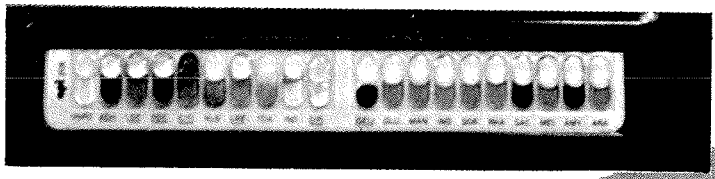
Volume 7 Número 1 Jan.– Mar. 1976

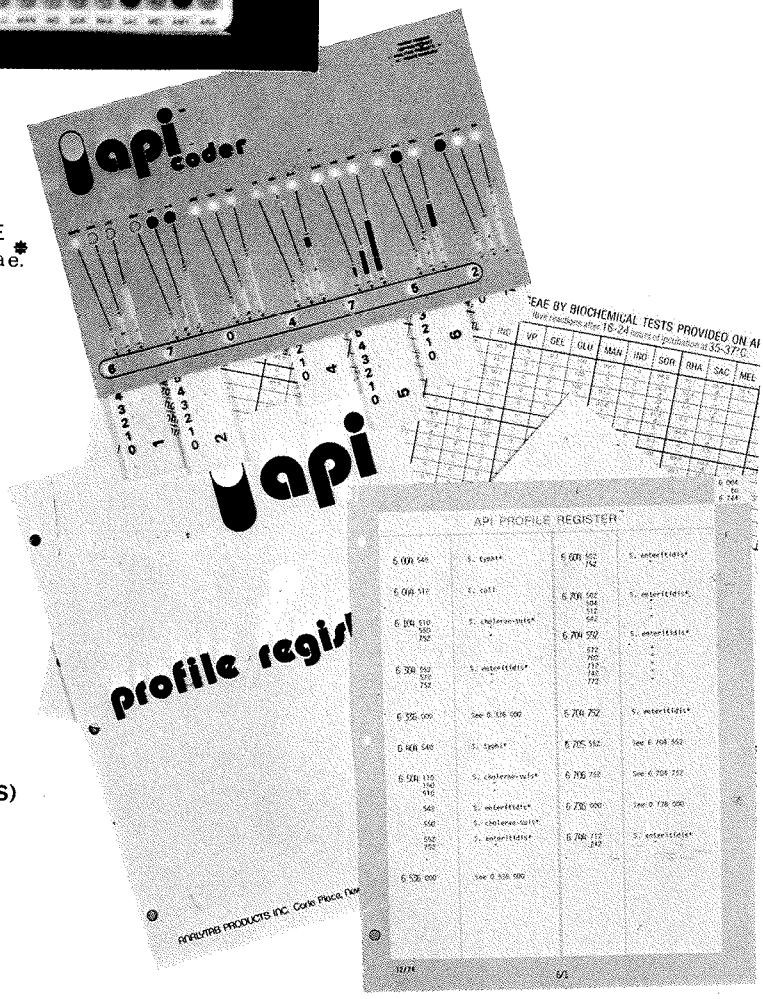
Revista de Microbiologia

Órgão Oficial da Sociedade Brasileira de Microbiologia
São Paulo – Brasil

api system

abriu o caminho da estandardização na identificação bacteriológica.





API 20 E e o SISTEMA DE LEITURA CODIFICADA põem à disposição dos laboratórios mais de 25.000 cepas registradas de ENTEROBACTERIACEAE e outros GRAM ⊖ não-Enterobacteriacea e.* Esse é o resultado de vários anos de colaboração entre utilizadores, centros de referência e os serviços de pesquisa API na França e na América. E, amanhã, esse número crescerá ainda mais com a sua participação.

*Pseudomonas, Aeromonas, Víbrio, Moraxela, etc.

Sempre com o mesmo princípio de microtécnica padronizada
API SYSTEM identifica também:

- **os germes anaeróbios (API 20 ANAERÓBIOS)**
- **os agentes das principais micoses encontradas em clínica (API 20 CÂNDIDA)**
- **germes os mais diversos (API 50 PESQUISA, API 50 LACTOBACILLUS)**
- **ou apenas as Enterobacteriaceae (API 10 SCREEN)**

BIOLAB-MÉRIEUX PRODUTOS PARA LABORATÓRIOS LTDA.

Rua do Resende, 96-A – 2.° andar – Tels.: 221-4089 e 242-0050
RIO DE JANEIRO, RJ. – 20.000 – ZC-06

SÃO PAULO – BRASÍLIA – PORTO ALEGRE – RECIFE – BELO HORIZONTE

Revista de Microbiologia

Órgão Oficial da Sociedade Brasileira de Microbiologia
São Paulo – Brasil

Aquisição por não-membros Assinatura anual para quatro números: Cr$ 120,00 para o Brasil; US$ 20.00 (via marítima) ou US$ 25.00 (via aérea) para o Exterior. Números avulsos: Cr$ 30,00 para o Brasil e US$ 6.50 (via aérea) ou US$ 5.00 (via marítima) para o Exterior. Cheques ou ordens de pagamento em nome da Sociedade Brasileira de Microbiologia, para o endereço do Editor.

Acquisition by non-members Annual subscription for four numbers: US$ 20.00 (surface mail) or US$ 25.00 (air mail). Single copies: US$ 6.50 (air mail) or US$ 5.00 (surface mail). Checks or money orders for Sociedade Brasileira de Microbiologia, addressed to the Editor's office.

Instruções aos autores

A Revista de Microbiologia publica trabalhos originais em todos os campos da microbiologia e da protozoologia. Revisões e atualizações serão publicadas a convite do conselho editorial.

Os manuscritos devem ser enviados em duplicata (original e uma cópia) para o Editor da Revista.

NORMAS GERAIS — Todo manuscrito submetido à Revista deve representar pesquisa original inédita e que não será publicada em outro órgão. Os trabalhos podem ser redigidos em português, espanhol ou inglês. Se aceitos pela Revista, não poderão ser reproduzidos, mesmo em partes, sem permissão oficial dos Editores.

A Revista segue, essencialmente, o "Style Manual for Biological Journals" (2ª edição, AIBS, 1964). Os símbolos genéticos devem seguir, essencialmente, as recomendações de Demerec & col. (*Genetics,* 54: 61, 1966). Abreviações bioquímicas devem seguir, essencialmente, as regras da IUPAC-IUB (*J. Biol. Chem.,* 241: 527, 1966). Atividade enzimática deve ser expressa em termos de unidades internacionais (Enzyme Nomenclature, Elsevier Publ. Co., 1965). Para pesos e volumes, devem ser usados os prefixos nano (n) e pico (p), ao invés de milimicro (mμ) e micromico ($\mu\mu$). Comprimentos devem ser expressos em micrômetro (μm; 10^{-6}m), ao invés de micro (μ); nanômetro (nm; 10^{-9}m), ao invés de milimicro (mμ); e Angstroms (A; 10^{-10}m). Partes por milhão (ppm) devem ser expressas como microgramos por mililitros (μg/ml) ou microlitros por litro (μ litros/litro). A Revista se reserva o direito de adaptar os manuscritos ao estilo adotado.

NOMENCLATURA DE MICRORGANISMOS — A combinação binomial, nome do gênero seguido do da espécie, é adotada e a nomenclatura apresentada no "Bergey's Manual of Determinative Bacteriology" (7ª ed., 1957) obedecida. Se o Autor divergir dessa nomenclatura, seu julgamento será respeitado, mas a classificação correspondente ao Manual de Bergey deverá ser mencionada a seguir, entre parênteses, à primeira vez em que o nome figurar no texto e no resumo. Quando um nome novo for proposto para um microrganismo, será solicitada a opinião de uma autoridade internacional em taxonomia.

FORMA DO MANUSCRITO — Os trabalhos devem ser datilografados em papel branco, comum, tamanho ofício, em espaço duplo ou triplo, deixando-se o mínimo de 3 cm de margens laterais, superior e inferior. Títulos de capítulos de primeira ordem devem ser centrados, incluídos essencialmente pela ordem: **Introdução, Material e Métodos, Resultados, Discussão, Agradecimentos, Referências Bibliográficas.** Os de subcapítulos devem ser junto à margem esquerda.

A *página-título* deverá conter apenas e tão somente: (i) o título do artigo, centrado usando-se letras maiúsculas somente quando obrigatórias, (ii) o(s) nome(s) do(s) autor(es) com maiúsculas e minúsculas normalmente usadas e (iii) afiliação como nota de rodapé, indicada por asterisco.

O *resumo* não deverá ultrapassar 250 palavras. Os trabalhos redigidos em inglês devem ser acompanhados de um título e resumo em português; os redigidos em português e espanhol, de um título e resumo em inglês. O resumo na língua do trabalho e o título com resumo na outra língua devem ser datilografados em página separada do restante do texto.

REFERÊNCIAS BIBLIOGRÁFICAS — No texto, as referências devem ser citadas por número. Os sobrenomes de dois autores devem ser separados por &; quando houver mais de dois autores, a separação é feita por ponto e vírgula e o último por &. Na secção **Referências Bibliográficas,** as citações devem ser por ordem alfabética de autor (ou da mesma combinação de autores) e, para um mesmo autor, em ordem cronológica, numeradas sequencialmente. Os artigos em periódicos devem ser citados na seguinte ordem: nome(s) do(s) autor(es) em maiúsculas, título do trabalho, nome do periódico (abreviado de acordo com o World Medical Periodicals), número do volume, número do fascículo (quando desejado; colocado entre parênteses), número da primeira e da última página do artigo, e ano da publicação.
Exemplo:

WEISER, O.L. & EMERSON, J.S. — Selective screening for quantitative urine cultures. *Amer. J. clin. Path.,* 45:649-650, 1966.

Quando puder ocorrer confusão com o título do periódico, o local da publicação deverá ser mencionado entre parênteses, após a abreviação. Exemplo: *Publ. Hlth Rep. (Wash.).*

Citações de livros devem ter a seguinte ordem: autor(es), título, edição, local, editores e data. Exemplo:

MILLER, S.E. — A textbook of clinical pathology. 6th ed. Baltimore, Williams & Wilkins, 1960.

Citações de "resumos", "dados não publicados", "comunicações pessoais" ou "no prelo" poderão ser adequadamente mencionadas no texto, mas não serão aceitas como referências bibliográficas.

TABELAS — Devem ser numeradas em arábico e datilografadas em páginas separadas. Os dados devem ser arranjados de maneira que colunas de material idêntico sejam lidas perpendicular e não transversalmente. Cabeçalhos e legendas devem ser suficientemente claros e compreensíveis, sem necessidade de consulta ao texto. São permitidas notas explanativas de rodapé indicadas por asteriscos, mas não descrições pormenorizadas dos experimentos. Materiais e métodos utilizados para a obtenção dos resultados devem ser incluídos na secção correspondente.

ILUSTRAÇÕES — Cada uma das duas vias do manuscrito deverá ser acompanhada de um conjunto completo de ilustrações (preferivelmente como fotografias). Os gráficos e desenhos devem ser concluídos, não requerendo qualquer montagem ou rearranjo. Nenhuma parte do gráfico deverá ser datilografada, devendo ser usado um e o mesmo normógrafo. As fotografias de ilustrações a traço devem ser feitas em papel de alto contraste apropriado. É recomendável submeter gráficos e desenhos no dobro da impressão final da Revista, prevendo-se todos os elementos da figura para as escalas de redução. Fotografias com retícula devem ser feitas em papel brilhante, com contraste adequado para reprodução na escala 1:1, observados os espaços máximos disponíveis na Revista e seus submúltiplos, reservando-se espaço suficiente para a legenda. Sempre que houver escala de aumento ou redução, esta deverá ser superposta ou desenhada diretamente na fotografia ou figura. Ver instruções sobre legendas, notas de rodapé, etc. sob TABELAS. Proteja as ilustrações durante o trânsito, com uma folha de papelão.

NOTAS BREVES — Não devem exceder a 500 palavras e o respectivo resumo não mais do que 25. O(s) autor(es) deve(m) consultar um número da Revista para familiarização com a forma e o estilo.

SEPARATAS — Serão fornecidas, gratuitamente, 10 separatas por artigo. Para encomendas maiores, o(s) autor(es) deve(m) entrar em entendimento com o Editor, correndo as despesas por conta dos interessados.

Revista de Microbiologia

Órgão Oficial da Sociedade Brasileira de Microbiologia

Volume 7 Janeiro-Março 1976 Número 1

Rev. Microbiol. (S. Paulo), 7(1)

CONTEÚDO — CONTENTS

Artigos originais

Revisão:

Sociedade Brasileira de Microbiologia

Sócios Patrocinadores

BIOLAB-MÉRIEUX – Produtos para Laboratórios Ltda.

Eli Lily do Brasil Ltda.

Indústria Química e Farmacêutica Schering S.A.

Dextranase bacteriana isolada de placa dental de humanos

T. da Costa, L.C.Bier & F. Gaida

Resumo

A hidrólise do dextrano em nove amostras de placa dental revelou que todas mostraram capacidade de desdobrá-lo. *Fusobacterium nucleatum* foi, dentre os microrganismos isolados, o mais frequente e estável no metabolismo do dextrano. Obteve-se uma enzima — dextranase — altamente ativa, tanto em relação ao polissacarídeo obtido do *Leuconostoc mesenteroides* (Nutritional Biochemicals Cor.), quanto em relação aos depósitos acumulados sobre bastões de vidro, por amostras de *Streptococcus mutans*. Esta enzima, estudada em maiores detalhas, mostrou-se associada à célula e capaz de atuar pela remoção de unidades de glicose do final da moléćula do dextrano, apresentando o ótimo de atividade no pH 6.8.

Summary

Bacterial dextranase isolated from human dental plaque

Dextran hydrolysis in dental plaque was investigated. All of the nine plaque samples studied showed hydrolytic ability. The search for the microorganisms responsible for such dextranase activity showed *Fusobacterium* as one of the more frequent and stable. *Fusobacterium*-dextranase was studied in more detail. The enzyme was found to be cell-associated, working by removing glucose units from the ends of the dextran molecule. The enzyme preparation exhibited a maximal activity at pH 6.8. Plaque-like deposits "in vitro" accumulated were partly dissolved.

Introdução

Muito embora seja referido qua algumas espécies de fungos do gênero *Penicillium* (10) produzem dextranases extracelulares, entre as bactérias somente membros dos gêneros *Bacteroides* (9) e *Lactobacillus* (10) são mencionados como produtores desta enzima.

A placa dental pode acumular polissacarídios quando exposto a soluções de sacarose (7, 11, 14) que, identificados como dextrano, seriam resistente ao ataque dos microrganismos salivares (8). Entretanto Wood (15) e Manly & col. (12) observaram que o dextrano pode ser parcialmente metabolizado na placa dental, quando na ausência de uma fonte exógena de substrato, sugerindo a presença de dextranase em algumas placas.

A presente investigação procura determinar as fontes desta enzima na placa dental e a estuda em maiores detalhes.

Material e Métodos

Isolamento de bactérias hidrolizadoras do dextrano — Amostras de placa dental foram obtidas de nove pacientes que exibiram baixa atividade de cárie. Dispersas em tampão fosfato (0.067 M, pH 7.2), em homogeneizador de tecido, foram a seguir diluídas e aliquotas de 0.1ml inoculadas no meio de cultura sugerido por Da Costa & col. (5).

As culturas foram incubadas de 2 a 3 dias, aeróbica e anaerobicamente e, a seguir, centrifugadas para remover o crescimento bacteriano misto. A hidrólise do dextrano foi determinada pela adição de 2 volumes de etanol ao sobrenadante das culturas livres de células. Tubos com caldo-dextrano não inoculados, serviram como controle. A não obtenção de um precipitado com etanol serviu como indicação de que o polissacarídio havia sido hidrolisado pelo crescimento bacteriano misto.

De uma amostra de placa, mostrando capacidade

* Departamento de Patologia, Secção de Microbiologia, Universidade Federal de Santa Maria — RS. Trabalho realizado com auxílio do CNPq — Conselho Nacional de Desenvolvimento Científico e Tecnológico.

hidrolítica, uma aliquota foi retirada, espalhada na superfície de agardextrano e incubada anaerobicamente, durante 72 horas. As colônias desenvolvidas foram recultivadas em caldo-dextrano e testadas em sua capacidade hidrolítica.

Uma amostra de *Fusobacterium* foi isolada e, estudada em maiores detalhes, serviu como fonte de dextranase.

Preparação da dextranase — Preparações enzimáticas foram obtidas após 3 dias de subcultivo de uma única amostra de *Fusobacterium*, em caldo-dextrano a 0.15% (p.m. 5-50 X 10^6 - NBC), incubada anaerobicamente. Essas culturas foram centrifugadas e o sedimento celular coletado, lavado 3 vezes e ressuspenso em tampão fosfato (0.067 M, pH 7.0) à densidade ótica de 1.5 (Espectrofotômetro Bausch Lomb), usando-se 540 nm como comprimento de onda.

A dextranase associada a célula pôde ser obtida numa forma solúvel. Vinte mililitros foram tratados com 1ml de Desoxicolato de sódio a 5%, durante 30 min., centrifugando-se a seguir para remover os restos celulares e, finalmente, dializando-se.

Medida da atividade enzimática — A atividade da dextranase foi determinada pela incubação de 4ml da preparação enzimática com 2ml de tampão fosfato (0.067 M, pH 6.8) e 2ml de uma solução de dextrano a 1%, em banho-maria a 35°C. Misturas de: (a) enzima inativada em água em ebulição mais dextrano; (b) dextrano e (c) dextranase, foram incluídas como controles.

Amostras da mistura reativa foram removidas periodicamente durante 60 min. de incubação. Após desproteinização com $ZnSO_4$ e $Ba(OH)_2$, os açúcares redutores foram analisados pelo método de Somogyi (16).

Os produtos formados durante a hidrólse foram analisados cromatograficamente em camada delgada, usando-se Kieselguhr (Merck). Amostras da mistura reativa foram aplicadas nas placas, utilizando-se como solvente acetato de etila, ácido acético e água (3:3:1). Os pontos correspondentes à migração das substâncias em estudo nos cromatogramas foram visualizados, borrifando-se uma solução de anilina difenilamina.

Estudou-se, também, a capacidade de preparações enzimáticas dialisadas hidrolisarem placas formadas "in vitro" pelo *Streptococcus mutans* amostra GS-5 (7), quando cultivado em caldo sacarose. Depósitos semelhantes a placas, acumulados sobre bastões de vidro, foram expostos a preparações enzimáticas e observados durante 60min..

Resultados

Bacterias hidrolisadoras de dextrano na placa dental — Verificamos que o dextrano pode ser hidrolizado por culturas bacterianas da placa dental. Nas amostras de placa estudadas, foi observada atividade hidrolítica nos tubos de cultura correspondentes as baixas diluições (10^{-3} e 10^{-4}); entretanto, em duas dessas amostras, a hidrólise foi também observada nos tubos de alta diluição (10^{-8}).

