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On the Interaction between Intrinsic Proteins and Phosphatidylglycerol in the Membrane of *Acholeplasma laidlawii*

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About 30% of the phosphatidylglycerol in oleic acid-enriched *Acholeplasma laidlawii* membranes are not hydrolyzed at temperatures below 10°C by phospholipase A_2 from porcine pancreas. Removal of 53% of the membrane proteins by proteolysis did not reduce the size of this inaccessible phosphatidylglycerol pool. However, modification of the membrane proteins with 2,4,6-trinitrobenzenesulfonic acid or glutaraldehyde did make an additional 70% of this protected pool of phosphatidylglycerol accessible to phospholipase A_2. Complete hydrolysis of phosphatidylglycerol at low incubation temperatures was achieved only after heat treatment of the membranes which resulted in an extensive aggregation of intrinsic membrane proteins as visualized by freeze-etch electron microscopy. Phospholipase A_2 from bee venom was more effective in hydrolyzing phosphatidylglycerol at low temperature than the pancreatic enzyme. These results show that the inaccessibility of phosphatidylglycerol is not due to resealing of isolated membranes, the presence of a crystalline phase in the membrane lipids, or a shielding effect of surface proteins. The protection against hydrolysis may be due to an interaction of phosphatidylglycerol with intrinsic membrane proteins which is stabilized at low temperatures. Increasing the temperature favors the exchange of protein-bound phosphatidylglycerol with other membrane lipids resulting in complete hydrolysis.

Based on the "fluid mosaic" model (1) as a general concept of biological membranes, questions may be raised regarding the role of lipids in the function of integral membrane proteins. Electron spin resonance studies have indicated the presence of "boundary lipids" in the cytochrome oxidase membrane (2, 3). Other studies have tended to support the presence of such specialized lipid in the vicinity of integral proteins in membranes (4–9). The significance of these boundary lipids in membrane function is suggested by studies which have shown that the activities of some membrane enzymes are dependent upon the presence of certain types of lipids (4, 10–19). It is therefore of considerable importance to examine further the existence of such lipid–protein interactions in biological systems. In this report, evidence is obtained by using phospholipase accessibility as the criterion to detect the existence of boundary lipids in *Acholeplasma laidlawii* membranes.

In *A. laidlawii* membranes, phosphatidylglycerol is one of the major lipids and can be hydrolyzed by exogenous phospholipase (20, 21). Studies with pancreatic phospholipase A_2 have shown that the fatty acid composition of this phospholipid and the incubation temperature are important parameters which determine both the rate and the extent of hydrolysis. First of all, the extent of phosphatidylglycerol hydrolysis parallels the amount of membrane lipid which is in the liquid–crystalline phase (21). Second, in membranes in which all the lipids are in the liquid–crystalline phase, hydrolysis is strongly temperature-dependent (20). This effect is most clearly shown in membranes isolated from cells which were grown in the presence of oleate or linoleate because in these membranes the lipid phase transi-

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tion takes place below 0°C. Incubation of such membranes between 0 and 25°C with a vast excess of pancreatic phospholipase A_2 yields a biphasic hydrolysis pattern (20). During the initial phase, 70% of the phosphatidylglycerol is instantaneously hydrolyzed. The residual 30% is hardly accessible below 10°C and can be hydrolyzed above this temperature at rates which increase with increasing incubation temperatures.

EXPERIMENTAL PROCEDURES

Acholeplasma laidlawii B was grown on a lipid-depleted tryptose medium supplemented with oleic acid to a final concentration of 0.12 mM (23). Phosphorus-containing lipids were labeled by addition of 1 mCi [^{32}P]orthophosphate/liter of medium. Cells were harvested after 24-h growth, and membranes were isolated according to the method described by van Golde *et al.* (23).

Membranes, suspended in 0.5 ml buffer containing 50 mM Tris adjusted with HCl to pH 7.5 were treated with 100 units of pure phospholipase A_2 from porcine pancreas (kindly provided by Dr. G. H. de Haas) in the presence of 25 mM $CaCl_2$. Incubation was carried out for 20 min at 5°C and stopped by adding 0.5 ml saturated EDTA disodium salt solution. Phospholipase A_2 from bee venom was a gift of Dr. R. F. A. Zwaal. Lipid analysis was carried out as described before (20). In all experiments, appropriate controls were carried out using unmodified membrane preparations.

