Reprinted from

Biochimica et Biophysica Acta
Elsevier Publishing Company, Amsterdam - **Printed in The Netherlands**
BBA 55963

STRUCTURE OF AN ABNORMAL PLASMA LIPOPROTEIN (LP-X) CHARACTERIZING OBSTRUCTIVE JAUNDICE*

D. SEIDEL, B. AGOSTINI[a] AND P. MÜLLER
Medizinische Universitätsklinik (Ludolph-Krehl-Klinik) and [a]Max-Planck-Institut für medizinische Forschung, Abteilung Physiologie, Heidelberg (Germany)
(Received July 22nd, 1971)

SUMMARY

The structure of the abnormal plasma lipoprotein (LP-X) characterizing obstructive jaundice was studied by electron microscopy and immunology. In the electron microscope negatively stained preparations of LP-X appear to consist of round particles of 300–700 Å with a tendency to aggregate and undergo remarkable structural changes. Studies with specific antibodies following phospholipase A_2 (EC 3.1.1.4) treatment indicate the presence of apolipoprotein X on the surface and of albumin in the core of the particles. Denaturation of the particles with phospholipase A_2 also suggests the presence of phospholipids on the surface, which is in agreement with the results obtained by X-ray diffraction analysis[18].

INTRODUCTION

It has been shown that the characteristic elevation of plasma lipids and plasma lipoproteins in patients with obstructive jaundice is due to the presence of a low density lipoprotein (*d* 1.006–1.063 g/ml) of abnormal composition and properties, designated LP-X[1–7].

The protein–lipid composition of LP-X is unique and distinguished by the presence of cholesterol almost entirely in unesterified form and by a high phospholipid and low protein content. LP-X contains 2.9% triglycerides, 2.4% cholesterol esters, 22.4% cholesterol, 66.5% phospholipids and 5.8% protein, so that the ratio of phospholipids to protein is 11.5 (ref. 4). Among the bile acids present (2–3%, w/w), lithocholic acid, known to be liver toxic, is represented in a relatively high amount. The protein composition of LP-X is unique, consisting of a combination of approx. 40%

Abbreviations: LP-X, lipoprotein occurring in obstructive jaundice; Apo-C, apolipoprotein C, protein moiety of lipoprotein C; Apo-A, apolipoprotein A, protein moiety of lipoprotein A; Apo-B, apolipoprotein B, protein moiety of lipoprotein B; Apo-X, apolipoprotein X, protein moiety of LP-X.

* A preliminary report was presented at the European Atherosclerosis Group Meeting, Paris, October 1970.

albumin (which corresponds to a content of 2.3% for the whole abnormal lipoprotein) and 60% of the characteristic non-albumin protein[7]. LP-X shows a positive immunoreaction with antibodies to very low density lipoprotein (d <1.006 g/ml) and *vice versa* but not with antibodies to α- or β-lipoprotein[4,7]. Very low density lipoprotein is known to have the peptide moieties of apolipoprotein C (Apo-C), apolipoprotein A (Apo-A) and apolipoprotein B (Apo-B)[8]. Since anti-LP-X serum does not react with Apo-A and Apo-B, similarity of apolipoprotein X (Apo-X) with Apo-C from very low density lipoprotein has been proposed[5,7]. The anti-LP-X serum reacts also with albumin, while anti-albumin serum shows no immunoreaction with intact LP-X[4-7]. This suggests that albumin could be masked in the intact particle.

In order to investigate this problem and the localisation of phospholipids, a structural study of LP-X was conducted by electron microscopy and further immunological investigation, both combined to enzymic degradation with phospholipase A_2.

MATERIAL AND METHODS

Isolation of LP-X

The LP-X was isolated from plasma of patients with obstructive jaundice as reported previously[4], by a procedure combining ultracentrifugation, heparin precipitation and ethanol fractionation. Material was stored at 0° in 0.9% NaCl, at pH 7.7.

Lipid and protein analysis

Esterified and free cholesterol of plasma and isolated lipoprotein fractions were estimated by the method of SPERRY AND WEBB[9], tryglicerides according to VAN HANDEL AND ZILVERSMIT[10] and phospholipids by the method of GERLACH AND DEUTICKE[11]. Protein was determined according to LOWRY *et al.*[12].

