was nearly 7 (Figure 3B). The differences between the values of K_1 of NPO calculated in the thiolysis and hydrolysis reactions were clearly due to the effect of HM incorporated in the vesicle on the distribution constant of NPO, as demonstrated in Table II. The values of k_{2m}/k_2° are only slightly different, varying from 10 to 70 with different vesicle sizes and counterions (Table III). In all cases, the calculated reactivities in the vesicles were higher than those in aqueous solution.

Discussion

Single bilayer vesicles, ranging from 200 Å to several micrometers in diameter, can be obtained by using several preparation methods.^{14,24,25} The size and the number of compartments of such vesicles have been determined by using a variety of physical techniques. In particular, DODAC and DODAB yield small (by sonication and ethanol injection)¹⁷ and large (chloroform injection)^{9,17} vesicles displaying a number of size-dependent properties. Small DODAC vesicles fuse upon addition of salt while large vesicles aggregate under the same conditions.²⁵ Differential osmometric behavior for both preparations has also been described.¹⁴ The extent of ion dissociation from the outer surface (α) of small vesicles can be 10 times higher than that of large vesicles.¹⁷ The capacity for solubilizing amphiphilic compounds is also affected by vesicle size.^{15b} The measured binding constant of a series of alcohols is consistently lower for large vesicles, indicating that the bilayer has a lower capacity for accepting (at least) these compounds.^{15b}

Qualitative differences in the effect of small and large vesicles upon the pK_{ap} of HM and on the rates of hydrolysis and thiolysis of *p*-nitrophenyl octanoate were evident upon inspection of the data (Figures 1–3). The apparent catalytic effect (k_2^{max}/k_2°) for both reactions was higher in small vesicles. In the hydrolysis of NPO the difference in k_2^{max}/k_2° (small/large) in both DODAC and DODAB is ca. 2 (Table I). In the thiolysis reaction the apparent catalytic effect obtained with small vesicles is larger by a factor of 5 (DODAB) to 10 (DODAC, Table III).

Understanding the nature of the size-dependent kinetic effects requires dissection of the observed effect into its various components. In (the related) micellar systems separation of the role of individual rate-modifying factors was possibly only by using models for the quantitative analysis of observed rate data.¹ Several models for the quantitative analysis of micellar modified reactions have been put forward over the past decades.^{1,10,26} The models—and the rationale for the analysis—varies. However, application of the models leads to the identification of the same factors as responsible for micellar effects on reaction. Intrinsic reactivity components, changes in the pK_a of dissociable nucleophiles, and (mainly) local concentration are the most important contributions to the observed effects. In selecting one particular model for the quantitative analysis of vesicle effects, i.e., PPIE, we are aware of the pitfalls in the use of this model.²⁶ Several reasons led us to use the PPIE model in this case. Apart from

its simplicity, the number of uncertainties in the reaction sites probed by the systems described here demands the use of a tractable model. As shown in the Results, we have assumed that the system can be described by a single vesicular rate constant. The intrinsic reactivity of OH^- is comparable at both the inner and outer surfaces of large vesicles.¹⁹ We have recently demonstrated, using the OH^- attack on 4-cyanopyridinium ions, that the reactivity of OH^- ion in the internal aqueous compartment of large vesicles is comparable to that in water.²⁷ Thus it is reasonable to use a single k_{2m} to analyze the vesicle data. Moreover, the outer area in small vesicles corresponds to about 70% of the total available reaction area, and thus it is to be expected that the major site of reaction occurs at the outer surface. Our choice of a value for the partial molar volume is also open for discussion. The definition of a reaction volume presupposes the exact knowledge of the reaction site(s). Even where the problem is simplified, as in the case of micelles, the exact definition of a reaction volume is debatable.^{2,11,26} For comparative purposes our choice of V as the partial molar volume of the aggregated amphiphile is reasonable. One major unknown, however, is the ion dissociation at the internal surface. The best-fit values of k_{2m} and K_1 were unaffected by values of α lower than 0.04. Thus, the best-fit values of the parameters obtained with large vesicles, which exhibit a low α , are little affected by our choice of α . On the other hand, the value of $K_{M/V}$ varied by as much as a factor of 2 when the value of α of the small vesicles was decreased from 0.2 to 0. We have thus preferred to use a simple well-tested model for the sake of comparison, rather than trying to apply a more sophisticated framework in a system that is poorly understood.

Hydrolysis and thiolysis reactions in small sonicated vesicles have been analyzed quantitatively in a limited number of systems.¹ The excellent fit of the PPIE model to the present data, using some independently determined parameters, ensures that the pseudophase model can be applied to vesicles. Although the rate of OH^- attack is increased in the presence of vesicles, this kinetic effect arises from a concentration of the reagents in the vesicles rather than from a decrease in the activation energy required for the attack. As in other amphiphile-modified alkaline hydrolysis,² the k_{2m}/k_2° was less than one indicating an inhibition of 3–7-fold of the reaction in the vesicles (Table I). In CTAB micelles the k_{2m}/k_2° ratio is 0.3 for the same reaction.² The calculated values for k_{2m} for small and large vesicles were comparable. Thus the difference in the k_2 vs [amphiphile] profiles arises mainly from differences in ion dissociation from—and substrate binding to—large and small vesicles.