Membrane suspensions (0.5 ml) in 50 mM Tris buffer (pH 7.5) were treated with trypsin, Pronase, or proteinase K (all preparations were obtained from Boehringer, Mannheim) at final enzyme concentrations of 0.1-0.2 mg/ml for periods ranging from 15 to 60 min at 37°C. At the end of the incubation, the mixture was diluted tenfold with ice-cold 50 mM Tris buffer (pH 7.5) and centrifuged at 40,000*g*. Membranes were washed three times and finally suspended in the same buffer. The degree of proteolytic digestion was measured according to Lowry *et al.* (24).

Membranes were resuspended in 0.1 M phosphate solution (pH 9) to a final concentration of about 3 mg protein/ml. Trinitrobenzenesulfonic acid (Sigma), freshly dissolved in 0.15 M phosphate solution adjusted with NaOH to pH 8.5, was added to the membrane suspension to give a final concentration of 20 mM. The reaction took place at 37°C under continuous stirring. About 5 min after addition of trinitrobenzenesulfonic acid the pH of the incubation mixture was decreased to 8.1 and remained at 8.1 for at least 120 min. At different time intervals, aliquots were taken from the incubation mixture and mixed with an ice-cold solution containing 20 mM lysine to neutralize the excess trinitrobenzenesulfonic acid. Membranes were washed four times with 50 mM Tris buffer (pH 7.5) and finally suspended in the same buffer. The extent of labeling with trinitrobenzenesulfonic acid was determined spectrophotometrically according to Habeeb (25).

Membrane suspensions in 50 mM $NaHCO_3$ solutions were treated with 2% glutaraldehyde for 30 min at 37°C while the pH of the mixture was kept at 8.0. The reaction was stopped with ice-cold 50 mM $NaHCO_3$ solution, and membranes were washed four times in 50 mM Tris buffer (pH 7.5) before incubating with

TABLE I

EFFECT OF REMOVAL OF MEMBRANE PROTEINS ON THE PROTECTED POOL OF PHOSPHATIDYLGLYCEROL IN OLEIC ACID-ENRICHED MEMBRANES[a]

Treatment	Duration of treatment (min)	Percentage of protein released from the membrane	Percentage of residual phosphatidylglycerol after phospholipase A_2 incubation
—	—	—	33
0.2 M KCl (twice)	—	4	31
Trypsin, 0.1 mg/ml	60	39	29
Trypsin, 0.2 mg/ml	60	45	29
Proteinase K, 0.2 mg/ml	60	40	26
Pronase, 0.1 mg/ml	15	47	34
Pronase, 0.1 mg/ml	30	50	33
Pronase, 0.1 mg/ml	60	53	33
Pronase, 0.2 mg/ml	60	53	33

[a] All membrane preparations were washed thoroughly (see Experimental Procedures) before addition of excess phospholipase A_2 at 5°C.

phospholipase. Mg^{2+}-ATPase activity and NADH oxidase activity were measured as described by Pollack *et al.* (26). Lysophospholipase was assayed according to the method of Aarsman and van den Bosch (27).

RESULTS

Effects of proteolytic digestion on phosphatidylglycerol hydrolysis. Efforts were made to remove membrane surface protein by washing with a 0.2 M KCl solution or by digestion with proteolytic enzymes (Table I). Membranes washed twice with a solution containing 0.2 M KCl hardly lost any protein. About half of the membrane protein was lost upon treatment with proteolytic enzymes, and this quantity appeared to be the maximal digestable amount (see Pronase treatment, Table I). However, even after such drastic treatment no significant change in the extent of hydrolysis of phosphatidylglycerol was observed. A maximal hydrolysis of about 70% of the total phosphatidylglycerol was achieved at incubation temperatures below 10°C for all preparations. From these results it can be concluded that the protection against hydrolysis is due to a shielding effect of intrinsic proteins rather than proteins covering the membrane surface. It has to be emphasized that after Pronase treatment followed by incubation with phospholipase A_2, a small amount of lysophosphatidylglycerol was detected. This is most likely due to a partial reduction of the membrane-bound lysophospholipase activity (23) by the Pronase treatment. Although the rapidly formed lysophosphatidylglycerol is only slowly hydrolyzed by the residual lysophospholipase, the accumulation of this detergent-like phospholipid does not enhance the hydrolysis of phosphatidylglycerol.