Uma fusobactéria, isolada e caracterizada bioquimicamente como *Fusobacterium nucleatum* (2), serviu como fonte de dextranase (5), por ser considerada como principal responsável pelo metabolismo do dextrano. Este microrganismo não produziu polissacarídio extracelular, quando cultivado em caldo-sacarose a 5%.

Esfregaços de culturas puras em caldo-dextrano, corados com solução de iodo, apresentaram uma coloração variável de púrpura a violeta, indicação provável de armazenamento de polissacarídio intracelular. A fusobactéria exibiu também intensa ação hidrolítica sobre o levano e o amido, quando cultivada em presença destes polímeros.

Características da dextranase fusobacteriana — Filtrados da cultura de *Fusobacterium* em caldo-dextrano não apresentaram atividade hidrolítica.

A enzima, revelando-se associada à célula, liberou, do dextrano, aproximadamente 700ng de açúcar redutor por h/ml de solução enzimática, mostrando-se também, nas condições empregadas, proporcional à sua concentração.

O ótimo da atividade enzimática está no pH 6.8, quando determinada em tampão fosfato. Soluções enzimáticas podem ser armazenadas durante várias semanas, sem marcante diminuição da atividade.

A ação da dextranase foi demonstrada parcialmente sobre placas artificialmente formadas "in vitro" por amostras de *Streptococcus mutans*. Quando estas placas foram incubadas, juntamente com a enzima, durante 60min., foram liberados 280ng de açúcares redutores por h/ml de solução enzimática.

Discussão

A importância da placa dental no início da lesão de cárie tem recebido maior atenção no últimos anos. Evidências acumularam-se de que a síntese de dextranos, por representantes da flora oral, desempenha um importante papel na sua formação (3, 8, 13, 15). Estes polímeros eram até bem pouco considerados como resistentes ao ataque bacteriano na cavidade oral (8). Entretanto, Wood (15) e mais recentemente Manly & col. (12) relataram seu desdobramento parcial na placa dental.

O presente trabalho, investigando o metabolismo do dextrano na cavidade oral, revelou como principal responsável pela hidrólise, uma fusobactéria. Propriedade semelhante foi exibida por cocos gram-positivos,

quando no isolamento primário, observando-se, entretanto, a perda dessa característica nos subcultivos.

Sugere-se que a dextranase fusobacteriana em estudo seja ligada à célula e atue pela remoção terminal de unidades de glicose da molécula do dextrano. Assim, uma contínua liberação de glicose ocorreria na superfície da célula, permitindo sua fácil utilização. Este mecanismo de ação seria, em parte, similar ao da dextranase produzida por bacteróides intestinais (1, 9) e o da frutan-hidrolase descrita por Da Costa & Gibbons (4). As unidades de glicose poderiam ainda ser parcialmente acumuladas intracelularmente, sob a forma de grânulos de reserva, conforme foi possível concluir da reação tintorial observada nos esfragaço preparados de cultura de *Fusobacterium* em caldo-dextrano, corados com solução de iodo.

Uma vez que a placa dental é capaz de sintetizar e parcialmente utilizar o dextrano, na falta de uma fonte exógena de substrato energético, pode-se admitir que este polissacarídio funcione como material de reserva, à semelhança da amilopectina (6) e do levano (4).

A presença de dextranase, em altas diluições de material de placa, está provavelmente relacionada a um aumento do número de fusobactérias naquelas placas. Estes resultados, entretanto, não excluem a possibilidade de placas dental sem dextranase, estando a explicação destas diferenças por ser determinada.

Referências Bibliográficas

1. BAYLEY, R.W. & CLARKE, R.T.J. — A Bacterial Dextranase. *Biochem. J.*, 72:49-54, 1959.
2. BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY — 8 th. ed. Edited by Buchanan, R.E. & Gibbons, N.E., pp.406-407. Williams and Wilkins, Batimore, 1974.
3. CRITCHLEY, P.; WOOD, J.M.; SAXTON, C.A. & LEACH, S.A. — The polymerization of dietary sugars by dental plaque. *Caries Res.*, 1:112-129, 1967.
4. DA COSTA, T. & GIBBONS, R.J. — Hydrolysis of levan by human plaque streptococci. *Arch. oral Biol.*, 13:125-128, 1968.
5. DA COSTA, T.; BIER, L.C. & GAIDA, F. — Dextran hydrolysis by a *Fusobacterium* strain isolated from human dental plaque. *Arch. oral Biol.*, 19:341-342, 1974.
6. GIBBONS, R.J. & SOCRANSKY, S.S. — Intracellular polysaccharide storage by organisms in dental plaque. Its relation to dental caries and microbial ecology of the oral cavity. *Arch. oral Biol.*, 7:73-80, 1962.
7. GIBBONS, R.J.; BERMAN, K.S.; KNOETTNER, P. & KAPSIMALIS, B. — Dental caries and alveolar bone loss in gnotobiotic rats infected with capsule forming streptococci of human origin. *Arch. oral Biol.*, 11:549-560, 1966.
8. GIBBONS, R.J. & BANGHART, S.B. — Synthesis of extracellular dextran by cariogenic bacteria and its presence in human dental plaque. *Arch. oral Biol.*, 12:11-24, 1967.
9. HEHRE, E.J. & SERY, T.W. — Dextran splitting anaerobic bacteria from the human intestine. *J.Bacteriol.*, 63:424-426, 1952.
10. HULTIN, E., & NORDSTROM, L. — Investigations on dextranase. *Acta Chem. Scand.*, 3:1405-1417, 1949.
11. MANLY, R.S. — Retention of carbohydrate from sugar solutions by salivary sediment. *J.Dent. Res.*, 40:379, 1961.
12. MANLY, R.S.; KERRING, J.H. & SKLAIR, I.R. — Polysaccharide metabolism of dental plaque. Printed Abstracts, I.A.D.R., 49th General Meeting, Chicago, 1971.
13. McDOUGALL, W.A. — Studies on the dental plaque. IV — Levans and the dental plaque. *Aust. dent. J.*, 9:1-15, 1964.
14. WOOD, J.M. — Plolysaccharide synthesis and utilization by dental plaque. Abstract 28, 12th General Meeting, I.A.D.R., 1964.
15. WOOD, J.M. — The amount, distribution and metabolism of soluble polysaccharides in human dental plaque. *Arch. oral Biol.*, 12:849-858, 1967.
16. SOMOGYI, M. — Determination of blood sugar. *J.Biol. Chem.*, 160:69-73, 1945.

Ineficácia da tobramicina *in vitro* sobre *Pseudomonas* gentamicina-resistentes

Marcelo Magalhães *& Adelma Véras*

RESUMO

Quarenta amostras gentamicina-resistentes de *Pseudomonas aeruginosa*, clinicamente significativas, foram testadas frente à tobramicina, pelos métodos das diluições e da difusão em agar. Os resultados foram concordantes e mostraram inequívoca resistência cruzada entre gentamicina e tobramicina. Isso sugere o emprego de apenas uma das drogas, nos antibiogramas rotineiros, em bacteriologia clínica.

SUMMARY

In vitro failure of tobramycin against gentamicin-resistant Pseudomonas

Forty gentamicin-resistant strains of *Pseudomonas aeruginosa*, clinically important, were tested against tobramycin by using both agar dilution and single-disc susceptibility techniques. The results were concordant and showed a distinct cross-resistance between gentamicin and tobramycin. This suggests that either one of the drugs might be employed for routine sensitivity testing, in clinical bacteriology.

Introdução

A tobramicina é um novo aminoglicosídeo, de espectro antibacteriano semelhante ao da gentamicina (3). Sua principal vantagem sobre a gentamicina reside na maior atividade *in vitro* contra as pseudomonas (4, 10, 11). Por outro lado, existem divergências de opinião sobre a ocorrência de resistência cruzada entre aquelas drogas (3, 5, 6, 8). Neste trabalho, empregando-se os métodos das diluições e da difusão em agar, concluiu-se pela ineficácia, *in vitro*, da tobramicina sobre linhagens gentamicina-resistentes de *Pseudomonas aeruginosa.*

Materiais e Métodos

Bactérias. Foram selecionadas 40 culturas de *P. aeruginosa*, clinicamente significativas, resistentes à gentamicina ao método de Bauer & Kirby (1). As amostras foram isoladas em diferentes hospitais e ambulatórios do Recife, e identificadas com o emprego dos seguintes testes (9): oxidação-fermentação da glicose, oxidase, motilidade, produção de pigmento, oxidação do gluconato, crescimento em citrato de Simmons, lisina e ornitina descarboxilase, arginina deidrolase, e crescimento em caldo de coração e cérebro a 42°C.

Antibióticos. Sulfato de tobramicina, da Eli Lilly & Co. e sulfato de gentamicina, da Schering Corporation.

Antibiogramas. Os níveis de resistência à gentamicina e tobramicina foram determinados pelo método das diluições em meio sólido. As drogas foram incorporadas ao Agar de Muller-Hinton (Difco), em concentrações 1 – 2 – 4 – 8 – 16 e 32 vêzes superiores à concentração inibitória mínima (CIM) da amostra susceptível padrão. Os resultados foram fornecidos em razões de resistência (RR), isto é, relacionando-se a CIM da cultura sob teste e a CIM da amostra padrão. Na avaliação da resistência, pelo método da difusão em agar, foram usados discos únicos de 6 mm contendo 10μg de tobramicina ou 10μg de gentamicina (1).

* Depto. de Microbiologia, Faculdade de Medicina, Cidade Universitária 50000 Recife, PE.
Os autores são gratos ao Dr. Barros Barreto, Diretor Médico da Eli Lily do Brasil pelo sulfato de tobramicina e literatura a respeito fornecidos.

RESULTADOS

Com referência à amostra susceptível de **Pseudomonas**, as CIMs foram de 8 e 2μg/ml e os diâmetros dos halos de inibição de 21 a 25 mm, respectivamente para gentamicina e tobramicina.

Os resultados comparativos entre a atividade da gentamicina e tobramicina, contra as 40 amostras gentamicina-resistentes de *P. aeruginosa*, estão sintetisados na Figura 1, e a correlação entre os métodos das diluições e dos discos, nos testes para a tobramicina, na Figura 2.

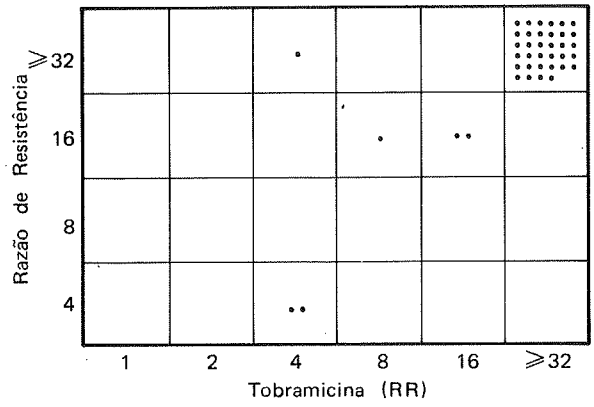


Fig. 1 — Atividade da tobramicina sobre 40 cepas gentamicina-resistentes de *Pseudomonas*. RR = razão de resistencia.

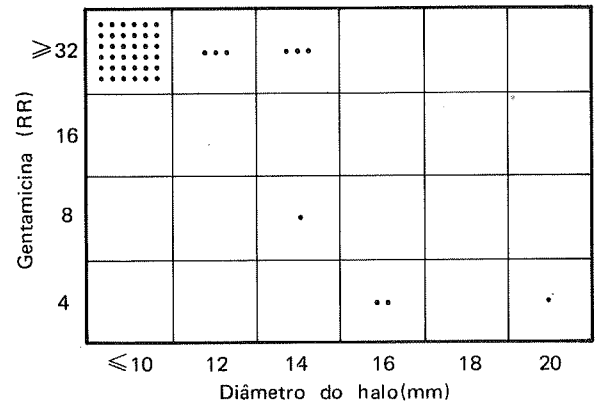


Fig. 2 — Correlação entre os métodos das diluições e dos discos (10μg) para a tobramicina.

DISCUSSÃO

Os resultados substanciam observações anteriores (4, 5, 6, 7), e indicam nítida resistência cruzada entre a gentamicina e a tobramicina. A esse respeito, é possível que as discordâncias existentes dependam do mecanísmo de resistência envolvido, cromosomal ou plasmidial, nos diferentes lotes de culturas examinadas. No Recife, pelo menos em relação às enterobactérias, a gentamicina-resistência é transferível e está vinculada ao plasmídio Gk (M. Magalhães, A. Véras & A. Dâmaso, no prelo) que governa a síntese de uma adeniltransferase que inativa, além da gentamicina, a kanamicina e a tobramicina (2). Embora não se tenha ainda caracterizado a natureza da gentamicina-resistência, seus elevados níveis em nossas culturas de *P. aeruginosa* sugerem o caráter plasmidial. É, portanto, improvavél que a tobramicina tenha possibilidades terapêuticas contra essas linhagens, apesar de sua maior atividade, *in vitro*, contra as cepas sensíveis.

A notável correlação entre os métodos das diluições e da difusão em agar apóia a sugestão (8) de utilizar-se um único disco, contendo 10μg de tobramicina, na avaliação rotineira da resistência bacteriana a essa droga. Por outro lado, a não inclusão de um número significativo de culturas susceptíveis, neste estudo, dificulta deduções sobre quais os diâmetros críticos, dos halos de inibição, indicativos de resistência ou susceptibilidade. Todavia, é questionável se há realmente necessidade de testar-se simultaneamente a tobramicina e a gentamicina em bacteriologia clínica. Nas recomendações da Food and Drug Administration, para a realização de antibiogramas rotineiros, está a introdução do conceito de família. Isto é, deve-se usar apenas um membro representativo de cada grupo de antibióticos relacionados (12). Este parece ser o caso da tobramicina e gentamicina. Essas drogas, além de idênticos espectros de atividade antibacteriana (3), apresentam inequívoca resistência cruzada, pelo menos, no sentido gentamicina-tobramicina.

Referências Bibliográficas

1. BAUER, A. W.; KIRBY, W. M. M.; SHERRIS, J. C. & TURCK, M. — Antibiotic susceptibility testing by a standardized single disc method. Amer. J. Clin. Pathol,. 45:493-496, 1966.
2. BENVENISTE, R. & DAVIES, J. — R-Factors mediated gentamicin resistance: a new enzyme which modifies aminoglycoside antibiotics. Febs Letters, 14:293-296, 1971.
3. BLACK, H. R. & GRIFFITH, R. S. — Preliminary studies with nebramycin factor 6. Antimicrob. Ag. Chemother., 1970, p. 314-321, 1971.
4. BODEY, G. P. & STEWART, D. — In vitro studies of tobramycin. Antimicrob. Ag. Chemother., 2:109-113, 1972.
5. BRITT, M. R.; GARIBALDI, R. A.; WILFERT, J. N. & SMITH, C. B. — In vitro activity of tobramycin and gentamicin. Antimicrob. Ag. Chemother., 2:236-241, 1972.
6. BRUSCH, J. L.; BARZA, M.; BERGERON, M. G. & WEINSTEIN, L. — Cross-resistance of *Pseudomanas* to gentamicin and tobramycin. Antimicrob. Ag. Chemother., 1:280-281, 1972.

7. CROWE, C. C. & SANDERS, E. — Is there complete cross-resistance of Gram-negative bacilli to gentamicin and tobramicin? Antimicrob. Ag. Chemother., 2:415-416, 1972.

8. DEL BENE, V. E. & FARRAR Jr., E. W. — Tobramycin: In vitro activity and comparison with kanamycin and gentamicin. Antimicrob. Ag. Chemother., 1:340-342, 1972.

9. HUGH, R. & GILARDI, G. L. — *In* LENNETTE, E. H.; SPAULDING, E. H. & TRUANT, J. P. — eds. — Manual of Clinical Microbiology 2nd. edition. American Society for Microbiology, Washinghton, 1974, p. 250-269.

10. LEVINSON, E. M.; KNIGHT, R. & KAYE, D. — In vitro evaluation of tobramycin, a new aminoglycoside antibiotic. Antimicrob. Ag. Chemother., 1:381-384, 1972.

11. MOLAVI, A.; BARZA, M.; COLE, W.; BERMAN, H. & WEINSTEIN, L. — In vitro assessment of tobramycin, a new aminoglycoside with anti-*Pseudomonas* activity. Chemotherapy, 18:7-16, 1973.

12. WRIGHT, W. W. — *In* BALOWS, A. — ed. — Current techniques for antibiotic susceptibility testing. Charles C. Thomas, Illinois, 1974, p. 26-46.

Microbial membranes I. Structure and molecular organization*

Luiz R. Travassos & W. de Souza

Summary

Review on membrane isolation, composition, structure and molecular organization, structure determination, with emphasis on the experiments with microorganisms.

Resumo

Membranas de microrganismos I. Estrutura e organização molecular

Revisão sobre o isolamento, composição, estrutura e organização de membranas, com ênfase nos experimentos com microrganismos.

Introduction

The formation of membranes is a major event in creation of a cell and hence of a unicellular microbe. Besides maintaining the cell contents apart from the suspension medium thus conferring individuality to the cytoplasmic elements, biological membranes determine the rate of passage of inorganic and organic molecules inward and outward the cell. This is accomplished by a selective process that involves either a facilitated diffusion or an active transport. Such a property makes the cell membrane an important structure able to determine the growth rate and the metabolic activity of cells in particular environments.

Membranes play a dynamic biochemical role in providing a supporting matrix for the location of proteins with varied enzymatic activities. Due in part to their lack of rigidity, tendence to coalesce and ability to accomodate a great variety of proteins, membranes are important agents in the biogenesis of specialized organelles such as mitochondria, chloroplasts, bacterial mesosomes and chromatophores. In forming intracellular compartments membranes enclose several molecules which have increased probability of reacting with each other thus favoring the metabolic process. The possibility of ranking reacting elements along a single supporting structure would also provide a basis for an efficient low-energy-consuming sequence of biochemical reactions. Depending on the nature of the membrane, compartments can also exist less active metabolically and sometimes acting only as storage parts. There are membranes that encircle nuclei of eukaryotic cells. In bacteria, mesosomes deeply intruded into the cytoplasm closely associate with the bacterial DNA possibly playing a role in the equal distribution of nucleic acid into each of the daughter cells.

Several functions of biological membranes are presently known. Their intimate structure and molecular organization are nevertheless far from being completely understood. Models have been proposed for membranes of animal and microbial cells based on certain aspects of membrane chemical composition and morphology. These models are still under study mainly with the introduction of new experimental methods only recently employed in the investigation of biological membranes.