Both heptyl mercaptan dissociation and NPO thiolysis in the presence of vesicles were well described by using the PPIE model. As in the case of hydrolysis the differential rate effects of small and large vesicles was clearly due to major differences in the association constants of the substrates and extent of ion dissociation from the surface(s). The differential effects of small and large vesicles on the pK_{ap} profiles of HM originate from variations in K_{HM} and $K_{M/V}$. The fit of the data led to the finding that the protonated HM is less soluble in large vesicles and that Cl^- (or Br^-) ion is displaced less efficiently by mercaptide ion in the large vesicles. These results suggest a more impenetrable character of the large vesicles. Comparable results were obtained for the distribution of NPO using independent methods (Tables I–III).

Increasing evidence shows that the variations in headgroup packing, resulting from changes in vesicle size, cause differences in the association constants for both amphiphilic substrates and ions.^{17,19} In ester thiolysis the apparent catalytic effects of vesicles are larger than those obtained with micelles.² The k_2^{max}/k_2° ratios obtained with small vesicles were larger than those observed with large vesicles by a factor of 10 (DODAC) or 6 (DODAB). In all cases the k_{2m} are 10–60 times larger than k_2° while in CTAB micelles the k_{2m}/k_2° ratio is 1.0.² Changes in the structure of the supramolecular aggregate are effectively discriminated by using thiolysis as a test reaction. Vesicles efficiently increase mercaptide ion reactivity at the interface and the reactivity can be further

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modulated by vesicle size by a factor of 2 (Table III). These findings suggest that for reactions that are more sensitive to medium composition the structure-dependent modulation can be even larger. The same amphiphile can modulate the reaction by a variety of different factors including subtle structural modifications. Structural changes due to monomer packing in vesicles of different diameters lead to differences in the characteristics of the interfacial reaction site. This additional reaction control mechanism, together with changing association constants and/or ion dissociation, can be of importance to fine tune reaction rates even in systems where the rate acceleration is of several million fold.

In conclusion we have demonstrated that the variation in vesicle size produces significant changes in the effect of these aggregates on reaction rates. The kinetic results were analyzed quantitatively,

and the values of the substrate binding parameters were confirmed by using an independent, nonkinetic, method. These data show that the rate of differences arise mainly from a variation in the capacity of the bilayer to solubilize substrate. Moreover we have demonstrated that the solubilization of one substrate, even at low substrate/amphiphile ratio, can affect the binding of a second substrate, specially in large, planar-like bilayers.

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Temperature Dependence of Emulsion Morphologies and the Dispersion Morphology Diagram

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The recently constructed "dispersion morphology diagram" predicts that for amphiphile/oil/water systems there exists a range of concentrations within which only OL/AQ ("oil-in-water") emulsions form and another range of concentrations for which the emulsion morphology is always AQ/OL ("water-in-oil"), regardless of whether the temperature is below the lower critical end-point temperature, above the upper critical end point, or between these two temperatures. These predictions contradict the PIT (phase inversion temperature) idea, that below the PIT amphiphile/oil/water systems form only "oil-in-water" emulsions and above the PIT they form only "water-in-oil" emulsions. By using electrical conductivity measurements to determine the emulsion morphologies at different temperatures for systems of constant composition, we show for the system studied that the previous predictions of the dispersion morphology diagram are correct.

Introduction

According to the PIT (phase inversion temperature) idea, below the PIT amphiphile/oil/water systems form O/W ("oil-in-water") emulsions and above the PIT they form W/O ("water-in-oil") emulsions.¹ It has been shown² that this belief is consistent with the so-called "Bancroft rule", that in an emulsion the phase of greater surfactant concentration is always the continuous phase.³⁻⁵

The temperature-dependent phase behavior of nonionic amphiphile/oil/water systems has been determined⁶⁻⁹ in great detail since the PIT idea was first stated. As a result, it is now known that a "phase inversion temperature" cannot be defined precisely in terms of emulsion behavior, because the idea is intimately connected with the formation of conjugate top, middle, and bottom phases, and these phases form over a range of temperatures.⁹ However, the phase inversion temperature can be given a precise definition; for example, it can be identified with the "optimum" temperature, at which the interfacial tension between the top and bottom phases passes through a minimum.⁹

Moreover, detailed knowledge of the temperature-dependent phase behavior of nonionic amphiphile/oil/water systems has made it possible to design experiments to clarify the relationships between phase behavior and emulsion behavior.¹⁰⁻¹³ In these experiments, electrical conductivities were measured to determine the emulsion morphologies, and emulsions were always prepared from conjugate (i.e. pre-equilibrated) phases.¹⁰⁻¹³ (For nonmultiple emulsions of

two phases, the emulsion morphology is defined by which phase is the continuous phase or, equivalently, which phase is the dispersed one.¹⁴)

On the basis of these measurements and others already in the literature, we constructed a model "dispersion morphology diagram", which predicts the relationships between the morphologies of nonmultiple dispersions and the phase behavior in amphiphile/oil/water temperature diagrams.¹¹ The dispersion morphology diagram includes all six nonmultiple dispersion

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