Effects of heat treatment on the extent of phosphatidylglycerol hydrolysis. The inaccessible pool of phosphatidylglycerol in

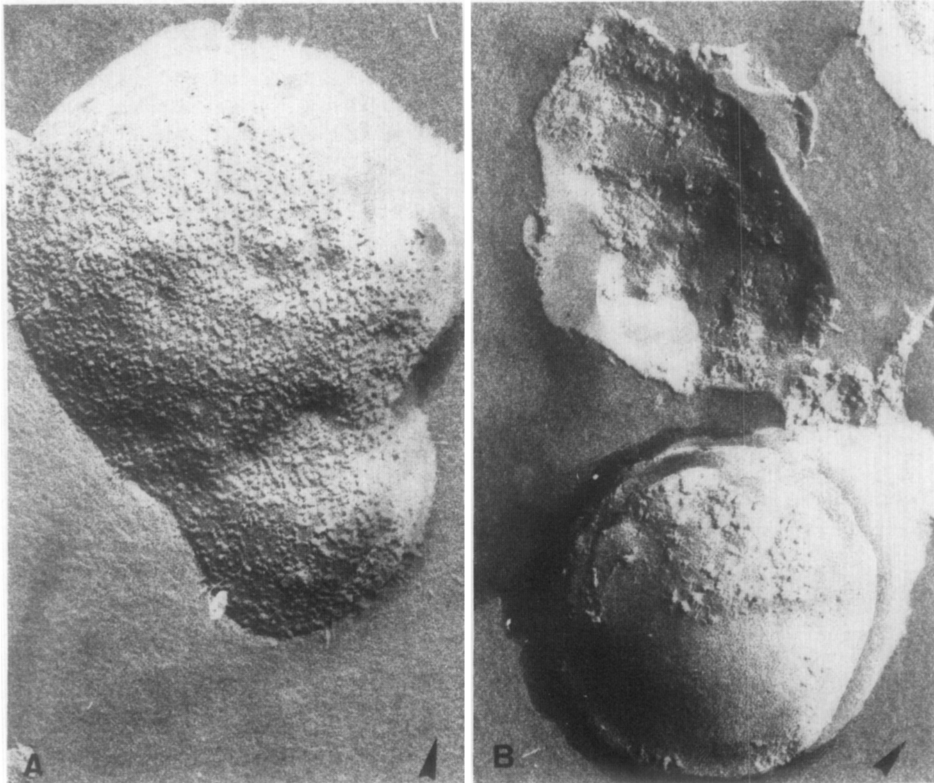

FIG. 1. Freeze-fracture faces of *Acholeplasma laidlawii* membranes, quenched from 23°C after fixation with glutaraldehyde. A, control; B, after incubation at 90°C. Magnification, ×80,000.

oleic acid-enriched membranes was reduced by subjecting the membranes to high temperature before incubation with phospholipase A_2. When a membrane preparation is preincubated for 5 min at 90°C, almost complete (96%) hydrolysis of phosphatidylglycerol at 5°C can be achieved. No lysophosphatidylglycerol can be detected at the end of the incubation, indicating that at least part of the lysophospholipase activity remained. The freeze-fracture plane of untreated oleic acid-enriched membranes at room temperature showed a homogeneous particle distribution (Fig. 1). In a heat-treated membrane preparation quenched from 22°C, aggregation of the particles was visible, suggesting that denaturation of the proteins was accompanied by clotting (Fig. 1B). Furthermore, the extent of protein digestion by Pronase was not significantly changed after the heat treatment, indicating that in spite of aggregation and denaturation, the proteins retained their intrinsic character in the membrane. It is obvious from these experiments that proteins are involved in the protection of phosphatidylglycerol against hydrolysis by phospholipase A_2.