Rothfield (138) so defines biological membranes: they "are continuous structures separating two aqueous phases. They are relatively impermeable to water-soluble compounds, show a characteristic tri-

* Departamento de Microbiologia Geral, Instituto de Microbiologia e Instituto de Biofísica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, 20.000.

laminar appearance when fixed sections are examined by electron microscopy, and contain significant amounts of lipids and proteins".

In the following sections some of the studies on membranes will be discussed. Emphasis is given to the experiments with microorganisms.

Isolation

a — Isolation of intact membrane fragments — The isolation of membranes from various types of cells is the primary step in the study of their chemical composition and structural organization. The procedures selected in this purpose must lead to membrane preparations not contaminated with adjacent structures originally present in the cell or with other materials accessible to the inner (cytoplasmic) or to the outer surface of the membrane. The most likely contaminants in bacteria and yeasts are components of the cell wall, ribosomes and other cytoplasmic materials. In some cases membranes are isolated completely free of contaminants. In other instances adjacent structures are so tightly bound to the membranes that pure preparations are hard to obtain. This is frequently the case with Gram-negative bacteria. The cell envelopes of these bacteria contain large amounts of lipopolysaccharide in addition to the usual bacterial mucopeptide. These outer layers are intimately associated with the inner lipid-protein membrane. Connections between specific regions of the cytoplasmic membrane and the inner layer of the cell wall can be seen in thin sections of plasmolysed cells of *Escherichia coli* (4). In freeze-etched preparations of bacilli (49, 102) strands or fibres seem to connect the inner layer of the cell wall with the membrane. Bridges extending from the outer regions of the cytoplasmic membrane are also visible in thin sections of *B. licheniformis* (136). These observations show that a close association between the cell wall and the membrane can occur in certain microorganisms.

The isolation of membranes from animal cells usually involves cell-disruption by osmotic lysis or by mechanical means. Methods for the mechanical disruption of cells include freezing and thawing, sonication and rapid decompression. The isolation of membranes is accomplished by differential centrifugation and density gradient sedimentation. In this latter procedure a material of known buoyant density can be predictably sedimented through a solution of variable density (gradient) until its density equilibrates with the density of the solvent. The material which forms a band at this point can be removed with relatively little contamination of adjoining materials as the centrifuge tubes are perforated at the bottom and the various bands drip out in succession. Gradients can be easily prepared by layering in the centrifuge tubes solutions of increasingly lower concentrations of appropriate solutes, e.g. sucrose. In bacteria, osmotic lysis can be used to isolate membranes from protoplasts. True protoplasts are not usually obtained from Gram-negative cells because of the close association of the cell wall components with the membrane. Upon removal of the cell wall from these bacteria residues of that structure usually remain attached to the membrane. These residues can be demonstrated through selective reactions using specific cell wall receptors. "Protoplasts" with remaining residues of the cell wall are called spheroplasts. Birdsell and Cota-Robles (9) apparently succeeded in separating the cytoplasmic membrane from the "outer membranes" of *E. coli:* the procedure involved the suspension of the bacteria in 0.5 M sucrose prior to the lysozyme-EDTA treatment generally used in spheroplasting bacteria. The "outer membranes" visualized in the electron microscope are thought to contain the lipopolysaccharide component of the cell wall (11). The lysozyme-EDTA treatment is a routine method for the removal of the cell wall components from Gram-negative bacteria. While lysozyme destroys the mucopeptide polymer, EDTA chelates divalent ions presumably important in maintaining ionic cross-links between the lipopolysaccharide and the other polymers of the cell envelope. The cytoplasmic membrane of *Salmonella typhimurium* was isolated from the "outer membranes" by use of equilibrium density gradient centrifugation of the total membrane fraction obtained by lysis of spheroplasts (109). Spheroplasts were prepared by the method of Birdsell-Cota-Robles (9). The "outer membrane" fraction and the cytoplasmic membrane from *S. typhimurium* differed markedly in their specific enzyme activities as well as in their over-all protein composition as determined by polyacrylamide gel electrophoresis in sodium dodecylsulfate. The ratios of phosphatidylglycerol to phosphatidylethanolamine and of cardiolipin to phosphatidylethanolamine in the "outer membrane" were approximately one half and 1/4 of those for the cytoplasmic membrane respectively.

Protoplasts from Gram-positive bacteria, e.g. *Micrococcus lysodeikticus* have been routinely used as sources of membranes. Membranes are also easily obtained from mycoplasmas and L-forms. Electron microscopy and chemical analysis of these microorganisms indicate that their cells contain no cell wall material external to the membrane (119). Membranes of mycoplasmas can be isolated by differential centrifugation of lysates of the microorganisms in hypotonic solutions (123).

Membranes from yeast cells were prepared by osmotic shock of yeast spheroplasts (13). Unlike bacteria yeast cell walls are not susceptible to

lysozyme attack; spheroplasts are usually prepared by treatment with a multiple enzyme preparation from the digestive juice of the snail *Helix pomatia*. Yeast spheroplasts can be lysed in suspension with ice-cold Tris buffer (pH 7.2) containing $MgCl_2$. The pellet of intact membranes and debris are then isolated by centrifuging at 20,000 g. Such a procedure has been used to prepare membranes from *Candida utilis* (40) and from *Saccharomyces cerevisiae* (78).

b — Isolation of membrane constituents — Membranes are composed mainly of lipids and proteins. While lipids are usually extracted in organic solvents under N_2 a more elaborated methodology is usually necessary for the extraction of membrane proteins. Since many of these proteins are enzymes the success of a given isolation procedure is followed by determining the specific enzymatic activity of the preparation.

Proteins found in biological membranes are special in their high percentage of hydrophobic residues which tend to dissolve in non-polar solvents rather than in water. Since non-polar compounds can induce reversible changes in protein structure (185) one should expect that the removal of protein hydrophobic residues from the lipid phase may change the special conformation of the protein. A change in the native conformation of the protein may be followed by the loss of its biological activity in the case of an enzyme. The question so arises as whether the methods used in the extraction of membrane proteins will or will not result in structural changes which may impair, in the case of enzymes, their catalytic activity.

In *Micrococcus lysodeikticus* the following procedures have been used for the isolation of bacterial enzymes from membranes (100):

1. *Solutions of low-ionic strength.* A Ca^{++} -dependent ATPase from *M. lysodeikticus* membranes was released by washing several times in 0.03 M Tris buffer (pH 7.5) and then with 0.003 M Tris (pH 7.5). The specific activities of the 5th and 6th washes were 4 to 5 times that of the preceding wash (99). ATPase appears in particles which are distributed throughout the bacterial membrane. The release of the ATPase particles by use of the above procedure was also followed by electron microscopy of the membrane fragments.

2. *Cation depletion by chelating agents (EDTA).* Treatment of membranes with 0.005 M EDTA in 0.03 M Tris buffer (pH 7.5) was used to release membrane NADH-dehydrogenase. The extracted material which contained also cardiolipin displayed a vesicular form.

3. *Detergents.* Cytochrome *c* was released from the bacterial membranes by dissociation in 0.5% sodium dodecylsulfate followed by ammonium sulfate precipitation. While cytochrome *c* is soluble, cytochromes a and *b* were precipitated. Deoxycholate (1%) has been used for extracting most of the membrane phospholipids and carotenoids leaving behind sheets of extracted membrane showing the absorption spectra of the cytochromes and retaining an activity for succinic dehydrogenase. Complete membrane solubilization was achieved with sodium dodecylsulfate, Nonidet P-40 (a polyoxyethylated alkyl phonel), Triton X-100 (1% with 0.04 M $AlCl_3$), or 10 M acetic acid (100). Cationic detergents are generally ineffective in solubilizing membranes (149).

4. *Organic solvents. n*-Butanol solubilizes enzymes such as ATPase in the active form but other enzymes, e.g. NADH-dehydrogenase are destroyed. 3-Pentanol extraction preserves 30–40% of the NADH-dehydrogenase activity while removes more than 90% of the lipids (100,146). Nachbar, Winkler and Salton (101) investigated the ability of several aliphatic alcohols in extracting ^{32}P-labelled phospholipids from bacterial membranes dispersed in water. The following order of efficiency of extraction was observed: *tert*-amylalcohol = *n*-butanol = *iso*-butanol = *sec*-butanol > *n*-amylalcohol > *iso*-amylalcohol > 3-pentanol. ATPase was well preserved upon extraction with several alcohols; NADH-dehydrogenase was still active following extraction with *tert*-amylalcohol.

Bacterial membrane proteins can also be solubilized by the so-called chaotropic agents (133). These are inorganic ions favoring the transfer of non-polar residues into an aqueous environment. This efect is possibly achieved due to the ability of chaotropic agents to disorder the structure of water. The relative effectiveness of some of these inorganic ions (140) is shown below:

$SCN^- > ClO_4^- > NO_3 > I^- > Br^- > Cl^- > SO_4^=$, CH_3COO^-, F^-

The various procedures so far discussed do not lead to a random solubilization of the components of the membrane (10,188). Concentrations of sodium dodecylsulfate sufficient to solubilize the NADH-2,6-dichlorophenolindophenol oxido-reductase from membranes of *Bacillus subtilis* do not solubilize the succinate dehydrogenase from this same microorganism. With higher concentrations of SDS, membrane protein is solubilized rather than the lipid. In some instances an enzymatic system can be restored by combining fractions obtained through extraction procedures which selectively solubilize certain components of the membrane. A procedure for the sequential extraction of fractions containing the primary dehydrogenase and the cytochromes of the NADH oxidase system of *B. megaterium*

membranes was developed (189). Several components were released from the original membrane structure being inactive unless combined in the presence of Mg^{++} ions. Supernatants A and B (Fig. 1) are inactive when assayed alone for enzymatic activity; they become active when joined in the presence of Mg^{++}. Under these conditions the NADH oxidase activity is similar to that of the sonicated membranes. The deoxycholate-KCl pellet is inactive. The restoration of the NADH oxidase activity is inhibited when either fraction is treated with phospholipase A indicating a requirement for a phospholipid in each fraction for the oxidase reconstitution (189)

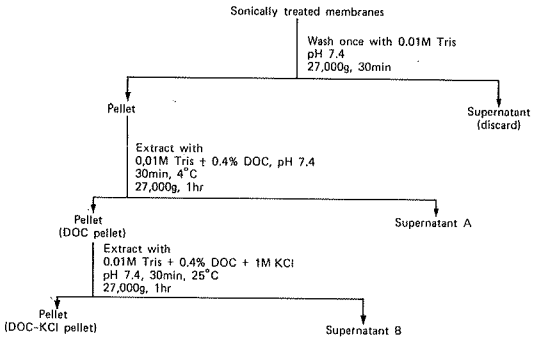


Fig. 1 - Sequential extraction of NADH oxidase components (after Yu and Wolin, ref. 189).

General composition of membranes

Analysis of ghosts from red blood cells showed a gross composition of approx. 60% proteins/approx. 40% lipids by weight (82). Van Deenen (28) reviewed the data on the composition of lipids in human erythrocytes: approximately 70% are phospholipids – lecithin, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, phosphatidic acid, inositol phosphoglycerides – and 30% is cholesterol. There are also small amounts of glycolipids. The proteins of red blood cell membranes vary in molecular weight with two major peaks at approx. 200,000 and 100,000 (132). About 95% of the mitochondrial lipid is phospholipid. Cholesterol, ubiquinone, tocopherol, carotenoids and neutral fat account for the remaining 5% (44). Phospholipids found in mitochondria are lecithin, phosphatidylethanolamine, cardiolipin. Other types account for 5% of the total phospholipid fraction. The so-called membrane "structural proteins" appear rather heterogenous in nature (150,151). Data are uniform in showing that animal membranes have a high percentage of phospholipids as well as a varied composition of proteins including molecules of several molecular weights. The types of phospholipids vary also in different organelles and in different species (3,85)

The lipid bilayer of erythrocytes has compositional asymmetry: choline phospholipids are present on the external side while amino phospholipids face the cytoplasm. It is probable that such an asymmetry occurs also in other membranes (17). Lecithin, sphingomyelin and glycolipid contain few polyunsaturated fatty acid residues. On the contrary, phosphatidylethanolamine and phosphatidylserine are rich in unsaturated fatty acids such as arachidonic acid. Bretscher (17) suggests that polyunsaturated residues in the inner half of the lipid bilayer provide a less ordered phase that may be a better solvent for proteins as compared to the outer half of the bilayer.

In bacteria, phosphatidylethanolamine is the common phospholipid. Noteworthy is the absence of lecithin in most bacteria with the exception of some species of *Agrobacterium* (42,62) and *Rhizobium* (81). In addition bacterial lipids which are located mainly on the plasma membrane and mesosome fractions include several unusual lipids. Some of them have been characterized: aminoacylesters of phosphatidylglycerol (52,80), a variety of glycosyl diglycerides, cyclopropane fatty acids in the phospholipids (in Gram-negative bacteria and *Lactobacillus),* branched chain fatty acids (in Gram-positive bacteria), branched high molecular weight fatty acids (in *Corynebacteria* and *Mycobacteria*). Glycosyl diglycerides are widely distributed in Gram-positive bacteria (154) and in mycoplasmas but are absent in Gram-negative bacteria. Cholesterol (and other sterols) present in the membranes of algae, fungi and membranes from higher organisms is not found in bacteria but occurs in mycoplasmas (161). The distribution of the various lipid categories in microorganisms is shown in Table 1.

The chemical composition of the membranes from *Micrococcus lysodeikticus* was studied in detail and was found to contain (% by weight): 49-64.5 (depending on the method) protein, 23.8 lipids, 4.2 carbohydrate, 1-2 RNA. Lipids included phospholipids, carotenoids and menaquinones (147). The area ratio of protein to lipid in several bacterial membranes – from *B. licheniformis* and *M. lysodeikticus* (148), *B. megaterium* (184), *Streptococcus faecalis* (155) and *Mycoplasma laidlawii* (125) – varied from 3.4 to 5.4. The areas were calculated considering that an amino acid occupies approximately 17Å^2 (184), a phospholipid approx. 70Å^2 and cholesterol approx. 38Å^2 (179). Besides phosphatidylethanolamine already referred to as a common phospholipid of bacteria, phosphatidylglycerol and diphosphatidylglycerol were identified in *B. subtilis* (10), *S. faecalis* (56), *Staphylococcus aureus* (183).

The content of RNA of bacterial membranes seems to be directly related to the concentration of magnesium in the membrane washings. By lowering the magnesium content of the solutions used

Table 1

Distribution of various lipid categories in microorganisms (modified from Law and Snyder, ref. 73)

Lipid	*Eubacteria*	*Mycobacteria*	*Fungi*	*Protozoa*
Saturated fatty acids	+	+	+	+
Monounsaturated fatty acids	+[a]	+	+	+
Polyunsaturated fatty acids	-	+, rare [b]	+	+
Cyclopropane fatty acids	+	+[c]	-	+[d]
Branched-chain fatty acids	+	+[e]	Rare	Rare
Phospholipids	+	+	+	+
Phosphonolipids	-	-	-	+
Triglycerides	Traces	Rare	+	+
Sterols	-	-	-	+[f]
Sphingolipids	Rare [g]	-	+	+
Aliphatic hydrocarbons	-	?	?	?
Saturated ethers	Rare	?	?	+
Glycolipids	+	+	+	+
Sulfolipids	?	?	?	+
Quinones	+	+	+	+

[a] A few species lack unsaturated acids, which are replaced by branched-chain cyclopropane acids.
[b] Some mycobacteria produce unconjugated polyene acids of a unique type
[c] Mycobacteria produce extremely long chain mono- or polycyclopropane acids
[d] Thus far found only in *Crithidia*
[e] The major fatty acid of mycobacterial phospholipids is 10-methylstearic acid (tuberculostearic acid)
[f] *Tetrahymena* lacks sterols but contains the triterpene tetrahymenol.
[g] Limited so far to anaerobic *Bacteroides*

in washing membranes from *B. licheniformis*, the RNA content decreased from 13-15% to 2-3% (137).

The composition of membranes from yeast species is given in Table 2. The yeast membrane lipids consist of triacylglycerols, phospholipids – lecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid – and sterols. Under certain conditions ergosterol is the major sterol component of yeast membrane lipids (rather than its precursor Δ5, 7, 22, 24 (28)-ergostatetraene-3β-ol) (55). Other sterol commonly found in yeast membranes is zymosterol. A large proportion of the sterols is found in the esterified form. Hunter and Rose (55) reported also the occurrence of cardiolipin and squalene in yeasts and Baraud, Maurice and Napias (2) identified galactosyldiglycerides in the plasma membrane from *S. cerevisiae*. Mitochondria from *S. cerevisiae* resemble mammalian mitochondria in their oxidative activity (79) but differ in their lipid composition. Yeast mitochondria contain a lower proportion of phospholipids and a higher proportion of triacylglycerols as well as a higher proportion of mono-unsaturated fatty acids in the place of the poly-unsaturated fatty acids of mammalian mitochondria.

Table 2

Chemical composition of membranes from *Saccharomyces cerevisiae* and *Candida utilis* (modified from Hunter and Rose, ref. 55)

	S. cerevisiae (13)	*C. utilis* (40)	*S. cerevisiae* (78)
Protein	46-47.5	38.5	49.3
Lipid	37.8-45.6	40.4	39.1
DNA	0.97	0	-
RNA	6.7	1.1	7.0
Carbohydrate	3.2	5.2	4.0-6.0
Ergosterol	5.6	-	6.0

The proteins present in the membranes of microorganisms (128) follow the same pattern of other systems, that is they comprise molecules of several molecular weights (66). Protein distribution is reproducible for a given type of membrane but differs for different membranes. Mirsky (96) counted 12 bands in gel electrophoresis of proteins from *B. megaterium* membranes but this is probably an underestimate of the real number because of the limitations of the technique employed. About three times as many bands were detected for *B. licheniformis* membrane proteins (128).

Carbohydrates are minor components of the membranes from microorganisms. In mycoplasmas they can determine the antigenic specificity of the

cell (83). In bacteria most of the surface antigens are located in the cell wall and capsule which coat the cytoplasmic membrane. In yeasts a detergent-extracted membrane preparation by the method of Matile, Moor and Muhlethaler (87) liberated globular particles composed of mannan-protein. These particles could be seen through electron microscopy of freeze-etched membranes.

a — Chemical composition and the environment — There are several reports on the influence of the environment on the chemical composition of microbial membranes. Some reports suggest that depending on their composition membranes are more resistant to osmotic lysis. Rod-like forms rather than the usual spherical forms appear in the lysozyme treated suspensions of *B. megaterium* and *B. subtilis* provided the cells are in pH 5. Rod-like forms are more resistant to osmotic lysis than the usual spherical protoplasts formed at neutral pH (57,108). The change in shape was correlated to the replacement of phosphatidylglycerol by glucosaminylphosphatidylglycerol in the membranes of *B. megaterium* and by the lysil derivative in *B. subtilis.* In *S. faecalis* and *S. aureus* (52,53) the lysil ester of phosphatidylglycerol accumulated at acid pH values. In addition, *S. faecalis* forms the alanyl ester of phosphatidylglycerol which is the main phospholipid irrespective of the pH value (68). When anaerobic growing cells of *S. aureus* were allowed to change to an aerobic environment the amount of phosphatidylglycerol in the cells increased 2 fold, that of diphosphatidylglycerol increased 1.6 fold whereas the amount of lysilphosphatidylglycerol remained constante (37).