Electron microscopy

LP-X (0.5–1.5 mg/ml in 0.9% NaCl) was prepared for study in the electron microscope at 0° and at 25° after 1 day to 6 weeks after isolation, with negative staining techniques. A 2% solution of ammonium molybdate, a 1% solution of phosphotungstic acid, both adjusted to pH 7.1 with KOH, and a 1% solution of uranyl acetate at pH 4.5–5 were used. Copper grids of 300 mesh covered with a collodion film were coated with carbon in a Siemens vacuum evaporator. Usually a drop of LP-X suspension was placed on the carbon surface of a grid. After 10–20 sec the grid was drained onto filter paper and immediately covered with a drop of stain for from 10 sec to 1 min, then excess stain was removed with filter paper. Alternatively, the staining solution was mixed with the specimen suspension in a test tube and then a grid was dipped into the mixture and excess liquid was removed. Samples of LP-X were also fixed for 15–20 min at 0° by addition of an equal volume of 2% OsO_4 or 6% glutaraldehyde, both in distilled water. A drop of the specimen was applied to a grid which was stained as above. Grids were dried in air and immediately examined in a Siemens Elmiskop 101 electron microscope equipped with a cooling device, at an accelerating voltage of 80 kV. Pictures were taken at magnifications ranging from $\times$20000 to 50000.

Coupling between LP-X and mercuryazoferritin

Unfixed LP-X was also studied with the electron microscope after incubation

with the electrodense SH-group reagent mercuryphenyl azoferritin, prepared according to HASSELBACH AND ELFVIN[13], by coupling *p*-diazomercuryphenyl to *N*-ethylmaleimide-treated ferritin. Cadmium-free ferritin from horse spleen was purchased from Calbiochem (Lucerne, Switzerland).The mercuryphenyl azoferritin in solution of 0.1 M KCl and 0.05 M K_2HPO_4, pH 8.5, was added in a 2-fold excess to the LP-X suspension for 2–3 h at 4°.

Treatment of LP-X with phospholipase A_2

LP-X was also observed in the electron microscope after treatment with phospholipase A_2 (*Crotalus adamanteus*, EC 3.1.1.4, F. G. CELO, Zweibrücken, Germany) essentially according to VAN DEENEN AND DE HAAS[14], adding 2–3 mg enzyme per mg LP-X in 0.9% NaCl solution, containing 3 mM calcium acetate, pH 7.0. The mixture was incubated at 37° for 2 h, with stirring. Under these conditions, with lecithin as substrate, the reaction is linear over 1 h. The sample to which no enzyme was added served as a control.

Treatment of LP-X with lysolecithin

For comparison LP-X was mixed with lysolecithin (Koch Light Laboratories, Colnbrooks Bucks, England) in a ratio of 0.02–0.05 mg/mg of LP-X, with gentle shaking. After 10–20 min incubation at 0° samples were prepared for electron microscope study.

Immunological methods

For immunological investigations native and phospholipase treated LP-X were studied by double immuno-diffusion, essentially according to the procedure introduced by OUCHTERLONY[15], in 1% agar gel prepared with barbital buffer, pH 8.6, ionic strength 0.05, against mono-specific antisera to human albumin (Behringerwerke AG, Marburg/Lahn, Germany) and against anti-Apo-X serum. This was prepared according to SEIDEL *et al.*[4] from rabbits 2 weeks after intraperitoneal injection of LP-X mixed with an equal volume of Freund's adjuvant.

RESULTS AND DISCUSSION

In the electron microscope LP-X preparations negatively stained at pH 7.1 with potassium phosphotungstate appear to consist of more or less spherical particles (Figs. 1a and 1b) with a diameter ranging from 300 to 700 Å, larger than the normal components of the low density lipoprotein fraction[16,17]. As for these, no evidence of any distinct surface features or subunit structure can be observed. Upon contact with each other, LP-X particles undergo distortion and changes in shape (Figs. 1c and 1d). One significant characteristic of LP-X particles is their great tendency to aggregate from a globular shape into little rolls or stacks of disc-like shaped structures. Groups of rolls appear to flow together to form myelin like figures (Fig. 1d). While our paper was in preparation this was shown also by HAMILTON *et al.*[18].