Effects of amino-group reacting agents on the extent of phosphatidylglycerol hydrolysis. Labeling of *A. laidlawii* membranes with trinitrobenzenesulfonic acid at 37°C (Fig. 2A) resulted in a rapid loss of activity of membrane-bound enzymes, e.g., NADH-oxidase and Mg^{2+}-dependent ATPase (Fig. 2B). The lysophospholipase activity is reduced much more slowly. The size of the protected pool of phosphatidylglycerol decreased with increasing trinitrobenzenesulfonic acid labeling, as can be seen from Fig. 2A and Table II. The trinitrobenzenesulfonic acid reaction reached a maximum after 2 h of incubation and resulted in a reduction of the protected pool to 10% of the total amount of phosphatidyl-

TABLE II

EFFECT OF NH_2-GROUP ATTACKING AGENTS ON THE PROTECTED PHOSPHATIDYLGLYCEROL POOL IN OLEIC ACID-ENRICHED MEMBRANES[a]

Treatment	Incubation time (min)	Percentage of residual phosphatidylglycerol after phospholipase A_2 incubation
—	—	33
TNBS, 20 mM	1	25
TNBS, 20 mM	30	17
TNBS, 20 mM	60	12
TNBS, 20 mM	120	10
Glutaraldehyde, 20%	30	10

[a] Membranes were treated at 37°C and washed extensively (see Experimental Procedures) before incubation with 200 units phospholipase A_2 at 5°C.

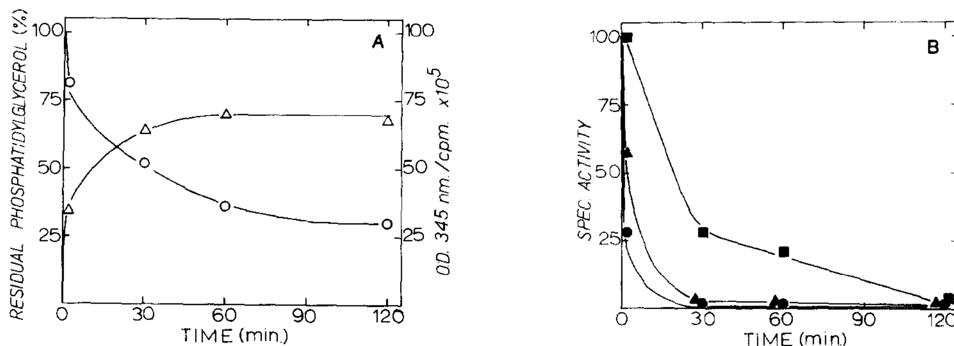

FIG. 2. A. Hydrolysis of the inaccessible pool of phosphatidylglycerol in membranes labeled to different extents with trinitrobenzenesulfonic acid (TNBS). After reaction with TNBS and extensive washing, each of the membrane preparations is treated with phospholipase A_2 at 5°C for 20 min. Phosphatidylglycerol hydrolysis is expressed as a percentage of the protected pool (found after treatment of control membranes with phospholipase A_2 at 5°C). ○, phosphatidylglycerol hydrolysis; △, the extent of TNBS labeling. B. Decrease in activity of membrane-bound enzymes due to labeling with TNBS. ■, lysophospholipase; ▲, NADH-oxidase; ●, Mg^{2+}-dependent ATPase.

glycerol (Table II). This figure can also be expressed by saying that 70% of the inaccessible phosphatidylglycerol pool is now unmasked by the trinitrobenzenesulfonic acid action. This effect was best observed when the trinitrobenzenesulfonic acid labeling was carried out at pH 8.1–8.5 and 37°C.

A similar experiment was done with glutaraldehyde. In this case, the protected pool was also reduced to 10% of the total membrane phosphatidylglycerol comparable to the effect of trinitrobenzenesulfonic acid (Table II). Since trinitrobenzenesulfonic acid and glutaraldehyde both react primarily with amino groups (39) and since all of the amino groups in *A. laidlawii* membranes originate from residues of membrane protein, it can therefore be concluded that the decrease in protection of phosphatidylglycerol against phospholipase attack resulted from perturbation of the normal lipid–protein interaction.