Depending on the conditions of growth the fatty acids of the cytoplasmic membranes from several Gram-positive bacteria — *B. licheniformis, M. lysodeikticus, Sarcina lutea* (22), *S. aureus* (183), *B. subtilis* (10), and *B. megaterium* (190) — are *iso* or *antiiso* C_{15} branch-chain acids: the ratio of *antiiso* to *iso* forms depends on the availability of precursors in the growth medium (61,173).

Mutants of *E. coli* unable to synthesize unsaturated fatty acids are also unable to grow in the absence of an appropriate supplement of those acids (156). Membrane phospholipids from such cells contain a much lower percentage of unsaturated residues, resulting in disturbances of several functions of the cell including membrane transport (38). The extreme case of replacement of membrane lipids by incorporation of externally supplied fatty acids occurs in mycoplasmas. *Mycoplasma laidlawii* will tolerate a replacement of nearly 90% of membrane acyl residues by stearic acid saturated groups (166).

Temperature is another environmental condition which frequently alters the lipid composition of membranes. Fatty acyl chains of several organisms become richer in unsaturated groups as growth temperature is lowered. While increasing the growth temperature does not result in the increase of the average chain length of the fatty acids, the amount of branching decreases (26).

b — The membranes of mycoplasmas — Due to the importance of these microorganisms in the study of the fundamental aspects of membrane structure a more detailed description of the composition of their membranes is given below.

Mycoplasma membranes have 50-59% protein, 32-40% lipid, 0.5-2% carbohydrate, 2-5% RNA, and about 1% DNA (120,121). The RNA and DNA were regarded as contaminants in the membrane preparations. Engelman and Morowitz (33) observed that glucosamine and galactosamine can be present in amounts as large as 5% in *Acholeplasma laidlawii* membranes as blocks of long-chain polysaccharides (98). Membrane-incorporated glucosamine does not seem to be covalently attached to membrane proteins. Apparently, it is utilized in the synthesis of a polymer weakly associated with the membrane which may reside on the exterior surface (170). Polyacrylamide gel electrophoresis of mycoplasma membrane proteins revealed 20 to 30 bands (83). The protein amino acid composition in mycoplasmas as compared with those in erythrocytes and *B. megaterium* is given in Table 3.

The polar lipids of *M. laidlawii* can be divided in the following fractions: 1) phosphatidylglycerol (36%); 2) *O*-amino acid ester of phosphatidylglycerol (6%); 3) monoglucosyldiglyceride (14%); 4) diglucosyldiglyceride (32%) and 5) a phosphorylated derivative of monoglucosyldiglyceride (the phosphate is esterified on one of the hydroxyls of glucose) (12%). Most of the polar lipids are glycolipids rather than phospholipids (174). Cholesterol represents 12-30% of the total lipid (1).

Membrane lipid and protein syntheses are not synchronized in non-growing cells of *A. laidlawii* (60). Accordingly, since mycoplasma membrane lipids can be drastically altered by growing the organisms with appropriate fatty supplements, it seems that proteins and lipids are largely independent in these membranes.

Mycoplasmas are more osmotically stable than bacterial protoplasts (122). By enriching mycoplasma membranes with specific fatty acids both characteristics — rate of glycerol permeability (89) and osmotic fragility (126,144) — could be associated with the predominance of a certain type of fatty acid in the following order: linoleic (18:2) > oleic (18:1 *cis*) > eladic (18:1 *trans*). *A. laidlawii* is much more osmotically fragile than other *Acholeplasma* species (83).

Table 3

Amino acid composition of membranes (in mole%) (after Maniloff and Morowitz, ref. 83)

Amino acid	*Mycoplasma gallisepticum*[a]	*Acholeplasma laidlawii* A[b]	B[c]	B[d]	*Erythrocytes*	*Bacillus megaterium*
Lysine	9.38	7.1	6.37	5.77	5.17	6.7
Histidine	1.41	1.4	1.46	1.32	2.31	1.6
Arginine	3.41	3.0	2.95	2.76	4.74	3.3
Aspartic acid	12.32	12.5	11.43	12.30	8.94	8.8
Threonine	6.21	7.5	6.77	7.90	5.75	5.7
Serine	6.70	6.4	6.40	7.44	7.20	5.5
Glutamic acid	10.34	9.4	8.23	9.59	13.34	7.2
Proline	4.42	3.9	3.60	4.29	4.84	5.0
Glycine	6.60	8.1	6.95	8.31	6.68	7.8
Alanine	7.48	8.1	8.23	9.62	7.71	7.0
Cysteine	0.30	tr	0.19	0.12	1.15	0.1
Valine	7.31	7.0	7.55	5.18	6.57	7.0
Methionine	1.83	2.3	2.33	2.15	1.86	2.3
Isoleucine	6.11	7.0	7.39	5.27	4.71	6.6
Leucine	8.60	9.5	9.79	8.96	11.50	8.6
Tyrosine	3.93	4.1	4.81	4.36	2.36	2.1
Phenylalamine	4.54	4.8	5.40	4.70	3.95	4.6
Tryptophan	-	2.3	-	-	1.43	-
Amide	-	6.4	-		-	3.3

[a] Cells grown on Tryptose medium
[b] Cells grown on peptone-yeast extract medium
[c] Cells grown on Tryptose medium
[d] Cells grown on beef-heart infusion medium

Traditional models for membrane structure and molecular organization

During the past years the generally accepted model for membrane structure has been the one proposed by Davson and Danielli (25,27). In this model lipids are disposed in a bilayer sandwiched between protein monolayers. Robertson (131) accepted the general idea of the Davson and Danielli model and in a recent review (132) points out the following aspects of membrane structure:

1. Lipids in membranes are in a liquid state and the ratios of different types of lipids are variable;
2. Lipids and proteins are bound electrostatically with the mediation of divalent cations;
3. Monolayers of proteins cover the lipid bilayer but the molecular architecture of membranes may be asymmetric i.e. there may be differences in the composition of the inner surface as compared with the outer surface of membranes.

Such a model is subject to criticism as discussed later.

In 1964 Benson (5) proposed an opposing model in which the membrane is made of closely packed globules of protein inserted randomically with lipid molecules. The main interactions among proteins and lipids were supposed to be hydrophobic in nature (5,6). This model does not accept the lipid bilayer as a common feature in all membranes. A third model suggests that membranes are composed of discrete subunits (45,160) without establishing any restrictions as to the nature of the lipid-protein interactions thus being in accordance with Benson's model.

In order to propose their models for membrane structure authors gave different interpretations to the available experimental data. Some proved to be quite illusive as it is the case with the interpretations to electron micrographs of membranes. In conventional electron microscopy of thin sections of fixed materials treated with heavy metals, membranes appear in a characteristic rail-road feature. A pair of dense lines (approx. 20Å thick) are separated by a light central zone approx. 35Å wide. This structure has been called a "unit membrane" (133) and should correspond to the proposed model of membrane structure by Davson and Danielli. The dense lines represented the protein monolayers

and the light central zone the lipid bilayer.

A specimen to be analysed in an electron microscope is often fixed with aldehydes like formaldehyde or glutaraldehyde. These aldehydes cross-link proteins making them insoluble. In addition the material can be fixed with OsO_4 or potassium permanganate previously to the addition of heavy metal atoms which will provide a high contrast to the preparation. Fixation with OsO_4 and with $KMnO_4$ may however alter markedly the structure of membrane proteins (75,76). The mechanism of the staining reactions after OsO_4 or $KMnO_4$ fixation is only partially understood (69). It is possible that staining occurs due to a reaction with membrane hydrophilic and not with hydrophobic groups no matter whether these groups are provided by proteins or lipids (157). It is noteworthy that membranes previously extracted for lipids have a staining pattern similar to that of the "intact" membrane. These arguments preclude a clear interpretation to the electron micrographs in support of the Davson-Danielli model.

Conflicting results arose also with preparations of membrane components. To provide an experimental basis for the Benson's model several attempts were made to isolate what should be the basic lipoprotein of the membrane. This reasoning followed a similar suggestion for a "structural protein" by Richardson, Hultin and Green (130). Razin, Morowitz and Terry (125) claimed to have isolated a lipoprotein subunit for the membrane of *Mycoplasma laidlawii*. This molecule was thought to be homogenous showing a single 3.3S schlieren peak when analysed in an ultracentrifuge. Engelman, Terry and Morowitz (34), however, showed later that components of the sodium dodecylsulfate-solubilized membrane could be resolved in more than one fraction by prolonged centrifugation in sucrose gradients.

Proteins exist in biological membranes which are closely associated with lipids. Such interactions may determine the native conformation of the protein. However, no matter how frequent is a particular association of this type in a membrane, there is no evidence so far for repeating lipoprotein subunits taking part in building up the membrane structure. If a close and often specific association of proteins and lipids sometimes occurs as in the case of lipid-dependent enzymes, it is also true that proteins and lipids are relatively free to undergo independent conformational changes in situ, translational migration, or replacement by exogenously supplied molecules not necessarily identical to the original ones present in the membrane (159). These observations are rather difficult to reconcile with the Davson-Danielli model in which the electrostatic interactions between proteins and lipids would confer a less versatile type of association and hence little independence of proteins to lipids. The dynamics of lipids and proteins in biological membranes is further discussed in another section.

New Methods for studying membrane structure

Recently, several methods have been introduced for the study of membrane structure and molecular organization. Among these methods the following gave a significant contribution to a better understanding of membranes: electron microscopy of freeze-etched materials, nuclear magnetic resonance spectroscopy, electron-spin resonance spectroscopy, fluorescence methods, circular dischroism and optical rotatory dispersion, X-ray diffraction, calorimetry in artificial and natural membranes, the action of phospholipases, immunochemical and other surface reactions. This section will briefly review these methods.

a — Electron microscopy of freeze-etched materials — This method was introduced by Steere (164). It was further studied by Moor & col. (97) and by Pinto da Silva and Branton (115). A fresh specimen is frozen in liquid Freon 22. The frozen specimen is fractured under vacuum at $-100^{\circ}C$ with a microtome knife and this is followed by sublimation of some of the water of the fractured surface. This surface is then shadowed with metal (e.g. evaporated platinum) and replicated. The fracture on the frozen specimen occurs at the point of greater weakness in the structure. Through electron microscopy of freeze-etched replicas a characteristic feature is evident for membranes from several types of cells, including microorganisms: a mosaic-like structure with a smooth matrix studded with fairly uniform-sized particles (15). It is probable that most of these particles are integral proteins deeply intercalated in the interior of the membrane and dispersed in the lipid matrix (157).

Micrographs of chloroplast lamellae prepared by the freeze-etching procedure seem to indicate that the cleavage plane occurs at the lipid-lipid interface of the lipid bilayer (16). Since the preparations show the outer surface of the membranes and also expose the inner hydrophobic face it is possible to study the topographic distribution of the particles referred to above. The inner face while smooth in the case of myelin (14) had, in chloroplast membranes, the same pattern of globular particles forming a mosaic as generally observed on the outer surface of most membranes. This is compatible with a structure in which globular proteins intercalate with the hydro-

carbon portion of the lipid bilayer rather than coating the bilayer at the periphery. Through the observation of freeze-etched preparations one can also study the surface orientation of components of the membrane whether towards the outer aqueous phase or towards the inner hydrophobic non-aqueous phase. In the study of the surface orientation of glycoproteins in human erythrocytes a phytohemagglutinin from *Phaseolus vulgaris* conjugated to ferritin was used to map the distribution of the glycoprotein receptors over the surfaces of intact erythrocytes. Cells were then freeze-etched and observed in the electron microscope. The phytohemagglutinin reacted on sites of the membrane overlying intramembranous particles. No label was found covering the particles of the inner face of the membrane. These observations suggest that the glycoprotein is oriented so that the oligosaccharides are exposed to the aqueous environment while the C-terminal segment of the protein interacts with other components in the interior of the membrane to form intramembranous particles (84)

b — Nuclear magnetic resonance spectroscopy — The nuclei of the constituent atoms of a given substance may behave like charged spinning bodies thus possessing a non-zero nuclear spin I. The following nuclei among others have this property: ^{1}H (I = 1/2), ^{2}H (I = 1), ^{13}C (I = 1/2), ^{14}N (I = 1). These nuclei behave like small magnets which can be lined up when placed in a magnetic field. In order to have magnetic properties the nuclei must have either an even mass but odd atomic number or an odd-numbered mass. Nuclei such as ^{12}C, ^{16}O and ^{32}S with an even mass and atomic numbers do not have magnetic properties. When a nucleus has a spin I of 1/2 — as is the case with the ^{1}H nucleus — it means that the magnitude of its magnetic moments can have only two equal but opposite values that correspond to spin quantum numbers +1/2 and -1/2. Nuclei with magnetic properties will assume different orientations depending on whether the nuclear magnets are aligned with or against the externally applied field. The energy required to change the original alignment of the magnetic nuclei when an external magnetic field is applied can be measured through nuclear magnetic resonance (NMR) spectroscopy. While the absorption of U.V. and visible radiations is followed by alterations in the electronic states of the molecule, and that of infra-red radiation by alterations in the vibrational--translational states, in the NMR spectroscopy radiation involved is in the radio-frequency or microwave region and affects the rotational energetic states of the molecule. Transitions observed in NMR spectra are between states separated by extremely small energy differences. The difference in energy between the states is proportional to the strengh H_0 of the applied magnetic field at the nucleus. It can be expressed as $\Delta E = \mu H_0/I$ where μ is the magnetic moment. Absorption of energy by the spins occurs when a radiofrequency field H_1 of frequency v_0 is applied perpendicular to H_0 but only when $hv_0 = \Delta E = \mu H_0/I$ where h is Planck's constant. NMR data can then be expressed in field units (H_0) or frequency units (v_0).

When an external magnetic field is applied at a compound it causes the local electrons to precess about the direction of this field. This precession which opposes in direction to the external field generates a local magnetic field the magnitude of which is approximately 10^6 times smaller than that of the external field. The magnitude of the local magnetic field depends upon the number of electrons surrounding the nucleus as well as on the molecular or bonding orbitals in which they exist. The magnetic field strength of a particular nucleus is then influenced by contributions from sources other than the nucleus itself. These small changes in the field cause the nucleus to absorb at a frequency slightly different from that initially predicted. The diamagnetic shielding effect is a measure of the magnitude of the locally induced fields which cause a displacement in the position of the resonance signals. Such a displacement is called chemical shift. Since the magnitude of the absolute shift is relatively small, the absolute value is multiplied by 10^6 thus giving the chemical shift in parts per million (ppm). This indicates a shift in the position of resonance of a given number of gauss per million gauss. Chemical shifts are measured with reference to a standard. For protons in organic molecules the common standard is tetramethylsilane $(CH_3)_4Si$.

The NMR spectra of complex molecules or structures like cell membranes will show signals from several groups which may have the characteristics of a high-resolution spectrum depending on the relative freedom of these groups in relation to adjacent groups and molecules. In simple lipid-water systems when lipids though in a bilayer arrangement are forming large aggregates no high-resolution NMR spectrum is obtained. Sonication of the lipids in this state is accompanied by a high-resolution NMR spectrum (19). In such systems — e.g. water dispersions of phosphatidylcholine or phosphatidylserine — the interaction of lipids and peptides can be studied. This has been done with cyclic polypeptides: valinomycin, alamethicin and gramicidin S. In presence of alamethicin the hydrocarbon chain signal is broadened due to a reduction in motion of the lipid chains (20). The effect of alamethicin (as well as of valinomycin but not of gramicidin S) is due to the interaction of the polypeptide with the hydrophobic parts of the lipids. Each molecule by sort of a cooperative action appears to inhibit the motion of a considerable number of lipid chains. In contrast high resolution signals from the $(CH_2)_2$, (CH_3) and N^+ $(CH_3)_3$ groups of the lipids are observed when biological membranes are treated with denaturing agents like trifluoroacetic adic and sodium dodecylsulfate. The NMR spectra of erythrocyte membrane preparations revealed that (21):

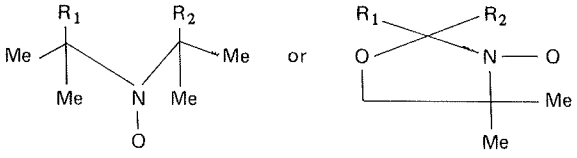
a — The protons of the sugar residues are located in an environment which allows considerable freedom of movement as interpreted by the narrow signals at 6.3 and 7.8 ppm.

b — The protons of the $N^+(CH_3)_3$ groups of lipids are in an environment which also allows freedom of molecular motion.

c — The lipid hydrocarbon chains in the membrane are restricted in their freedom of molecular motion; this is probably due to the effect exerted by adjacent lipid molecules or proteins.

These results suggest that most of the polar groups of the lipids as well as the carbohydrate residues of the proteins are directed to the aqueous environment thus keeping their freedom of molecular motion. The hydrocarbon chains of lipids should be restricted in molecular motion because they are closely packed through hydrophobic interactions with other lipids and with the hydrophobic portions of proteins. If the Davson-Danielli's model is true then a completely different result should be obtained as far as the relative freedom of the polar groups of lipids is concerned. These groups should be restricted in their molecular motion because of the ionic bonding with the charged groups of the proteins. This effect can be achieved in artificial systems with high concentrations of gramicidin S. In a system consisting of a sonicated aqueous dispersion of phosphatidylcholine, gramicidin S causes the choline signal to be reduced indicating that the polypeptide interacts primarily with the head polar groups of lipid molecules (20).

c — Electron spin resonance — Another useful spectroscopic method in the study of the molecular organization of membranes is the electron spin resonance of materials labelled with paramagnetic reporter molecules (spin-labels). The label can be either covalently attached to the normal groups of the membrane constituents or be diffused into certain hydrophobic regions in the case of label molecules with a non-polar moiety. Common labels are certain organic free radicals like the nitroxides with the general structure:



The N-O group has the unpaired electron that renders the molecule paramagnetic and hence detectable by electron spin resonance spectroscopy (ESR).