No differences were observed in the electron microscope between samples stained on a grid and those stained in a test tube.

The pH of the staining solutions does not seem to play any significant role in the aggregation of the particles, as similar patterns can be obtained when LP-X is

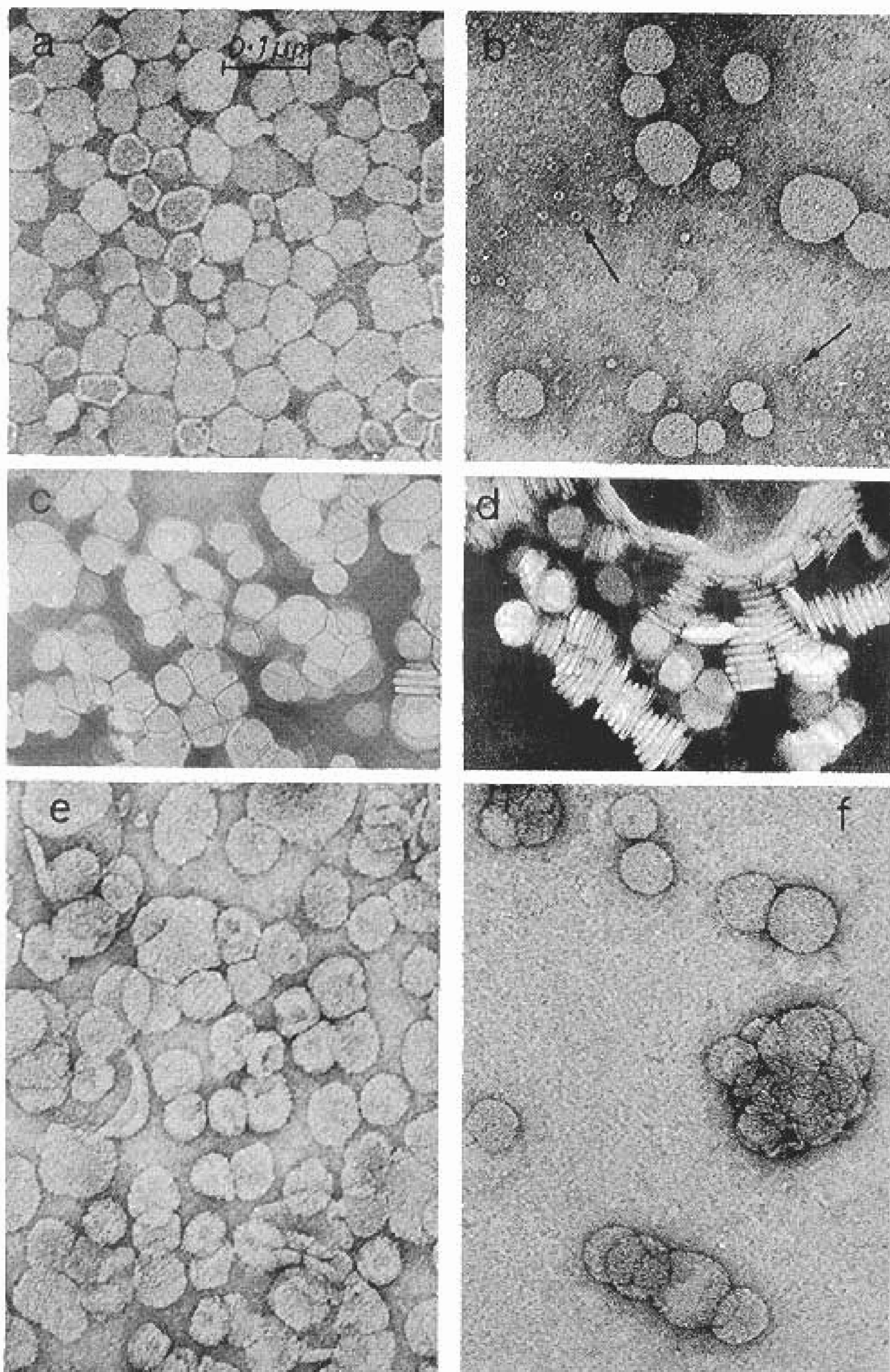

Fig. 1. Electron micrographs of negatively stained LP-X. (a) 10-day-old preparation of LP-X fixed with OsO_4 and stained at 25°. (b) Same preparation as in (a) incubated with mercuryphenyl azoferritin for 2 h; arrows indicate that ferritin granula are independent from LP-X particles; no fixation; staining at 0°. (c) Native LP-X particles stained at 0°. (d) Same preparation as in (a); no fixation; staining at 25°. (e) 12-day-old preparation of LP-X following glutaraldehyde fixation; staining at 0°. (f) Same preparation as in (e; no fixation; staining at 25°. (a–d) 1% potassium phosphotungstate, pH 7.1. (e–f) 1% uranyl acetate, pH 4.5. (a–f) × 120000.

stained at pH 4.5 with uranyl acetate (Figs. 1e and 1f). Conversely great differences can be observed even under the same pH of the staining solution (*cf.* also Figs. 1a–1d). Comparison of different areas of Fig. 1 shows that the results obtained seem to be independent of the age of the preparation or different staining conditions and fixation. Images obtained with ammonium molybdate staining are also similar to those reported in Fig. 1. In contrast to another lipoprotein isolated from abnormal egg yolk[19] aggregation of LP-X particles appear to be also independent of temperature.

In the electron microscope no binding activity of LP-X particles to the SH-groups reagent mercuryphenyl azoferritin can be observed (Fig. 1b). This suggests that insufficient free SH-groups are present on the surface of the particles to be detectable by this technique, however, without excluding the presence of protein. The localisation of the characteristic non albumin protein moiety (Apo-X) in the LP-X particles could be established by immunological study. As shown in Fig. 3, the positive immunoprecipitation reaction between anti-Apo-X serum and the native LP-X particles indicates that Apo-X is located on the surface. Thus, the failure of LP-X particles to bind the SH-groups reagent mercuryphenyl azoferritin (Fig. 1b) can be explained, since Apo-X is known to be devoid of cysteine[7].

Treatment with phospholipase A_2 denaturates the particles (Fig. 2), which may indicate that the LP-X surface consists of phospholipids in addition to Apo-X. However, small contaminations of proteolytic activity cannot be excluded and may play an additional role. In the electron micrograph, which illustrates the most usual finding,