Hydrolysis with phospholipase A_2 from bee venom. Oleic acid-enriched membranes were incubated with phospholipase A_2 from porcine pancreas at 5° for 30 min which resulted in 75% hydrolysis of the total phosphatidylglycerol. At this stage, half of the incubation mixture was incubated for another 30 min with 60 units of phospholipase A_2 from bee venom; to the other half the same amount of pancreatic phospholipase was added. At different time intervals samples were taken from both incubations and analyzed for residual phosphatidylglycerol.

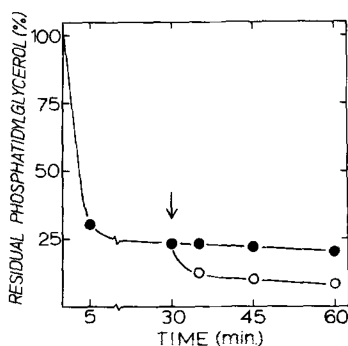

FIG. 3. Treatment of oleic acid-enriched membranes with phospholipase A_2 from porcine pancreas (●) and bee venom (○). For details, see Results.

As illustrated in Fig. 3, no significant increase in hydrolysis took place in the incubation with phospholipase from porcine pancrease. However, immediately after the addition of bee venom phospholipase, there was an additional hydrolysis of 10% phosphatidylglycerol. Further hydrolysis proceeded slowly, and after 30 min incubation with this enzyme 8% of the original phosphatidylglycerol was left.

DISCUSSION

A number of reasons might be given for the incomplete hydrolysis of phosphatidylglycerol in oleic acid-enriched membranes at low temperature. A plausible explanation is that formation of resealed membrane vesicles occurs during isolation. The existence of membrane vesicles from *A. laidlawii* has been reported by Fedotov *et al.* (28). Several observations, however, indicate that membrane preparations obtained by osmotic lysis are free of resealed structures. Electron micrographs indicate that resealing usually does not take place in membrane preparations of *A. laidlawii* (3). Furthermore with the fluorescent probes ACMA (9-amino-6-chloro-2-methoxyacridine) and merocyanin 450, we have not been able to observe any quenching of the signal after addition of ATP or glucose to *A. laidlawii* membranes (unpublished results). This indicates that no potential difference can be created over the membrane, which implies that there is no resealing or that the preparation contains only right-side-out vesicles. The latter possibility is most unlikely since two membrane-bound enzymes, Mg^{2+}-dependent ATPase and NADH-oxidase which are located on the cytoplasmic side of the membrane (30), can easily be assayed in our membrane preparations. Such results constitute good indications that these membranes do not form resealed vesicles. Finally, highly purified bee venom phospholipase A_2 free of melittin can hydrolyze phosphatidylglycerol to a larger extent than pancreatic phospholipase when membranes are incubated at 5°C (Fig. 3). Since the membrane is not freely permea-

ble to bee venom phospholipase A_2, this observation represents additional evidence that the inaccessible pool of phosphatidylglycerol is not due to inaccessible compartments of resealed membrane sacs.

Protection against phospholipase A_2 action can also occur when the lipids are present in the highly ordered gel state as we have previously shown (20, 21). Membranes of *A. laidlawii* grown on oleic acid do not show a gel- to liquid-crystalline-phase transition in the temperature range from 0 to 40°C, indicating that the lipids in this membrane are in the liquid-crystalline state. Therefore the observed inaccessibility of phosphatidylglycerol at 5°C cannot be due to the presence of solid domains of lipid in the membrane. Furthermore, a possible lateral phase separation of phosphatidylglycerol caused by Ca^{2+} ions, as was demonstrated in model systems (31), is not likely because the extent of phosphatidylglycerol hydrolysis is not dependent on the Ca^{2+} concentrations (20).