An unpaired electron has a spin and a magnetic moment with two possible orientations in a magnetic field corresponding to the magnetic quantum numbers +1/2 and -1/2. The states defined by the two orientations differ in

$$\Delta E = (h\gamma/2\pi)\ H$$

where γ is the gyromagnetic ratio of the electron. The frequency of the radiation being absorbed by the transition is given by

$$v = (\gamma/2\pi)\ H$$

For free electrons γ is about 10^3 times larger than that for the protons; the frequency is then 10^3 times that of protons at the same magnetic field.

When the spin label is introduced into the membrane it will give signals according to the orientation and spatial freedom of the label as well as to the polarity of the environment. The motion of doxyl (4',4' - dimethyloxazolidine-*N*-oxyl) - fatty acids — such as the 5-doxylstearic acid — was studied in membranes of *Mycoplasma* organisms (143). As it was emphasized before, cells of *Mycoplasma* offer a good model for these studies because the composition of their membranes can be easily altered by growing the organisms in appropriate media. Membranes with a high degree of unsaturation of the lipids were obtained when *Mycoplasma* thrived in presence of *cis*-Δ^9-octadecenoic acid at low incubation temperatures. Membranes with high and low degrees of unsaturation could then be compared in their ability to limit the motion of the previously diffused-in spin labels. The mobility of the label was higher in membranes with a high percentage of unsaturated lipids than in membranes with more saturated lipids (58). In addition membranes containing the *cis* isomer of Δ^9-octadecenoic provided an environment in which the spin label was more mobile than when the *trans* isomer predominated. Among several conclusions that could be drawn by studies of the mobility of spin labels the fact that the lipids (at least in part) in biological membranes have a fluid nature is certainly one of basic importance.

d — Thermal transitions — Steim & col. (166) studied the absorption of heat by lipids in a bilayer. Curves were obtained which could be correlated to the melting of the carbon chains of the lipids in the bilayers. Isolated membranes from *Mycoplasma* when studied bu this technique exhibited the same characteristic curves of lipid bilayers.

The technique involves a differential thermal analysis in which the sample is heated along with an inert material and the temperatures of the two materials recorded. If a thermal reaction occurs in the sample a differential temperature increase is measured in relation to the reference material. Peaks so recorded can indicate whether the transition is endothermic or exothermic. Upon studying the transition temperatures in membranes from *Mycoplasma laidlawii* it was concluded that at least 75% of the membrane lipids contributed to the transition: such a result is in accordance with an extensive bilayer of lipids in the membrane structure.

e — X-Ray diffraction — Interference between scattered x-rays is observed when $2D\sin\theta = n\lambda$, where D is the distance between electron-dense regions of the sample, λ is the wavelenght of the x-ray source (usually 1.54Å for ^{2}Cu radiation), and 2θ is the angle between the incident beam and n^{th} maximun of the scattered beam.

For hexagonally arranged lipid chains the size of the ordered region is determined by the width of the inter-hydrocarbonchain diffraction bands. The interchain distance in phospholipids is approximately

4.8Å for hexagonal packing. Such a structure gives by the x-ray diffraction method a sharp peak at D = 4.2Å. Upon increasing temperature the hydrocarbon packing is more irregular and a diffuse scattering maximum at 4.6Å replaces the sharp 4.2Å peak. Similarly, a lipid phase change is evidenced by a change of the high-angle peak from the sharp 4.2Å hexagonal packing diffraction to a broad 4.6Å peak (64).

Thermal phase transitions as detected by x-rays have been studied in *A. laidlawii* B and *E. coli* K12 membranes (31,32,35).

f — Fluorescence — Emission of light can occur when an electron returns from an excited state (e.g. π, π^* or n, π^*) to its ground state. Excited states are formed upon introducing energy through light or chemical reactions. The energy of the fluorescent light is generally lower than that of the absorbed light and hence emission will take place on the long-wave-length side of the absorption band. This can be expressed by

$$E = h\nu = h\frac{c}{\lambda}$$

where E is the energy of the transition, h is Planck's constant, v is the frequency of light, c is the velocity of light and λ the wavelength of light. The energy of the excited state can be transferred to another molecule by collisional quenching or by a vibrational coupling interaction between the excited states of two molecules, one acting as the energy donor and the other as the acceptor. This can be summarized as follows:

1 — $A \xrightarrow{h\nu} A^*$ A = ground state molecule, A* = excited state molecule

$A^* \longrightarrow A + h\nu_1$ (fluorescence)

$A^* \xrightarrow{Q+} Q + A$ (quenching)

Q = quenching molecule
v and v_1 = frequencies of and emitted light

2 — $A \xrightarrow{h\nu} A^*$

$A^* + B \longrightarrow A + B^*$

$B^* \longrightarrow B + h\nu_1$ (fluorescence)

A* = excited state donor
B* = excited state acceptor

While the quantum yield of the intrinsic fluorescence of proteins is low — possibly due to collisional quenching energy transfer reactions — extrinsic fluorescence can be introduced in proteins through covalently or non-covalently bound fluorescent probes. The most employed is l-anilino-naphthalene-8-sulfonate which has a very low fluorescence quantum yield in solution; when this substance binds to proteins, however, the quantum yield of the dye increases up to 200-fold (167).

1-Anilino-naphthalene-8-sulfonate interacts with erythrocyte membranes (36) probably by dissolving into the membrane lipids. Such an interaction could be related then to the hydrophobic phase determined mainly by lipids: one can predict that treatment of membranes with lipases will reduce the fluorescence quantum yield of the dye. These properties make fluorescence techniques quite useful in determining small conformational changes in the membrane. Probes like 1-anilino-naphthalene-8-sulfonate respond to localized changes and indicate transitions in proteins that are not detected by other methods. On the other hand, intact structures or cells can be examined by this procedure (20).

g — Optical rotatory dispersion and circular dichroism — When plane-polarized light passes through a solution of an optically active compound the plane of polarization is rotated. The angle of rotation is a function of the light's wavelength — the differences in rotation with varying wavelengths determine the optical rotatory dispersion. When the light of a given wavelength is absorbed by the molecule the curve of rotatory dispersion becomes inverse. Plane-polarized light can be resolved in two circularly polarized components by passage through an appropriate quarter wave plate (for details see ref. 7). When light passes through an optically active material one of the circularly polarized components may be absorbed to a greater extent than the other. In consequence the resolution of this unequal absorption will be characterized by a vibration which is no longer linearly polarized but rather elliptically polarized. In circular dichroism the molar ellipticity is proportional to the difference in the molar extinction coefficients for the two hands of light — $\epsilon_l - \epsilon_r$ (*). The relation between the ellipticity and the difference in the molar extinction coefficients for left and right circularly polarized light is

$$[\theta]_\lambda = 2.303\,\frac{4500}{\pi}\,(\epsilon_l - \epsilon_r) = 3300\,(\epsilon_l - \epsilon_r)$$

where $[\theta]_\lambda$ is the molecular ellipticity at a given wavelength λ. In practice the difference in optical density for the two circularly polarized components, which is measured directly, is converted to a difference in extinction coefficients. The main difference between the optical rotatory dispersion and circular dichroism spectra is that ORD measurements can be made at wavelengths distant from the absorption bands while CD spectra are confined to wavelengths of the absorption bands.

CD spectra of polypeptides are influenced by their conformation so that three spectra are obtained in the case of the α-helix, the antiparallel pleated sheet or β-conformation, or the random coil structures.

The general features of the ORD spectra of α-helical and random coil polypeptides are known (8,12). A characteristic trough at 233nm is observed with the right-handed α-helix with a value of $[m'] = -15000$ deg cm^2/decimole. The CD spectra of α-helical and random coil polypeptides were recorded by Holzwarth *et al.* (50,51). The helix of helical polypeptides usually shows bands at 222, 206 to 207 and 190 to 192nm with maximum ellipticities of -29000 to -31000, -26000 to -31000 and 52600 to 55000 deg cm^2/decimole respectively (8). The ORD and CD spectra of red blood cell membranes (74), Ehrlich ascites tumor plasma membrane (182), mitochondria and sub-mitochondrial particles (178) show several features in common. In the ORD

spectra a curve resembling that of the right-handed α-helix is observed in the far U.V. with the characteristic 233nm trough displaced by 4 to 6nm: the depth of the trough at 237 to 239nm suggests a helix content of 20 to 25% in membrane fragments and about 5% in whole mitochondria. The CD spectra show also a shifted n-π^* band located at 225mn rather than at the characteristic wavelength of 222nm. Wallach and Zahler (182) have suggested a model of hydrophobic interactions and helical segments which may account for the shift in the n-π^* trough. Urry, Mednieckes and Bejnarowitz (178) proposed that the optical activity of membrane phospholipids may contribute to the observed anomalies in the spectra.

Recently, Singer (157) reviewed the data so far obtained in the study of the optical activity of membrane proteins. At least 40% of the proteins in biological membranes have a helical structure being in a globular form. Since globular proteins have variable but significant amounts of β-structure it was not a surprise to find some β-structure in the proteins of intact membranes (181). Infrared studies (82) indicate also the absence of significant amounts of β-structure in membrane proteins. As discussed by Singer (157) the Davson-Danielli model predicts a predominant β-structure conformation of membrane proteins which opposes the ORD and CD results.

h – Action of phospholipases – The most promising phospholipase for studying membrane structure is phospholipase C because unlike other phospholipases it does not liberate products with detergent properties. Phospholipase C catalyses the hydrolysis of phospholipids liberating the phosphorylamine moieties. Lenard and Singer (76) showed that a crude preparation of phospholipase C liberated about 70% of the phosphorus from intact red cell membranes without disrupting the membrane. Circular dichroism spectra of the membrane proteins were not altered after phospholipase C treatment. Two major conclusions could be drawn from these experiments: a) the polar heads of the phospholipids in intact membranes are accessible to the enzyme and are probably not linked electrostaticlly to the proteins; b) if there are electrostatic bonds between lipids and proteins they are not important for stabilizing the membrane since the removal of 70% of the polar heads from the lipids leaves the membrane undisrupted. The fact that the CD spectra of the membrane proteins are not altered after phospholipase C treatment suggests that proteins and lipids are somewhat independent and thereby an alteration in the lipid constituents is not necessarily followed by a conformational change in the proteins.

i – Immunochemical and other surface reactions – Surface reactions are useful to locate certain components of the membrane orienting their active groups to the outside milieu. If surface reactions are coupled with electron microscopy techniques one may also discern whether a certain component detected by specific reactions is present on the inner or on the outer surface of the membrane. In fact, by observing the whole membrane sac with certain parts of the preparation folded back it is possible to analyze at the same time both the inner and the outer surfaces. By use of concanavalin A – which reacts with α-D-glucopyranosyl or α-D-mannopyranosyl residues (118) – labelled with ferritin it was possible to demonstrate that the oligosaccharides in membranes are directed to the outside milieu and are asymmetrically distributed (105). Depending on the presence of suitable receptors, ricin (which reacts with β-D-galactopyranosyl and sterically related residues) can also be used in such experiments. Ferritin conjugates of concanavalin A and ricin have been used to locate covalently-bound saccharide residues on membrane fragments from a myeloma-cell homogenate (48). The ferritin-concanavalin A conjugate appeared bound to the inner surface of the smooth-membrane vesicles as well as to a single surface of the membrane fragments of the rough endoplasmic reticulum. The results obtained support the scheme suggested by Palade (111) for the biosynthesis of membranes: a membrane "assembly line" is the precursor in the formation of vesicles which fuse with the previously existing cell membrane in such a way that the inner surface of the membrane vesicles become the outer surface of the newly synthesized cell membrane (48).

To determine the distribution of specific proteins in red cell membranes several immunological systems coupled to electron microscopy were used. Indirect systems using human red cells 0, $Rh^+ + I^{125}$ -antibodies to the Rh_0(D) antigen + ferritin-conjugated goat antibodies to human γ-globulin (104) or mouse erythrocytes + antibodies to the H-2^b histocompatibility alloantigen + ferritin-conjugated antibodies against 7S mouse γ-globulin (103), were used. In the former system ferritin was distributed in discrete clusters in random array suggesting that the Rh_0(D) antigen – an integral protein (43) – is distributed randomically throughout the human erythrocyte membrane. In the latter system clusters of ferritin formed patches on the membrane outer surface.

Immunological techniques were also used to prove the mobility of certain antigens in the membrane. This mobility would provide support for a more dynamic structure of biological membranes. Human and mouse cells were induced to fuse – as heterokaryons – by use of a Sendai virus as the fusing

(*) The term Cotton effect includes the three related phenomena: circular dichroism, inverse curve of rotatory dispersion and ellipticity.

agent. Immunofluorescence techniques were used to follow the distribution of the antigens from both cells. After fusion of the cells, mouse and human antigens were initially segregated in different halves of the heterokaryon membrane; on incubating at 37°C the antigens gradually intermixed. By lowering the temperature below 15°C the intermixing was inhibited. It was suggested that the intermixing of antigens resulted from the diffusion of components within the membrane (39). Diffusion of the antigens (proteins) depended on the relative fluidity of the lipid phase which should have an apparent viscosity about 10^3 to 10^4-fold that of water (159). Lateral diffusion of lipids also occurs. The rate at which lateral diffusion of lipids takes place was determined by Kornberg and McConnell (70,71). Phospholipids have an estimated neighbour exchange rate of less than 10^{-6} second. Such a motion is slightly affected by adding cholesterol to the bilayer (29). Lipids as well as proteins do not flip-flop or rotate accross the membrane. Lateral motion or rotaion of these components in the plane of the bilayer is a likely event (17).

Dynamics of lipids and proteins in biological membranes

The interaction of lipids and proteins on one side and the relative independence of these components in biological membranes on the other side are fundamental aspects to be accounted for in the proposition of a model for the structural organization of membranes. The changes in the conformation of membrane proteins induced by external ligands is another aspects motivating further study: such changes may influence cell's normal physiology and viability.

Some membrane-bound enzymes and antigens require lipids, usually specific phospholipids, for the expression of their activities (177). This requirement suggests a close association of proteins and lipids which however may not be a general characteristics of the majority of membrane proteins. Indeed the experiments with phospholipase C suggest that phospholipids and proteins do not generally interact strongly; they appear to be largely independent (159). It is possible that in most cases lipids act mainly as a matrix in which proteins embed. In such a structure proteins are free to undergo translational diffusion within the membrane. One imposing characteristics of membrane proteins besides the fact of assuming a globular conformation is the presence of a larger percentage of non-polar groups in their amino acids. This has been confirmed for several membrane proteins which generally contained 47 to 60% of non-polar amino acids, about 28 to 34% of acidic and polar amino acids, and 7 to 11% of basic amino acids (46). Evidence that a hydrophobic region in red-cell membrane glycoproteins does exist was obtained by Winzler (186). A hydrophobic sequence of 23 residues has recently been determined in these molecules (152).

Since the effective viscosity of the lipid bilayer varies depending on the particular lipid composition of membranes, the migration of proteins in the case of translational diffusion may occur at different rates. It is also possible that conformation changes of proteins only take place at a certain range of viscosity values for the lipid bilayer. It is well known that the presence of double bonds in the alkyl chains lowers the melting point of the lipids. Double bonds provide a steric hindrance to the tight packing of alkyl chains and in consequence the ordered arrangement of methylene groups. The axial symmetry of the alkyl chains of phospholipids thus disturbed by the occurrence of *cis*-double bonds permits a lower temperature for a order-to-disorder phase transition. The strict correlation between the physical state of membrane lipids and the biological activity of cells may explain why fatty acyl chains of bacteria, fungi, plants, insects and marine organisms become richer in unsaturated groups as the growth temperature is lowered (47,77,86,112). The organisms are able to adjust their membrane lipid composition so as to prevent lipid fluidity from decreasing below a level critical to the favorable functioning of membrane-bound enzymes. The transition temperature is usually slightly below the growth temperature: cells try to grow on the disorder side of the order-disorder phase transition (90).

The correlation of lipid fluidity and the physiological state of membranes has been questioned recently by Oldfield (107). Some organisms contain crystalline or gel-state lipids in addition to the fluid liquid crystalline domains. The plasma membranes of *A. laidlawii* B and *E. coli* undergo reversible thermotropic gel-to-liquid crystal phase transitions of low cooperativity (90,165,166). In unsupplemented or stearate-supplemented cells of *A. laidlawii* B, the thermal transition encompasses the growth temperature – 37°C. The transition extends from 35 to 73°C. As discussed by Oldfield (107) lipids are predominantly in a crystalline gel state at 37°C. For *E. coli* thermal transition extends from 15 to 43°C. Both crystalline gel ("solid") and liquid crystalline ("liquid") phases coexist on the transition. Engelman (32) by use of x-rays observed also that the termal phase transition in palmitate-supplemented membranes from *A. laidlawii* B encompasses the growth temperature of 37°C. Some microorganisms particularly those that lack cholesterol appear to be capable of growing with large quantities of their lipids in a rigid crystalline gel state (107). However, as pointed out by Singer (158) there are numerous reports establishing that the molecular components

of many cell membranes are rotationally and translationally mobile and that such mobility has profound biological significance. The evidences discussed by Oldfield (107) could also be interpreted as a limitation of the x-rays method in determining the low temperature end of the phase transition. An error of 10°C may be unavoidable thus casting doubt on whether the growth temperature (37°C) is indeed close to the low temperature end or in the middle of the phase transition (158). Recently, Petit and Edidin (113) presented evidence for the occurrence of phase separations in mammalian membranes at temperatures below physiological. Apparently the cell membrane may retain its integrity with solidified portions. It is possible that patches of solid lipids exist in mammalian membranes at 37°C. They could control the mobility of several membrane-intercalated receptors(110).

Membrane-intercalated particles can move translationally at least in some membranes as a function of pH (114) or temperature (162). The mobility of membrane components and the influence of colchicine was studied in the ciliate protozoan *Tetrahymena pyriformis* (187). As shown by freeze--etch electron microscopy colchicine impairs the temperature-induced translational and vertical mobility of membrane-intercalating particles of freeze--fractured alveolar membranes lying just below the plasma membrane of the protozoan. Presumably, colchicine which is a lipophilic drug, make membrane lipids more rigid. A similar effect has been described for cholesterol (72,142).

In the case of a more specific association of lipids and proteins it is possible that lipids act as cofactors inducing conformational changes in the proteins. Non-polar compounds usually can induce reversible changes in protein structure (185). That some associations of proteins and lipids are relatively specific is shown in several experiments in which the activity of a particular enzyme is enhanced by the addition of specific lipids. The ion-dependent ATPase activity of red blood cell membranes deprived of lipids was found to be restored with the addition of phosphatidylserine more than with other phospholipids (106,135). Dioleyl-phosphatidylethanolamine is over 600 times more effective than dioleyl-phosphatidylcholine in restoring galactosyltransferase activity in *Salmonella typhimurium* (141).