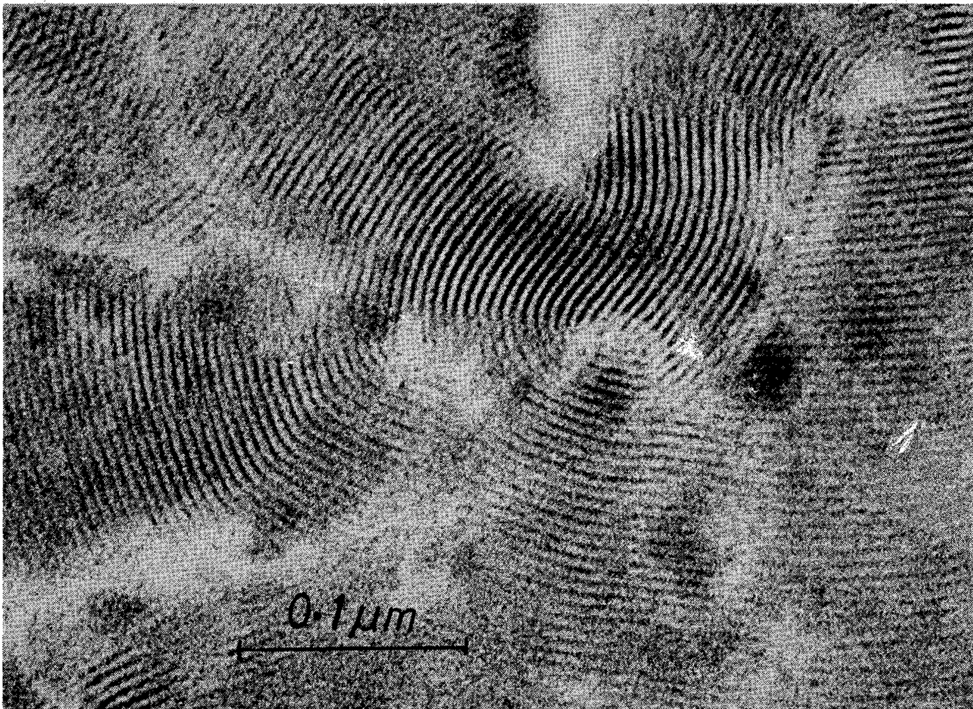

Fig. 2. Electron micrograph of LP-X following treatment with phospholipase A_2; staining at 0° with 1% potassium phosphotungstate, pH 7.1. × 300000.

LP-X particles appear completely replaced by a periodic pattern of dense and light bands, having a maximum width of 20–25 Å, arranged in the form of a finger print configuration. The feature appears to be rather unusual and may be likened to the atypical images occasionally observed on negatively stained equimolar dispersions of lecithin and cholesterol[20] or to those of lecithin and other lipids in water (for review see refs. 20 and 21). This is in agreement with the chemical composition of LP-X, which is distinguished by a high content of phospholipids (predominantly lecithin) and cholesterol[4–7].

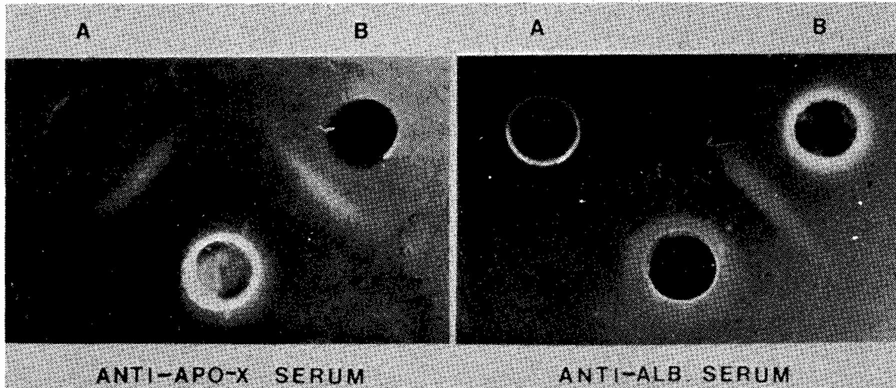

Fig. 3. Immunodiffusion pattern in 1% agar gel of native (A) and phospholipase A_2 treated LP-X (B), against anti-Apo-X serum (left), against anti-albumin serum (right).

The immunodiffusion pattern of native LP-X also changes, following phospholipase A_2 treatment. Whereas native LP-X shows no immunoreaction with anti-albumin serum, phospholipase A_2 denaturated LP-X gives a positive immunoprecipitation reaction not only with antibodies to Apo-X, but also with antibodies to albumin (Fig. 3). This indicates that treatment with phospholipase A_2 also releases albumin, which apparently is masked in the core of the native particles. At the time of writing, a paper appeared by HAMILTON *et al.*[18] which corroborates our suggestion that phospholipids are located on the surface of LP-X particles. These authors proposed also on the basis of X-ray diffraction analysis that the abnormal particles are surrounded by a continuous lipid bilayer of phospholipids and cholesterol, with the fatty acid chains and cholesterol at the center of the membrane. The protein moiety of LP-X has not been located by X-ray diffraction analysis. According to the observations of these authors, who used a gel filtration technique, LP-X contains in addition to a small amount of serum albumin the several small glycoproteins (normally found in very low density lipoprotein and high density lipoprotein), giving a total protein content in the range of that reported by other investigators[4,7]. As described by SEIDEL *et al.*[7], LP-X is a complex lipoprotein consisting of albumin in combination with several non-identical polypeptides, similar to those of Apo-C (from very low density lipoprotein), and like the latter with high affinity for lipid binding. Our finding on localisation of Apo-X on the surface of LP-X by immunology suggests that it may combine to form the wall of the particle with cholesterol and phospholipids. This is in agreement with the observation of PAPAHADJOPOULOS AND MILLER[22], that lamellar structures of lecithin

can be dispersed in small vesicular particles in the range of 200–1000 Å in diameter by addition of certain protein.