These results show that proteins of the membrane are the most likely to be involved in protecting phosphatidylglycerol against hydrolysis. Furthermore, results from high salt and protease treatments indicate that it is the intrinsic membrane proteins that are involved and not the peripheral proteins which are located on the surface of the membrane. This is in contrast with the *Mycoplasma hominis* membrane in which surface proteins have a shielding effect on membrane lipids (32). About 80–90% of the proteins in *A. laidlawii* belong to the category of integral membrane proteins (33, 34). We propose that the protective effect of this type of proteins on phosphatidylglycerol hydrolysis in *A. laidlawii* membrane is due to a short-range lipid–protein interaction. Evidence for the interaction of proteins and at least part of the lipids has been described for *A. laidlawii* membranes (35–37) and other membrane systems (2–9).

In the present membrane system, the protected pool of phosphatidylglycerol has a rather intriguing temperature dependence. Protection is effective at low temperatures, but with increasing temperatures the protected pool becomes accessible to phospholipase A_2 hydrolysis. This phenomenon can be explained by diffusion of phosphatidylglycerol out of the protected region (i.e., boundary lipid) into the bulk phase. At low temperatures, this diffusion is sufficiently slow so that it becomes the rate-limiting step of phosphatidylglycerol hydrolysis. Such a diffusion phenomenon is entirely consistent with previously reported findings (3).

The protein–lipid interactions we are discussing here is apparently disrupted as a result of chemical modification of membrane proteins. Heat treatment results in protein aggregation without, however, additional exposure of the proteins to Pronase. It appears that the proteins remain in the membrane but have lost their capacity to bind phosphatidylglycerol. Also trinitrobenzenesulfonic acid treatment and glutaraldehyde fixation results in a disturbed lipid–protein interaction. Treatment with trinitrobenzenesulfonic acid, which reacts only with the membrane proteins and some amino group containing polycarbohydrates (38), results in a loss of positively charged groups (39); a similar effect is obtained with glutaraldehyde. It is feasible therefore that the interaction between protein and the negatively charged phosphatidylglycerol has an electrostatic character. On the other hand, considering the intrinsic nature of the protein involved, it strongly indicates that apolar interaction may also be important. In any case, the lipid protein interaction is manifested in the altered characteristic of a pool of phosphatidylglycerol in the membrane. It might be a decreased lateral mobility, an increase in packing density, or both which lead to an effective protection against phospholipase attack at low temperatures. The results obtained with bee venom phospholipase A_2 (Fig. 3) support the idea of an increased packing density of lipid molecules around the integral proteins. This interpretation is suggested by the fact that bee venom phospholipase A_2 can act on phosphatidylcholine monolayers at higher surface pressures than phospholipase A_2 from porcine pancrease (40).