The interaction of proteins and phospholipids, as they occur in biological membranes, was also studied with sonicated phosphatidylcholine vesicles and ATPase. These vesicles do not fuse spontaneously to form larger vesicles (54). However the addition of an ATPase derived from *Streptococcus faecalis* membranes induces the formation of larger vesicles. The latter might be preceded by complete disruption of the smaller vesicles by the ATPase foloowed by reaggregation. Experiments with vesicles formed in the presence of ^{3}H-sucrose showed that this interpretation is unlike: the interaction of the ATPase with phospholipid vesicles is quite specific; the enzyme combines with the vesicles to form larger membrane vesicles which are more permeable to solute molecules (129). As opposing the ATPase--phospholipid interaction, the lytic polypeptide mellitin disrupts phospholipid multilamellae generating fragmented structures (153).

Polypeptides can interact with other polypeptides forming clusters. These may be important in the case of associated enzymatic activity. Functional clusters may be essential in respect to the biochemical activity of mitochondrial membranes. Recently, Steck (163) studied the reaction of human red blood cell membranes with several protein cross-linking reagents. Soluble monomeric proteins were not complexed under the mild conditions used but chains of oligomers (such as hemoglobin) were linked. Each agent studied (among them formaldehyde, glutaraldehyde and mild oxidants) caused alterations which could be assessed by electrophoresis of the polypeptides on polyacrylamide gels with sodium dodecylsulfate. Results suggest that some membrane polypetides may occur in specific oligomeric associations rather than in a random array (163). Soluble multimeric proteins are often allosteric, achieving their effects by quaternary arrangements of the subunits. Quaternary arrangements can occur upon binding a ligand to one of the subunits. These arrangements can on the other hand amplify and project the effects of a ligand binding so as to influence other sites at a considerable distance away from the original site of the structural change. This may be a common response in biological membranes. As discussed by Singer (157) two examples are relevant in understanding the influence of an arrangement in a multimeric protein-complex structure on its biological response and on that of another proteins of the membrane. In the first example the binding of a ligand such as acetylcholine interferes with the ion permeability of the post--synaptic membrane of a nerve-muscle junction (117). The binding of acetylcholine may induce a quaternary rearrangement of the different subunits of a protein complex molecule so as to change the ion-permeability properties of a pore extending down the central axis of the aggregate. In the second example, specific hormones stimulate the activity of the activity of the membrane-bound adenylcyclase (134). To explain this effect one might imagine the presence of two different sub-units: one is the allosteric sub-unit which reacts with the hormone on the outer surface of the membrane; the other is the catalytic sub-unit which would be on the cytoplasmic side of the membrane. The binding of the hormone to the

allosteric sub-unit would result in a quaternary arrangement affecting the catalytic sub-unit on the other side of the membrane (157).

Colicin E_1 which is adsorbed at receptor sites on the surface of sensitive bacterial cells without penetrating the cell causes the inhibition of other sites essential for oxidative phosphorylation, DNA and protein synthesis. There is indirect evidence that colicin can induce a cooperative change in the bacterial membrane: it would act as a nucleus for the aggregation of other proteins migrating from random points of the membrane. Apparently the cooperative change affects systems connected with the synthesis (and degradation) of ATP which underlie the membrane in these bacteria. The fluorescent probe 1-anilino-8-naphthalene sulfonate binds to *E. coli* showing an enhanced fluorescence. Upon adding colicin E_1 there is an increase of about 100% in the fluorescence of the bound probe (ANS). The kinetics of the colicin-induced fluorescence increase is similar to that of the ATP decrease in these bacteria. It is interesting that the phosphorylation uncoupler p-trifluoromethoxy-carbonylcyanide-phenylhydrazone also causes a large change in the fluorescence of bound ANS (24).

These are only a few experiments pointing out the dynamic role of lipids and proteins in biological membranes. This aspect will be further discussed in a forthcoming review on membrane transport.

Experiments with microorganisms supporting certain concepts of membrane structure and molecular organization

Apart from many experiments which are aimed mainly at isolating membranes and determining their chemical composition there are several other reports on the use of microorganisms in a more fundamental type of research, i.e. the determination of the structural organization of membranes. Some of these reports are discussed below.

Lipids and proteins in microbial membranes show the same dual type of interrelationship: on one hand they seem to be largely independent so that cells can build membranes within a wide range of lipid to protein ratios; on the other hand there are several examples of specific interactions of proteins and lipids necessary for their biological activity. Mycoplasmas are the choice organisms to study the independence of lipids to proteins in membranes. As stressed before the composition of lipids from *M. laidlawii* can be changed in a wide range depending on the fatty acid supplements of the medium. Membranes of chloramphenicol-treated cells of mycoplasma (60) have a significantly lower buoyant density than that of untreated cells showing taht a substancial increase in the ratio of lipid to protein had been achieved with no apparent effect on membrane function.

In the case of a lipid requirement for the expression of the catalytic activity of a membrane-bound enzyme there are several reports indicating that specific lipid-protein interactions are common in bacteria. In *Mycobacterium avium* Tobari (172) observed that the activation of a FAD-dependent malate dehydrogenase by cardiolipin was rather specific since other phospholipids (e.g. lecithin) were inactive. An unusual feature of the lipid requirments for the activity of this enzyme was the need for saturated rather than unsaturated fatty acids. On the contrary in most cases studied unsaturated fatty acids were required (59,139). Enzyme-specific phospholipid interactions might involve both polar and non-polar (hydrophobic) interactions.

The apparent independence of many proteins and lipids is reflected in the independence of their biosynthesis. This was clearly shown in *Bacillus subtilis.* Mutants of these bacteria were selected for glycerol auxotrophy and the inability to use glycerol as a source of carbon. Cells with both mutations incorporated glycerol entirely into the lipid fraction; phospholipid synthesis stopped immediately with the deprival of glycerol (94,95). The incorporation of labelled amino acids into the membrane proteins of these mutants of *B. subtilis* continued in the absence of lipid synthesis suggesting that a co-ordination of membrane protein and lipid syntheses does not occur.

Variations in the lipid composition of membranes can also be obtained by supplementing different unsaturated fatty acids to mutants deficient in fatty acid desaturase activity. Yeast mutants of this kind were obtained bearing membranes varying particularly in the positions of the unsaturated bonds along their lipid alkyl chains (65). Cultures of this mutant were obtained by supplementing with either stearolic acid or with octadecenoic acids having a *cis* double bond away 6,9 or 11 carbons from the acyl groups (30). Cells with different fatty acid compositions were then studied with spin-labelled stearic acids having a *N*-oxyloxazolidine ring located at 4, 6, 9, or 12 carbons away from the acyl group. Differences in the molecular motion of each spin label were observed as a function of the unsaturated bonds: characteristic order to disorder phase transitions were inferred from results of temperature dependence. Double bonds inhibit the ordered packing of lipid alkyl chains between the unsaturation and the terminal methyl group; the region between the acyl groups and the unsaturated sites are unaffected (30).

Experiments with a facultative thermophilic bacillus showed that the microorganism synthesized a

greater proportion of *iso*-fatty acids at 55°C than of the *anti-iso* fatty acids which predominated at 37°C (18). *Iso* fatty acids have melting points significantly higher than the *anti-iso* fatty acids of equai number of carbon atoms. Since this property could be correlated to the thermophily of the organism spin-labeling studies were conducted to determine the rigidity or viscosity of the membranes of this bacteria. The label employed was nitroxide stearate. Electron spin resonance spectroscopy showed that cells grown at 55°C formed a membrane more rigid and viscous than at 37°C.

The influence of the fatty acid composition on the distribution of membrane proteins was studied in *M. laidlawii* (116). Membrane lipids were enriched either in saturated (palmitic or stearic acids) or in unsaturated (palmitoleic or oleic acids) fatty acids. Protein composition analysed by sodium dodecyl-sulfate-acrylamide-gel electrophoresis and its relative abundance were not affected by fatty acid variation. Apparently only one high molecular weight protein was more prominent in membranes rich in saturated fatty acids than in those enriched in unsaturated fatty acids.

In membrane preparations of *Rhizobium meliloti,* Mackenzie and Jordan (81) studied the amino acid composition of membrane proteins: as expected a high percentage of hydrophobic residues emerged. Electrophoresis of proteins from *R. meliloti* membranes showed a wide range o molecular weights. These results seem to confirm that a repeating unit in the structure of biological membranes is unlike: proteins of several molecular weights interact with the lipid bilayer mainly throught their hydrophobic residues.

Membranes from animal and microbial cells are rich in membrane-bound particles which can be visualized by freeze-fracture electron microscopy. It is probable that these particles are proteins. Tourtellote & col. (175) observed a significant difference in the distribution of particles between membranes rich in saturated as compared with unsaturated fatty acids. In membranes rich in stearate the particles occupied approximately 10% of the fracture-exposed membrane surface while in oleate-rich membranes the particles occupied approximately 20% of the surface area. Further studies are necessary to reconcile the results of Pisetsky and Terry (116) with those of Tourtellote *et al.* (175) if one assumes that membrane particles are mainly proteic in nature. Recently, Tourtellote and Zupnik (176) showed that membranes from *Acholeplasma laidlawii* incubated in presence of puromycin or with the omission of amino acids had fewer particles between 50 and 100A in the hydrophobic fracture plan of the freeze-etched preparations than in that of the controls. These results suggest that membrane-bound particles are indeed proteins.

Choules and Bjorklund (23) studied the membrane protein conformation in *A. laidlawii* establishing percentages of 23 to 31% for the α-helix, 30 to 57% for the β (pleated sheet) and 13-45% for the random coil structures. Since other membranes studied did not contain significant amounts of the β-structure (180), the results of Choules and Bjorklund (23) must be taken with caution and may be due in part to the difficulty in interpreting band shifts in complex materials.

The rate of enzymatic hydrolysis of *E. coli* phospholipids either extracted from membranes or associated with the membrane protein was studied by using a purified phospholipase C of *Bacillus cereus* (88). The rate and extent of digestion of the phospholipids was the same in both preparations. It was concluded that phospholipids are readily accessible to the hydrolytic enzyme even when incorporated into the membrane structure. Although some proteins such as the glycerol 3-phosphate dehydrogenase and the succinic dehydrogenase were unaffected in their activities after the hydrolysis of 95% of membrane phospholipids in *E. coli,* other enzymes had a reduced activity following the same treatment.

Membranes from *A. laidlawii, M. gallisepticum* and *Mycoplasma mycoides* can be solubilized by detergents such as sodium dodecylsulfate (SDS). The solubilization is based on the formation of protein-SDS and lipid-SDS complexes. Upon removing SDS by dialysis membrane reaggregates are obtained with many of the characteristics of the original membrane (125,127,145). The formation of reaggregates is a function of the Mg^{++} concentration: at 20mM Mg^{++} the lipid-protein ratio of reaggregates is the same as in the native membrane. Although the fluidity of the reaggregates was comparable to that of the original membrane — as determined by electron spin resonance spectroscopy (143) — a major difference emerged: the absence of particles on the fracture face of freeze-etched reaggregated membranes (171). Assuming that these particles are proteins it is possible that in the process of reaggregation the membrane proteins were reassembled incorrectly. NMR-spectroscopy (91,92) and fluorescent probes (93) suggested that certain protein-reactive sites in reaggregated membranes were not primarily exposed in the original membrane indicating a change in conformation of some proteins or an alteration in their *in situ* interaction with lipids.

As a proof of the lack of specificity of lipids in their interaction with many proteins, one could attempt to hybridize lipids and proteins from membranes of different organisms. Hybrid membranes were successfully formed by combining lipids and proteins from *A. laidlawii* and *M. gallisepticum* (124).

The lipid globular-protein mosaic model for membrane structure

This model was conceived in order to conciliate most of the recent experimental data on membrane structure but its theoretical basis is determined mainly by thermodynamic restrictions.

There are two kinds of non-covalent interactions which are important in building up membranes: hydrophobic and hydrophilic. Hydrophobic bonds have a precise thermodynamic meaning (63). In order to proceed, chemical reactions require a net reduction in the free energy G of the system. Variations in free energy (ΔG) are defined by: $\Delta G = \Delta H - T\Delta S$ where ΔH is the enthalpy change, T is the absolute temperature and ΔS the entropy change.

How can these thermodynamic concepts be applied to membrane structure? Let us analyze the formation of lipid bilayers. A chain of $-CH_2-$ groups (as present in fatty acids) induces the formation of ordered "cages" of hydrogen-bonded water molecules around it; upon removing the $-CH_2-$ groups these cages become disordered which can be expressed thermodynamically as an increase in entropy. As the $-CH_2-$ groups are removed from water they associate themselves with a concomitant decrease in the ΔS values. However the increase in entropy resulting from the first step (i.e. removal of $-CH_2-$ groups from water) is of greater magnitude than the decrease in entropy in the second step (i.e. association of the $-CH_2-$ groups) so that a net increase in entropy results at a constant temperature (63). With a larger $T\Delta S$ and a low ΔH term a decrease in the free energy follows allowing the reaction to proceed. There is a decrease of free energy of about 10^3 cal/mole for each $-CH_2-$ group transferred from H_2O to a non-aqueous milieu (168,169). Lipids are probably in their lowest free energy state when forming bilayers in which the hydrophobic groups associate in a non aqueous milieu and the polar groups are oriented to the water phase. A similar study can be done concerning the variations in the free energy of membrane proteins. Membrane proteins usually contain several non-polar residues which tend to be sequestered from the aqueous milieu: to achieve the lowest free-energy state a particular polypeptide should adopt a three dimensional structure (conformation) representing the thermodynamically most favorable structure. This is determined by its particular constitution in amino acids. During the process of folding in an aqueous environment a protein assumes a structure in which the interior is completely free of water molecules and ionic residues. The sum of the ΔG contributions resulting from the burial of non-polar groups in the interior of the protein structure is a large negative number and should be considered the main source of stabilization of the native conformation of a protein in an aqueous milieu (169). Interpeptide hydrogen bonds could also be an important source in stabilizing folded protein molecules. In terms of variations of free energy, Klotz and Farnham (67) showed however that an interpeptide hydrogen bond has about the same free energy in an aqueous or in a non-polar environment. As pointed out by Singer (157) there may be hydrogen bonds in the unfolded state of a protein which are not formed in the folded conformation – e.g. hydrogen bonds to water molecules in the unfolded state. These bonds do not necessarily correspond in number to the interpeptide hydrogen-bonds formed in the folding process so that a decrease in the free energy as related to hydrogen bonding may not be a prominent factor in stabilizing membrane proteins.

As opposed to the Davson-Danielli model the lipid globular-protein mosaic model does not consider the electrostatic interactions between charged groups of lipids and proteins as a major source of stabilization of membrane structure. The general idea of the Davson-Danielli model is on the other hand thermodynamically unfavorable. Assuming that the proteins cover the lipid bilayer on both sides and that the electrostatic interactions are the prominent bonds linking proteins to lipids, the free energy of the buried ionic groups should be lower than the free energy of ionic groups in contact with water. This is most unlikely. The free energy required to bury ion groups in a non-aqueous milieu, either throught the formation of ion pairs (positively + + negatively-charged ions) or through protonation or dissociation is expected to be relatively large; ion pairs are in a much lower free energy in contact with water than with any other solvent.

To maximize the main sources of stabilization of membrane constituents, i.e. the hydrophilic and hydrophobic interactions (157,159) the membrane should have a structure in which the ionic groups of proteins are accomodated in parallel with the ionic groups of phospholipids; the lipid ionic groups and hydrophobic chains in the bilayer should move opening space to accomodate proteins in a globular conformation. In this conformation the protein non-polar groups are buried in a non-aqueous milieu along with the lipid hydrocarbon chains.

Besides being thermodynamically favorable the lipid globular-protein mosaic model apparently conforms with most of the experimental data on membranes from several organisms. Two important properties of the lipid-protein association arise from this model which are in accordance with the thermodynamically restrictions discussed above. Proteins with well defined polar and non-polar regions like the glycoproteins cannot rotate from one surface

to the other in the intact membrane. Anionic phospholipids also do not undergo such rotations in synthetic phospholipid vesicles (70,71). There are on the other hand no apparent thermodynamic restrictions for the free translational movements of some proteins in the plane of the membrane (39) as well as of lipids (29).

Although the lipid globular-protein model provides the most suitable explanation for many of the experimental results discussed here, it is evident that a considerable effort must still be done to detail the structure and molecular organization of membranes from microorganisms and other cells.

Acknowledgements

L.R.T is a fellow of the Brazilian National Research Council. We thank Dr. K. O. Lloyd for reading the manuscript in its preliminary form. This review was inspired by a series of lectures by S. J. Singer at the College of Physicians and Surgeons, Columbia University, New York (1972).

NOTE:

This review covers literature up to 1973. Some pertinent points raised in a recent review by Singer (195) are summarized below.

Several proteins or complexes were listed as peripheral thus having the following properties: a) Dissociation from membrane by mild treatments such as high ionic strength and metal ion chelating agents; b) Solubility usually free of lipids and c) After dissociation from membrane peripheral proteins are soluble and molecularly dispersed in neutral aqueous buffers. Among them are: cytochrome c, HPr protein (on the cytoplasmic surface of bacterial membranes), ribosomes, nectin (on the plasma membranes of *Streptococcus faecalis*), periplasmic binding proteins (plasma membrane of Gram-negative bacteria). Highly soluble peripheral proteins apparently attach to the membrane by specific binding to particular integral proteins. They remain completely outside the lipid matrix and react with the protruding region of the integral protein into the aqueous phase. Binding of peripheral proteins may involve reaction with the hydrophilic ends of the membrane specific--amphipathic-integral proteins. The possible attachment of peripheral proteins to the polar heads of lipids does not explain the high degree of specificity of peripheral protein binding to certain membrane surfaces.

Integral proteins are probably not completely embedded within the hydrophobic region of the membrane. For complete burial, proteins should contain very few ionic groups. A most hydrophobic integral protein such as the C_{55}-isoprenoid alcohol phosphokinase of *Staphylococcus aureus* (194) still has between 16 and 32 ionic residues per molecule of 17,000 daltons. Total free energy to bury most of these residues could be as much as several hundred kilocalories per mole (157, 195).

Membrane fluidity and the possibility of lateral mobility of membrane components may be restricted in certain membranes or in certain regions of the membrane. In a halobacterium there appear plaques of proteins with possibly some lipid chains in the interstices between the protein molecules (191). These plaques occur as a mosaic in the randomly ordered membrane matrix. Such protein-protein interactions can restrict mobility of membrane components and alter the fluidity of the membrane. Peripheral protein attachments to integral proteins can also inhibit mobility of membrane components.