Adding small amounts of lysolecithin before staining with potassium phosphotungstate alters native LP-X particles which flow irregularly together to form spherulites surrounded by elongated structures and asymmetric stacks of discs or lamellar structures similar to those observed in negatively stained dispersions of phospholipids, mixed or not with other lipids, in water[20,21]. This confirms that LP-X is very vulnerable to undergo structural transformation. Lysolecithin has been shown to interact with lecithin–cholesterol leaflets and appears to release segments of bimolecular lipids producing disc-like structures[20] which have been likened to the abnormal particles of LP-X[18].

In summary, the electron microscope study shows that the native LP-X appears to be a spherical particle with a diameter ranging from 300 to 700 Å, having a great tendency to aggregate. The presented data indicate the presence of apolipoprotein X on the surface and of albumin in the core of the particles. Localisation of phospholipids on the surface is suggested by phospholipase A treatment of LP-X and is supported also by X-ray diffraction analysis[18] so that it can be proposed that the wall of the particles is formed by lecithin and cholesterol together with Apo-X. Further studies are necessary to ascertain the localisation of the neutral lipids of LP-X.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. The authors thank Mr. K. Jelinek, Max-Planck-Institut für medizinische Forschung, Heidelberg, for technical assistance.

REFERENCES

1 H. A. EDER, E. M. RUSS, R. A. R. PRITCHETT, M. M. WILBER AND D. P. BARR, *J. Clin. Invest.*, 34 (1955) 1147.
2 E. M. RUSS, J. RAYMUNT AND D. P. BARR, *J. Clin. Invest.*, 35 (1956) 133.
3 S. SWITZER, *J. Clin. Invest.*, 46 (1967) 1855.
4 D. SEIDEL, P. ALAUPOVIC AND R. H. FURMAN, *J. Clin. Invest.*, 48 (1969) 1211.
5 P. ALAUPOVIC, D. SEIDEL, W. J. MCCONATHY AND R. H. FURMAN, *FEBS Lett.*, 4, 2 (1969) 113.
6 G. L. MILLS, D. SEIDEL AND P. ALAUPOVIC, *Clin. Chim. Acta*, 26 (1969) 239.
7 D. SEIDEL, P. ALAUPOVIC, R. H. FURMAN AND W. J. MCCONATHY, *J. Clin. Invest.*, 49 (1970) 2396.
8 A. GUSTAFSON, P. ALAUPOVIC AND R. H. FURMAN, *Biochemistry*, 5 (1966) 632.
9 W. M. SPERRY AND M. WEBB, *J. Biol. Chem.*, 187 (1950) 97.
10 E. VAN HANDEL AND D. B. ZILVERSMIT, *J. Lab. Clin. Med.*, 50 (1957) 152.
11 E. GERLACH AND B. DEUTICKE, *Biochem. Z.*, 337 (1963) 477.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 W. HASSELBACH AND L. ELFVIN, *J. Ultrastruct. Res.*, 17 (1967) 598.
14 L. L. M. VAN DEENEN AND G. H. DE HAAS, *Biochim. Biophys. Acta*, 70 (1963) 538.
15 Ö. OUCHTERLONY, *Acta Pathol. Microbiol. Scand.*, 32 (1953) 231.
16 G. M. FORTE, A. V. NICHOLS AND R. M. GLAESER, *Chem. Phys. Lipids*, 2 (1968) 398.
17 A. V. NICHOLS, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 1128.
18 R. L. HAMILTON, R. J. HAVEL, J. P. KANE, A. E. BLAUROCK AND T. SATA, *Science*, 172 (1971) 475.
19 R. W. BURLEY, *Biochim. Biophys. Acta*, 210 (1970) 347.
20 A. D. BANGHAM AND R. W. HORNE, *J. Mol. Biol.*, 8 (1964) 660.
21 A. M. GLAUBERT AND J. A. LUCY, in A. J. DALTON AND F. HAGENAU, *The Membranes*, Academic Press, New York, 1968, p. 1.
22 D. PAPAHADJOPOULOS AND N. MILLER, *Biochim. Biophys. Acta*, 135 (1967) 624.