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REFERENCES

1. SINGER, S. J., AND NICOLSON, G. L. (1972) *Science* **175**, 720–731.
2. JOST, P. C., GRIFFITH, O. H., CAPALDI, R. A., AND VANDERKOOI, G. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 480–484.
3. JOST, P. C., NADAKAVUKASEN, K. K., AND GRIFFITH, O. H. (1977) *Biochemistry* **16**, 3110–3114.
4. NAKAMURA, M., AND OHNISHI, S. (1975) *J. Biochem. (Tokyo)* **78**, 1039–1045.
5. TRÄUBLE, H., AND OVERATH, P. (1973) *Biochim. Biophys. Acta* **307**, 491–512.
6. CULLIS, P. R. (1976) *FEBS Lett.* **68**, 173–176.
7. BRÛLET, P., AND MCCONNELL, H. M. (1976) *Biochem. Biophys. Res. Commun.* **68**, 363–368.
8. DAHLQUIST, F. G., MUCHMORE, D. C., DAVIS, J. H., AND BLOOM, M. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5435–5437.
9. WARREN, G. B., HOUSLAY, M. D., METCALFE, J. C., AND BIRDSALL, N. J. M. (1975) *Nature (London)* **255**, 684–687.
10. BEVERS, E. M., SNOEK, G. T., OP DEN KAMP, J. A. F., AND VAN DEENEN, L. L. M. (1977) *Biochim. Biophys. Acta* **467**, 346–356.
11. COLEMAN, R. (1973) *Biochim. Biophys. Acta* **300**, 1–30.
12. GOLDMAN, S. S., AND ALBERS, R. W. (1973) *J. Biol. Chem.* **248**, 867–874.
13. WARREN, G. B., TOON, P. A., BIRDSALL, N. J. M., LEE, A. G., AND METCALFE, J. C. (1974) *Biochemistry* **13**, 5501–5507.
14. HESKETH, T. R., SMITH, G. A., HOUSLAY, M. D., MCGILL, K. A., BIRDSALL, N. J. M., METCALFE, J. C., AND WARREN, G. B. (1976) *Biochemistry* **15**, 4145–4151.
15. ROELOFSEN, B., AND SCHATZMANN, H. J. (1977) *Biochim. Biophys. Acta* **464**, 17–36.
16. MANDERSLOOT, J. G., ROELOFSEN, B., AND DE GIER, J. (1978) *Biochim. Biophys. Acta* **508**, 478–486.
17. WHEELER, K. P., AND WALKER, J. A. (1975) *Biochem. J.* **146**, 723–727.
18. GRISHAM, C., AND BARNETT, R. (1972) *Biochim. Biophys. Acta* **266**, 613–624.
19. HALLINAN, T. (1974) *Biochem. Soc. Trans.* **2**, 817–821.
20. BEVERS, E. M., SINGAL, S. A., OP DEN KAMP, J. A. F., AND VAN DEENEN, L. L. M. (1977) *Biochemistry* **16**, 1290–1295.
21. BEVERS, E. M., OP DEN KAMP, J. A. F., AND VAN DEENEN, L. L. M. (1978) *Eur. J. Biochem.* **84**, 35–42.
22. DE KRUIJFF, B., DEMEL, R. A., AND VAN DEENEN, L. L. M. (1972) *Biochim. Biophys. Acta* **339**, 331–347.
23. VAN GOLDE, L. M. G., MCELHANEY, R. N., AND VAN DEENEN, L. L. M. (1971) *Biochim. Biophys. Acta* **231**, 245–249.
24. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
25. HABEEB, A. F. S. A. (1966) *Anal. Biochem.* **14**, 328–336.
26. POLLACK, J. D., RAZIN, S., AND CLEVERDON, R. C. (1965) *J. Bacteriol.* **90**, 617–622.
27. AARSMAN, A. J., AND VAN DEN BOSCH, H. (1977) *FEBS Lett.* **79**, 317–320.
28. FEDOTOV, N. S., PANCHENKO, L. F., AND TARSHIS, M. A. (1975) *Folia Microbiol.* **20**, 488–495.
29. ROTTEM, S., STEIN, O., AND RAZIN, S. (1968) *Arch. Biochem. Biophys.* **125**, 46–56.
30. NE'EMAN, Z., AND RAZIN, S. (1975) *Biochim. Biophys. Acta* **375**, 54–68.
31. VAN DIJCK, P. W. M., VERVERGAERT, P. H. J. TH., VERKLEIJ, A. J., AND VAN DEENEN, L. L. M. (1975) *Biochim. Biophys. Acta* **406**, 465–475.
32. ROTTEM, S., HASIN, M., AND RAZIN, S. (1973) *Biochim. Biophys. Acta* **323**, 520–531.
33. RAZIN, S. (1972) *Biochim. Biophys. Acta* **265**, 241–296.
34. TOURTELLOTTE, M. E., AND ZUPNIK, J. S. (1973) *Science* **179**, 84–85.
35. TOURTELLOTTE, M. E., BRANTON, D., AND KEITH, A. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 909–916.
36. ROTTEM, S., HUBBELL, W. L., HAYFLICK, L., AND MCCONNELL, H. M. (1970) *Biochim. Biophys. Acta* **219**, 104–113.
37. ROTTEM, S., AND SAMUNI, A. (1973) *Biochim. Biophys. Acta* **298**, 32–38.
38. GILLIAM, J. H., AND MOROWITZ, H. J. (1972) *Biochim. Biophys. Acta* **274**, 353–363.
39. KOBLIN, D. D., AND WANG, H. H. (1976) *Biochem. Pharmacol.* **25**, 1405–1413.
40. DEMEL, R. A., GEURTS VAN KESSEL, W. S. M., ZWAAL, R. F. A., ROELOFSEN, B., AND VAN DEENEN, L. L. M. (1975) *Biochim. Biophys. Acta* **406**, 97–107.