Membrane asymmetry has been suggested in respect to the protein components. In some cells membrane asymmetry is indispensable to explain certain functions of the membrane. This is the case with the mitochondrial inner membrane. The asymmetry of the components of the electron transport chain in such membrane is related to the chemiosmotic hypothesis of Mitchell (193). Asymmetrical distribution of phospholipids although not considered in some liquid phase separation experiments (192, 196) may exist in *E. coli* membranes (195). In this bacterium phospholipids present are: phosphatidylethanolamine (70-80%), phosphatidylglycerol (5-15%) and cardiolipin (5-15%). Different arrangements of these phospholipids in the two layers of the lipid bilayer introduce an asymmetry which may be important in determining the biochemical properties of the bilayer.

The thermodynamic highly unfavourable rotation of proteins carrying ionic residues accross the hydrophobic interior of the membrane is again stressed. A model for the translocation of low molecular weight substrates in active transport is proposed based on the presence of peripheral proteins (195). The membrane contains aggregates of specific integral proteins which span the membrane and form water-filled pores. Such pores, closed to the diffusion of the substrates, open as a result of the quaternary arrangements assumed by the integral proteins. These arrangements depend upon the conformation of the specifically attached periplasmic proteins. In other words, the periplasmic protein changes in conformation resulting from specific substrate binding trigger the rearrangement of the integral proteins so as to open the pore accross the membrane which permits substrate translocation.

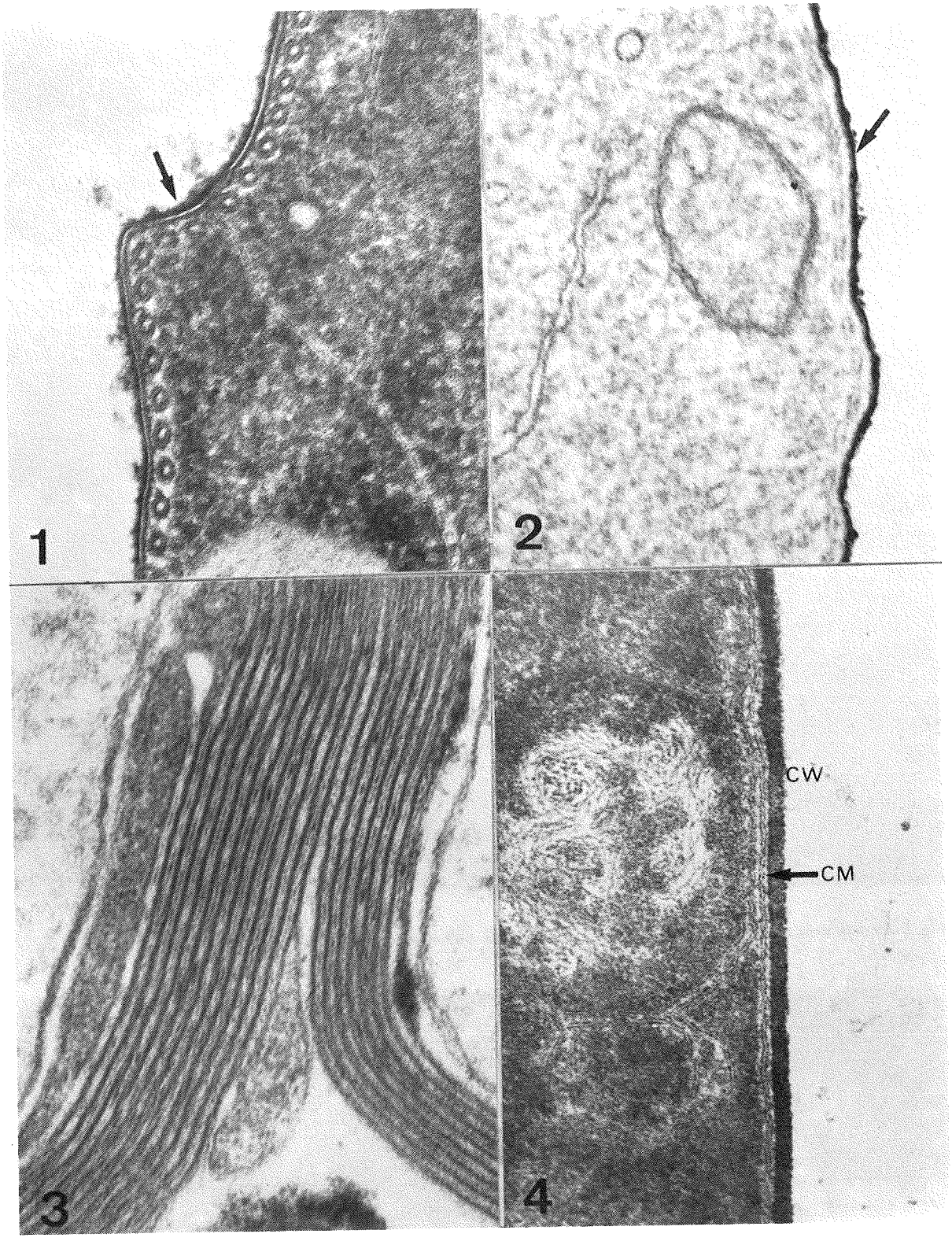


Fig. 1 Trilaminar aspect of the cell membrane. *Trypanosoma cruzi* was fixed with tannic acid and glutaraldehyde. X 140,000. (Micrograph taken by T.C.B. Soares and W. de Souza)
Fig. 2 Detection of carbohydrate residues on membrane surface of *Herpetomonas sp*. by use of a cytochemical method. The arrow indicates a dense material located on the outer face of the cell membrane. X 100,000. (Micrograph taken by W. de Souza, M.M. Bunn and J. Angluster).
Fig. 3 Myelin sheat from the optic nerve. X 150,000. (Micrograph taken by J.N. Hokoc, E.O. Cruz and R.D. Machado).
Fig. 4 Surface structures of *Bacillus licheniformis*. CW = Cell wall. CM = cell membrane. X 100,000. (Micrograph taken by L. Rabinovitch, M.L. Pereira, R.D. Machado and W. de Souza).

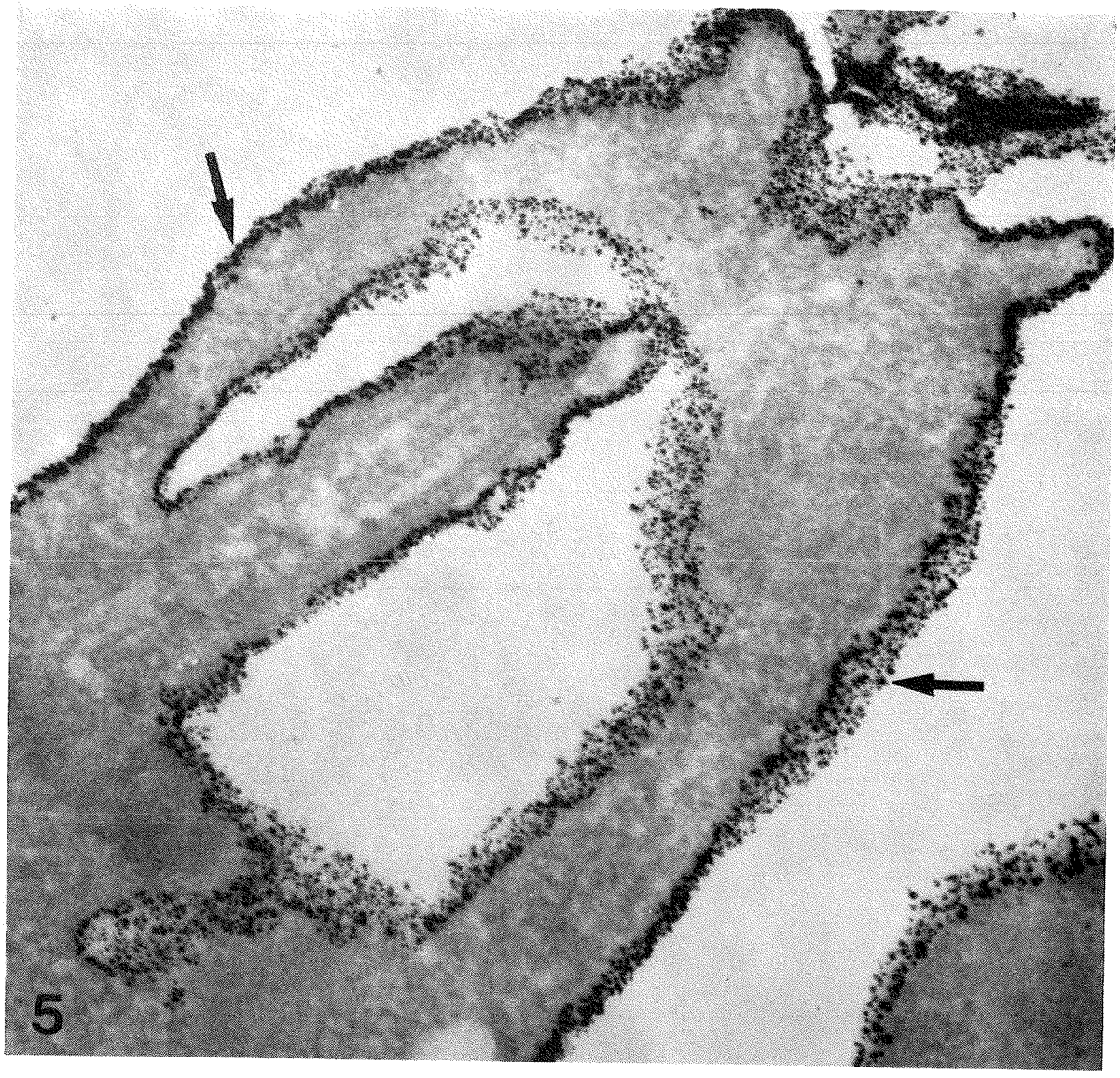


Fig. 5 Anionic sites on the cell surface of *Herpetomonas* sp. as detected by the colloidal iron method. X 80,000.

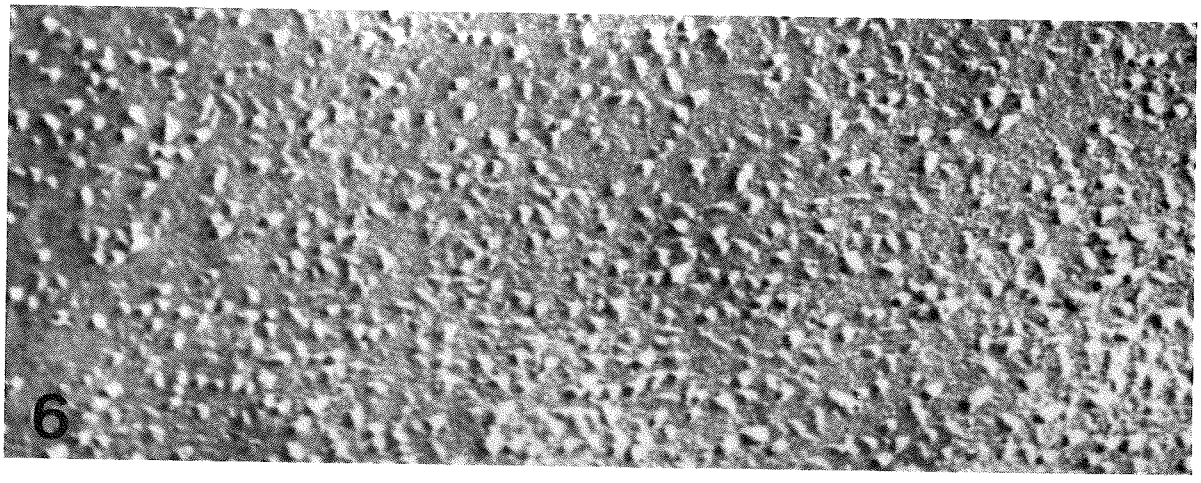


Fig. 6 Intramembranous particles from the cell membrane of *Trypanosoma cruzi*. Freeze-fracture technique. X 180,000. (Micrograph taken by A. Martinez-Palomo, A. Gonzales-Robles and W. de Souza).

References

Due to the numerous citations in this review the bibliography does not follow the general rules adopted in this journal.

1. ARGAMAN, M. & RAZIN, S. — *J. Gen. Microbiol.*, 38:153, 1965.
2. BARAUD, J., MAURICE, A. & NAPIAS, C. — *Bull. Soc. Chim. Biol.*, 52:421, 1965.
3. BARTLEY, W. — *in* Metabolism and physiological significance of lipids; R.M.C. Dawson, D.N. Rhodes, eds., p. 369. John Wiley Sons, Inc. London, 1964.
4. BAYER, M.E. — *J. Gen. Microbiol.*, 53:395, 1968.
5. BENSON, A.A. — *Annu. Rev. Plant Physiol.*, 15:1, 1964.
6. BENSON, A.A. — *J. Amer. Oil Chem. Soc.*, 43:265, 1966.
7. BEYCHOK, S. — *Science*, 154:1288, 1966.
8. BEYCHOK, S. — *Annu. Rev. Biochem.*, 37:437, 1968.
9. BIRDSELL, D.C. & COTA-ROBLES, E.H. — *J. Bacteriol.*, 93:427, 1967.
10. BISHOP, D.J., RUTBERG, L. & SAMUELSON, B. — *Eur. J. Biochem.*, 2:454, 1967.
11. BLADEN, H. & MERGENHAGEN, S.E. — *J. Bacteriol.*, 88:1482, 1964.
12. BLOUT, E.R., SCHMIER, I., SIMMONS, N.S. — *J. Amer. Chem. Soc.*, 84:319, 1962.
13. BOULTON, A.A. — *Exptl. Cell Res.*, 37:343, 1965.
14. BRANTON, D. — *Exptl. Cell Res.*, 45:703, 1967.
15. BRANTON, D. — *Annu. Rev. Plant Physiol.*, 20:209, 1969.
16. BRANTON, D. & PARK, R.B. — *J. Ultrastructure Res.*, 19:283, 1967.
17. BRETSCHER, M S. — *Science*, 181:622, 1973.
18. CHAN, M., VIRMANI, Y.P., HIMES, R.H. & AKAGI, J.M. — *J. Bacteriol.*, 113:322, 1973.
19. CHAPMAN, D. & CHEN, S. — *Chem. Phys. Lipids*, 8:318, 1972.
20. CHAPMAN, D. & DODD, G.H. — *in* Structure and function of biological membranes; L.I. Rothfield, ed., p. 13. Academic Press, Inc. New York, 1971.
21. CHAPMAN, D., KAMET, V.B., DE GIER, J. & PENKETT, S.A. — *J. Mol. Biol.*, 31:101, 1968.
22. CHO, K.Y. & SALTON, M.R.J. — *Biochim. Biophys. Acta*, 116:73, 1966.
23. CHOULES, G.L. & BJORKLUND, R.F. — *Biochemistry*, 9:4759, 1970.
24. CRAMER, W.A. & PHILLIPS, S.K. — *J. Bacteriol.*, 104:819, 1970.
25. DANIELLI, J.F. & DAVSON, H.A. — *J. Cell Comp. Physiol.*, 9:89, 1936.
26. DARON, H.H. — *J. Bacteriol.*, 101:145, 1970.
27. DAVSON, H.A. & DANIELLI, J.F. — *J. Cell Comp. Physiol.*, 5:495, 1935.
28. van DEENEN, L.L.M. — *Progr. Chem. Fats*, 8:1, 1965.
29. DEVAUX, P. & McCONNELL, H.M. — *J. Amer. Chem. Soc.*, 94:4475, 1972.
30. ELETR, S. & KEITH, A.D. — *Proc. Nat. Acad. Sci. U.S.*, 69:1353, 1972.
31. ENGELMAN, D.M. — *in* Membrane models and the function of biological membranes; L. Bolis and B.A. Pethica, eds., p. 203. John Wiley Sons, Inc., New York, 1968.
32. ENGELMAN, D.M. — *J. Mol. Biol.*, 58:153, 1971.
33. ENGELMAN, D.M. & MOROWITZ, H.J. — *Biochim. Biophys. Acta*, 150:385, 1968.
34. ENGELMAN, D.M., TERRY, T., & MOROWITZ, H.J. — *Biochim. Biophys. Acta*, 135:381, 1967.
35. ESFAHANI, M., LIMBRICK, A.R., KNUTTON, S., OKA, T., & WAKIL, S.J. — *Proc. Nat. Acad. Sci. U.S.*, 68:3180, 1971.
36. FREEDMAN, R.B. & RADDA, G.K. — *FEBS Letters*, 3:150, 1969.
37. FRERMAN, F.E. & WHITE, D.C. — *J. Bacteriol.*, 94:1868, 1967.
38. FOX, C.F. — *Proc. Nat. Acad. Sci. U.S.*, 63:850, 1969.
39. FRYE, C.D. & EDIDIN, M. — *J. Cell Sci.*, 7:319, 1970.
40. GARCIA-MENDOZA, C. & VILLANUEVA, J.R. — *Biochim. Biophys. Acta*, 135:189, 1967.
41. GLASER, M. & SINGER, S.J. — *Biochemistry*, 10:1780, 1971.
42. GOLDFINE, H. & ELLIS, M.E. — *J. Bacteriol.*, 87:8, 1964.
43. GREEN, F.A. — *Immunochemistry*, 4:247, 1967
44. GREEN, D.E. & FLEISCHER, S. — *in* Metabolism and physiological significance of lipids; R.M.C. Dawson and D.M. Rhodes, eds., p. 580. John Wiley Sons, Inc. New York, 1964.
45. GREEN, D.E. & PERDUE, J.F. — *Proc. Nat. Acad. Sci. U.S.*, 55:1295, 1966.
46. GUIDOTTI, G. — *Annu. Rev. Biochem.*, 41:731, 1972.
47. HILDICH, T.P. & WILLIAMS, P.N. — The chemical constitution of natural fats, p. 246 and 254. John Wiley Sons, Inc. New York, 1964.
48. HIRANO, H., PARKHOUSE, B., NICOLSON, G.L., LENNOX, E.S. & SINGER, S.J. — *Proc. Nat. Acad. Sci. U.S.*, 69:2945, 1972.
49. HOLT, S.C. & LEADBETTER, E.R. — *Bact. Rev.*, 33:346, 1969.
50. HOLZWARTH, G., DOTY, P. — *J. Amer. Chem. Soc.*, 87:218, 1965.
51. HOLZWARTH, G., GRATZER, W.B. & DOTY, P. — *J. Amer. Chem. Soc.*, 84:3194, 1962.
52. HOUTSMULLER, U.M.T. & van DEENEN, L.L.M. — *Biochim. Biophys. Acta*, 84:96, 1964.
53. HOUTSMULLER, U.M.T. & van DEENEN, L.L.M. — *Biochim. Biophys. Acta*, 106:564, 1965.
54. HUANG, C. — *Biochemistry*, 8:344, 1969.
55. HUNTER, K. & ROSE, A.H. — *in* The Yeasts; A.H. Rose and J.S. Harrison, eds., p. 211. Academic Press, Inc. New York, 1971.

56. IBBOT, F.A. & ABRAMS, A. – *Biochemistry,* 3:2008, 1964.

57. van ITERSON, W. & OP DEN KAMP, J.A.F. – *J. Bacteriol.,* 99:304, 1969.

58. JOST, P., WAGGONER, A.S. & GRIFFITH, D.H. – *in* Structure and function of biological membranes; L.I. Rothfield, ed., p. 83. Academic Press Inc., New York, 1971.

59. JURTSHUK, P., SEKUZU, I., GREEN, D.E. – *Biochem. Biophys. Res. Commun.,* 6:76, 1961.

60. KAHANE, I. & RAZIN, S. – *Biochim. Biophys. Acta,* 183:79, 1969.

61. KANEDA, T. – *J. Bacteriol.,* 93:894, 1967.

62. KANESHIRO, T. & MARR, A.G. – *J. Lipid Res.,* 3:184, 1962.

63. KAUZMANN, W. – *Adv. Prot. Chem.,* 14:1, 1959.

64. KEITH, A.D. & MEHLHORN, R.J. – *in* Membrane molecular biology; C.F. Fox and A.D. Keith, eds., p. 117. Sinauer Assoc. Inc. Pub., Stamford, Conn. 1972.

65. KEITH, A.D., RESNICK, M.R. & HALEY, A.B. – *J. Bacteriol.,* 98:415, 1969.

66. KIEHN, E.D. & HOLLAND, J.J. – *Proc. Nat. Acad. Sci. U.S.,* 61:1370, 1968.

67. KLOTZ, I.M. & FARNHAM, S.B. – *Biochemistry,* 7:3879, 1968.

68. KOCUN, F.J. – *Biochim. Biophys. Acta,* 202:277, 1970.

69. KORN, E.D. – *Science,* 153:1491, 1966.

70. KORNBERG, R.D. & McCONNELL, H.M. – *Biochemistry,* 10:1111, 1971.

71. KORNBERG, R.D. & McCONNELL, H.M. – *Proc. Nat. Acad. Sci. U.S.,* 68:2564, 1971.

72. KROES, J., OSTWALD, R. & KEITH, A. – *Biochim. Biophys. Acta,* 274:71, 1972.

73. LAW, J.H. & SNYDER, W.R. – *in* Membrane molecular biology; C.F. Fox and A.D. Keith, eds., p. 3. Sinauer Assoc. Inc. Pub., Stamford, Conn., 1972.

74. LENARD, J. & SINGER, S.J. – *Proc. Nat. Acad. Sci. U.S.,* 56:1828, 1966.

75. LENARD, J. & SINGER, S.J. – *J. Cell Biol.,* 37:117, 1968.

76. LENARD, J. & SINGER, S.J. – *Science,* 159:738, 1968.

77. LEWIS, R.W. – *Comp. Biochem. Physiol.,* 6:75, 1962.

78. LONGLEY, R.P., ROSE, A.H. & KNIGHTS, B.A. – *Biochem. J.,* 108:410, 1968.

79. LUKINS, H.B., JOLLOW, D., WALLACE, P.G. & LINNANE, A.W. – *Austr. J. Exp. Biol. Med. Sci.,* 46:651, 1968.

80. McFARLANE, M.G. – *Adv. Lipid Res.,* 2:91, 1964.

81. MACKENZIE, C.D. & JORDAN, D.C. – Abst. 72nd Ann. Meeting Amer. Soc. Microbiol., P–250, p. 177, Philadelphia, Penna., 1972.

82. MADDY, A.H. & MALCOLM, B.R. – *Science,* 150:1616, 1965.

83. MANILOFF, J. & MOROWITZ, H.J. – *Bact. Rev.,* 36:263, 1972.

84. MARCHESI, V.T., TILLACK, T.W., JACKSON, R.L., SEGREST, J.P. & SCOTT, R.E. – *Proc. Nat. Acad. Sci. U.S.,* 69:1445, 1972.

85. MARINETTI, G.V., ERBLAND, J. & STOTZ, F. – *J. Biol. Chem.,* 233:562, 1958.

86. MARR, A.G. & INGRAHAM, J.L. – *J. Bacteriol.,* 84:1260, 1962.

87. MATILE, P., MOOR, H. & MUHLETHALER, K. – *Arch. Mikrobiol.,* 58:201, 1967.

88. MAVIS, R.D., BELL, R.M. & VAGELOS, P.R. – *J. Biol. Chem.,* 247:2835, 1972.

89. McELHANEY, R.N., DE GIER, J. & van DEENEN, L.L.M. – *Biochim. Biophys. Acta,* 219:245, 1970.

90. MELCHIOR, D.J., MOROWITZ, H.J., STURTEVANT, J.M., & TSONG, T.Y. – *Biochim. Biophys. Acta,* 219:114, 1970.

91. METCALFE, J.C., BIRDSALL, N.J.M., FEENEY, J., LEE, A.G., LEVINE, Y.K. & PARTINGTON, P. – *Nature,* 233:199, 1971.

92. METCALFE, J.C., METCALFE, S.M. & ENGELMAN, D.M. – *Biochim. Biophys. Acta,* 241:412, 1971.

93. METCALFE, S.M., METCALFE, J.C. & ENGELMAN, D.M. – *Biochim. Biophys. Acta,* 241:422, 1971.

94. MINDICH, L. – *J. Mol. Biol.,* 49:415, 1970.

95. MINDICH, L. – *J. Mol. Biol.,* 49:433, 1971.

96. MIRSKY, R. – *Biochemistry,* 8:1164, 1969.

97. MOOR, H., MUHLETHALER, K., WALDNER, H. & FREY-WYSSLING, A. – *J. Biophys. Biochem. Cytol.,* 10:1, 1961.

98. MOROWITZ, H.J. & TERRY, T.M. – *Biochim. Biophys. Acta,* 183:276, 1969.

99. MUÑOZ, E., NACHBAR, M.S., SCHOR, M.T. & SALTON, M.R.J. – *Biochem. Biophys. Res. Commun.,* 32:539, 1968.

100. NACHBAR, M.S. & SALTON, M.R.J. – *in* Surface chemistry of biological systems. Plenum Press, New York, 1970.

101. NACHBAR, M.S., WINKLER, W.J. & SALTON, M.R.J. – *Biochim. Biophys. Acta,* 274:83, 1972.

102. NANNINGA, N. – *J. Cell Biol.,* 39:251, 1968.

103. NICOLSON, G.L., HYMAN, R. & SINGER, S.J. – *J. Cell Biol.,* 50:905, 1971.

104. NICOLSON, G.L., MASOUREDIS, S.P. & SINGER, S.J. – *Proc. Nat. Acad. Sci. U.S.,* 68:1416, 1971.

105. NICOLSON, G.L. & SINGER, S.J. – *Proc. Nat. Acad. Sci. U.S.,* 68:942, 1971.

106. OHNISHI, T. & KAWAMURA, H. – *J. Biochem. (Tokyo),* 56:377, 1964.

107. OLDFIELD, E. – *Science,* 180:982, 1973.

108. OP DEN KAMP, J.A.F., van ITERSON, W. & van DEENEN, L.L.M. – *Biochim. Biophys. Acta,* 135:862, 1967.

109. OSBORN, M.J., GANDER, J.E., PARISI, E. & CARSON, J. – *J. Biol. Chem.,* 247:3962, 1972.

110. PAGANO, R.E., CHERRY, R.J. & CHAPMAN, D. – *Science,* 181:557, 1973.

111. PALADE, G. — *in* Subcellular particles; T. Hayashi, ed., p. 64. Ronald Press, New York, 1959.

112. PEARSON, L.K. & RAPER, H.S. — *Biochem. J.*, 21:875, 1927.

113. PETIT, V.A. & EDIDIN, M. — *Science*, 184:1183, 1974.

114. PINTO DA SILVA, P. — *J. Cell Biol.*, 53:777, 1972.

115. PINTO DA SILVA, P. & BRANTON, D. — *J. Cell Biol.*, 45:598, 1970.

116. PISETSKY, D. & TERRY, T.M. — *Biochim. Biophys. Acta*, 274:95, 1972.

117. PODLESKI, T.R. & CHANGEUX, J.P. — *in* Fundamental concepts in drug-receptor interactions; D.J. Triggle, J.F. Danielli and J.F. Moran, eds., p. 93. Academic Press Inc., New York, 1969.

118. PORETZ, R.D. & GOLDSTEIN, I.J. — *Biochemistry*, 9:2870, 1970.

119. RAZIN, S. — Recent Progr. in Microbiology, Symp. Int. Congr. Microbiology, 8th, p. 526. Montreal, Canadá, 1963.

120. RAZIN, S. — *Ann. New York Acad. Sci.*, 143:115, 1967.

121. RAZIN, S. — *Annu. Rev. Microbiol.*, 23:317, 1969.

122. RAZIN, S. — *in* The mycoplasmatales and the L-phase of bacteria; L. Hayflick, ed., p. 317. Appleton-Century-Crofts, New York, 1969.

123. RAZIN, S., ARGAMAN, M. & AVIGAN, J. — *J. Gen. Microbiol.*, 33:477, 1963.

124. RAZIN, S. & KAHANE, I. — *Nature*, 223:863, 1969.

125. RAZIN, S., MOROWITZ, H.J. & TERRY, T.T. — *Proc. Nat. Acad. Sci. U.S.*, 54:219, 1965.

126. RAZIN, S., TOURTELLOTE, M.E., McELHANEY, R.N. & POLLACK, J.D. — *J. Bacteriol.*, 91:609, 1966.

127. RAZIN, S., NE'EMAN, Z. & OHAD, I. — *Biochim. Biophys. Acta*, 193:277, 1969.

128. REAVELEY, D.A. & BURGE, R.E. — *in* Advances in Microbial Physiology, vol. 7, p. 1; A.H. Rose and D.W. Tempest, eds., Academic Press Inc., London, 1972.

129. REDWOOD, W.R. & WEIS, P. — *Biochim. Biophys. Acta*, 332:11, 1973.

130. RICHARDSON, S.H., HULTIN, H.O. & GREEN, D.E. — *Proc. Nat. Acad. Sci. U.S.*, 50:821, 1963.

131. ROBERTSON, J.D. — *Progr. Biophys. Biophys. Chem.*, 10:343, 1960.

132. ROBERTSON, J.D. — *Arch. Int. Med.*, 129:202, 1972.

133. ROBERTSON, J., MOLDOW, C. & ROTHFIELD, L. — *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 30:1119, 1971.

134. ROBISON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. — *in* Fundamental concepts in drug-receptor interactions; D.J. Triggle, J.F. Danielli and J.F. Moran, eds., p. 59. Academic Press Inc., New York, 1969.

135. ROELOFSEN, B., BAADENHUYZEN, H. & van DEENEN, L.L.M. — *Nature*, 121:1379, 1966.

136. ROGERS, H.J. — *Bact. Rev.*, 34:194, 1970.

137. ROGERS, H.J., REAVELEY, D.A. & BURDETT, I.D.J. — *in* Protides of the biological fluids; H. Peeters, ed., vol. 15, p. 303. Elsevier Pub. Co., Amsterdam, 1967.

138. ROTHFIELD, L.I. — *in* Structure and function of biological membranes; L.I. Rothfield, ed., p. 3. Academic Press Inc., New York, 1971.

139. ROTHFIELD, L. & PEARLMAN, M. — *J. Biol. Chem.*, 241:1386, 1966.

140. ROTHFIELD, L. & ROMEO, D. — *in* Structure and function of biological membranes; L.I. Rothfield, ed., p. 251. Academic Press Inc., New York, 1971.

141. ROTHFIELD, L., WEISER, M. & ENDO, A. — *J. Gen. Physiol.*, 54:27, 1969.

142. ROTHMAN, J.E. & ENGELMAN, D.M. — *Nature New Biology*, 237:42, 1972.

143. ROTTEM, S., HUBBELL, W.L., HAYFLICK, L. & McCONNELL, H.M. — *Biochim. Biophys. Acta*, 219:104, 1970.

144. ROTTEM, S. & PANOS, C. — *J. Gen. Microbiol.*, 59:317, 1969.

145. ROTTEM, S., STEIN, O. & RAZIN, S. — *Arch. Biochem. Biophys.*, 125:46, 1968.

146. SALTON, M.R.J. — *Trans. New York Acad. Sci.*, 29:764, 1967.

147. SALTON, M.R.J. — *in* Microbial protoplasts and L-forms; L.B. Guze, ed., p. 144. Williams and Wilkins, Co. Baltimore, 1968.

148. SALTON, M.R.J. & FREER, J.H. — *Biochim. Biophys. Acta*, 197:531, 1965.

149. SALTON, M.R.J. & NETSCHEY, A. — *Biochim. Biophys. Acta*, 107:539, 1965.

150. SCHATZ, G. & SALTZGABER, J. — *Biochim. Biophys. Acta*, 180:186, 1969.

151. SENIOR, A.E. & McLENNAN, D.H. — *J. Biol. Chem.*, 245:5086, 1970.

152. SEGREST, J.P., JACKSON, R.L., MARCHESI, V.T., GUYER, R.B., TERRY, W. — *Biochem. Biophys. Res. Commun.*, 49:964, 1972.

153. SESSA, G., FREER, J.H., COLACICCO, G. & WEISSMAN, G. — *J. Biol. Chem.*, 244:3575, 1969.

154. SHAW, N. & BADDILEY, J. — *Nature*, 217:142, 1968.

155. SHOCKMAN, G.D., KOLB, J.M., BAKAY, B., CONOVER, M.J. & TOENNIES, G. — *J. Bacteriol.*, 85:168, 1968.

156. SILBERT, D. & VAGELOS, P.R. — *Proc. Nat. Acad. Sci. U.S.*, 58:1579, 1967.

157. SINGER, S.J. — *in* Structure and function of biological membranes; L.I. Rothfield, ed., p. 145. Academic Press, Inc., New York, 1971.

158. SINGER, S.J. — *Science*, 180:983, 1973.

159. SINGER, S.J. & NICOLSON, G.L. — *Science*, 175:720, 1972.

160. SJOSTRAND, F.S. — *J. Ultrastructure Res.*, 9:561, 1963.

161. SMITH, P.F. — *in* Recent Progr. Microbiology, Symp. Int. Congr. Microbiol., 8th, p. 518. Montreal, Canadá, 1963.

162. SPETH, V. & WUNDERLICH, F. — *Biochim. Biophys.*

Acta, 291:621, 1973.

163. STECK, T.L. – *J. Mol. Biol.,* 66:295, 1972.

164. STEERE, R.L. – *J. Biophys. Biochem. Cytol.,* 3:45, 1957.

165. STEIM, J.M. – *in* Liquid crystals and ordered fluids; R.S. Porter and J.F. Johnson, eds., p. 1. Plenum Press, New York, 1970.

166. STEIM, J., REINERT, J.C., TOURTELLOTE, M.E., McELHANEY, R.N. & RADER, R.L. – *Proc. Nat. Acad. Sci. U.S.,* 63:104, 1969.

167. STRYER, L. – *J. Mol. Biol.,* 13:269, 1965.

168. TANFORD, C. – Physical chemistry of macromolecules, p. 130. John Wiley Sons, Inc. New York, 1961.

169. TANFORD, C. – *J. Amer. Chem. Soc.,* 84:4240, 1962.

170. TERRY, T.M. & ZUPNIK, J.S. – *Biochim. Biophys. Acta,* 219:144, 1970.

171. TILLACK, T.W., CARTER, R. & RAZIN, S. – *Biochim. Biophys. Acta,* 219:123, 1970.

172. TOBARI, J. – *Biochem. Biophys. Res. Commun.,* 15:50, 1964.

173. TORNABENE, T.G., BENNETT, E.O. & ORO, J. – *J. Bacteriol.,* 94:344.

174. TOURTELLOTE, M.E. – *in* Membrane molecular biology; C.F. Fox and A.D. Keith, eds., p. 439. Sinauer Assoc. Inc. Pub., Stamford, Conn., 1972.

175. TOURTELLOTE, M.E., BRANTON, D. & KEITH, A. – *Proc. Nat. Acad. Sci. U.S.,* 66:909, 1970.

176. TOURTELLOTE, M.E. & ZUPNIK, J.S. – *Science,* 179:84, 1973.

177. TRIGGLE, D. – Recent Progr. Surface Sci., 3:273, 1970.

178. URRY, D.W., MEDNIECKS, M. & BEJNAROWITZ, E. – *Proc. Nat. Acad. Sci. U.S.,* 57:1043, 1967.

179. VANDENHEUVEL, F.A. – *J. Amer. Oil Chem. Soc.,* 40:455, 1963.

180. WALLACH, D.F.H. & GORDON, A.S. – *in* Regulatory functions of biological membranes; J. Jarnefelt, ed., p. 87. Elsevier Pub. Co., New York, 1968.

181. WALLACH, D.F.H., GRAHAM, J.M. & FERNBACH, B.R. – *Arch. Biochem. Biophys.,* 131:322, 1969.

182. WALLACH, D.F.H. & ZAHLER, P.H. – *Proc. Nat. Acad. Sci. U.S.,* 56:1552, 1966.

183. WARD, J.B. & PERKINS, H.R. – *Biochem. J.,* 106:391, 1968.

184. WEIBULL, C. & BERGSTROM, L. – *Biochim. Biophys. Acta,* 30:340, 1958.

185. WETTLAUFER, D.B. & LOVRIEN, R. – *J. Biol. Chem.,* 239:596, 1964.

186. WINZLER, R.J. – *in* Red cell membrane; G.A. Jamieson and T.J. Greenwalt, eds., p. 157. Lippincott, Philadelphia, 1969.

187. WUNDERLICH, F., MULLER, R. & SPETH, V. – *Science,* 182:1136, 1973.

188. YU, L. & WOLIN, M.J. – *J. Bacteriol.,* 103:467, 1970.

189. YU, L. & WOLIN, M.J. – *J. Bacteriol.,* 109:59, 1972.

190. YUDKIN, M.D. – *Biochem. J.,* 98:923, 1966.

191. BLAUROCK, A.E. & STOECKENIUS, W. – *Nature New Biol.,* 233:152, 1971.

192. LINDEN, C.D., WRIGHT, K.L., McCONNELL, H.M. & FOX, C.F. – *Proc. Nat. Acad. Sci. USA,* 70:2271, 1973.

193. MITCHELL, P. – *Biol. Rev.,* 41:445, 1966.

194. SANDERMANN, H. & STROMINGER, J.L. – *Proc. Nat. Acad. Sci. USA,* 68:2441, 1971.

195. SINGER, S.J. – *Annu. Rev. Biochem.,* 43:805, 1974.

196. TRAUBLE, H. & OVERATH, P. – *Biochim. Biophys. Acta,* 307:491, 1973.