BBA 55900

SOME STUDIES ON THE METABOLISM OF PHOSPHOLIPIDS IN PLASMA MEMBRANES FROM RAT LIVER

E. J. VICTORIA*, L. M. G. VAN GOLDE, K. Y. HOSTETLER, G. L. SCHERPHOF AND L. L. M. VAN DEENEN

Biochemisch Laboratorium, State University of Utrecht, Vondellaan 26, Utrecht (The Netherlands)

(Received March 9th, 1971)

SUMMARY

1. Rat liver plasma membranes were isolated by rate-isopycnic zonal centrifugation. A method is described for the size 14 zonal rotor. The isolated membranes had an isopycnic banding density of 41.2% sucrose (w/w). On the basis of studies with eleven marker enzymes and electron microscopy, at least 90% of the fraction consisted of plasma membranes. The electron micrographs showed a predominantly vesicular appearance with few junctional complexes.

2. Plasma membranes exhibited virtually no CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase activity and thus appear incapable of significant *in vitro* synthesis of phosphatidylcholine by the cytidine nucleotide pathway.

3. Phosphatidylglycerol was the major product of *sn*-glycerol-3-phosphate esterification by plasma membranes in the presence of CDP-diglyceride at pH 7.5. The product was identified by thin-layer chromatography using pure phosphatidylglycerol as a reference compound, and by paper chromatography of the products obtained by hydrolysis with phospholipase D.

4. Incubation of plasma membranes with labeled exogenous phospholipids in the presence of Ca^{2+} resulted in the formation of fatty acids and monoacylphosphoglycerides. Analyses of the reaction products indicated that the phospholipase A mainly attacks the 2-position of the phosphoglyceride molecule. Plasma membrane phospholipase A also acted on labeled endogenous phosphatidylethanolamine in the presence of Ca^{2+}.

5. A partial characterization of the phospholipase indicated that it required Ca^{2+} for optimal activity, was insensitive to *N*-ethylmaleimide and deoxycholate, and was relatively heat stable. Its pH optimum was 8.0. The following was the preferred order of hydrolysis: phosphatidylethanolamine > phosphatidylglycerol > > > phosphatidylcholine.

6. Lysophospholipase was not detected in rat liver plasma membranes under

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

* Present address: Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.).

conditions in which maximal activity of this enzyme was observed in rat liver 100 000 $\times g$ supernatant.

INTRODUCTION

In the past few years considerable interest has been focused on the study of isolated plasma membranes from solid tissues[1]. Most of these studies have involved membranes obtained from liver. Although most workers have used the flotation technique introduced by NEVILLE[2], rate-isopycnic zonal centrifugation has recently been applied to the isolation of plasma membranes[3].

The lipid composition of liver plasma membranes is extensively documented in the literature[4–10]. Few studies, however, have been concerned with metabolic rather than analytic aspects of plasma membrane lipids. In 1968 it was demonstrated that plasma membranes could incorporate long-chain acyl-CoA esters, primarily into phospholipids[7]. It was also reported that the plasma membranes could acylate exogenous lysolecithins[11]. Acyltransferases catalyzing the conversion of monoacyl phosphoglycerides into the diacyl compounds have been reported in microsomes[12] and mitochondria[13–15]. Because such an acylation mechanism, combined with (a) phospholipase(s) could represent a mechanism involved in the renewal of membrane phospholipids[16–18], it was thought of interest to investigate whether the plasma membranes possess phospholipase A activity and, if present, to assess the positional specificity of this enzyme.

A phospholipase acting preferentially on the 2-position has been demonstrated in rat liver mitochondria[19,20] whereas microsomes were shown to possess a phospholipase A attacking preferentially the 1-position of phosphoglycerides[21,22]. Lysosomes of rat liver were found to contain both phospholipase A_1 and A_2 activities[23]. While this manuscript was in preparation, TORQUEBIAU *et al.*[24] described the presence of phospholipase A activity towards exogenous substrates in plasma membranes. In the present study, it was also investigated whether the enzyme acted on endogenous substrate as well, and the positional specificity of the enzyme was determined. No significant *de novo* synthesis of the major phospholipids in plasma membranes has been reported. Although most of the enzymes involved in *de novo* synthesis of phospholipids are located in the endoplasmic reticulum[25], phosphatidylglycerol biosynthesis has been reported to occur predominantly in the mitochondrion[26]. Phosphatidylglycerol was recently reported to be present also in plasma membranes[8]. In the present study, the possible role of the plasma membrane in the biosynthesis of phosphatidylglycerol was investigated.

MATERIALS AND METHODS

Isolation of plasma membranes by rate-isopycnic zonal centrifugation

Plasma membranes were isolated from the livers of 250–300-g fed male Wistar rats. The basic procedure is that of ANDERSON *et al.*[3], but it was modified for the use with a Beckman Ti14 rotor. For each run, 15 g of liver were homogenized with 25 strokes of a loose-fitting Dounce homogenizer in about 100 ml of 1 mM $NaHCO_3$, pH 7.6. The homogenate was filtered through eight layers of cheese cloth and the

filtrate diluted with the bicarbonate solution to 750 ml. This suspension was centrifuged at 25800 *g*'min in the GSA rotor of a RC2-B Sorvall centrifuge. After removal of the supernatant, the loose pellets were combined by suspension in a total volume of 50 ml of bicarbonate solution with five strokes of the Dounce homogenizer. This material was used for zonal centrifugation.

With a Beckman 141 High Capacity gradient pump, a 450-ml density gradient ranging from 19 to 35% sucrose (w/w) was delivered into a Ti14 zonal rotor rotating at 3000 rev./min in a Beckman L-2 65K centrifuge. Overlay and cushion solutions used were 1 mM $NaHCO_3$ and 45% sucrose (w/w), respectively. Following the injection of 50 ml of sample material and 50 ml of overlay into the rotor core, the rotor was accelerated to 5000 rev./min. After 10 min, the rotor was decelerated to 3000 rev./min and ten 40-ml fractions were displaced from the rotor core with cushion solution. The outflow transmittance was continuously monitored at 254 nm with an LKB Uvicord II recorder. After injection of 15 ml of overlay, the rotor was accelerated to 20000 rev./min for 15 min. Upon deceleration to 3000 rev./min, the rotor was again unloaded. Refractive indices were measured at 20° in a Bausch and Lomb Abbe-3L refractometer. The plasma membrane-containing fractions were pooled and diluted 1:1 (v/v) with bicarbonate solution and sedimented at $105000 \times g$ for 60 min in the Beckman No. 30 rotor. The pellets were stored at −20°. The yield was approx. 0.5 mg protein per g wet liver.

Other subcellular fractions

Mitochondrial and microsomal fractions were isolated in 0.25 M sucrose by classical differential centrifugation.

Marker enzymes

The following enzymes were used as markers and assayed as described in the references: succinate:cytochrome *c* reductase[27] (EC 1.3.99.1); monoamine oxidase[28] (EC 1.4.3.4, substrate kynuramine); rotenone-insensitive NADH:cytochrome *c* reductase[29] (EC 1.6.2.1); rotenone-insensitive NADPH:cytochrome *c* reductase[29] (EC 1.6.2.3); acid deoxyribonuclease[30] (EC 2.1.4.6); CDP-choline:1,2 diacyl-*sn*-glycerol cholinephosphotransferase[15] (EC 2.7.8.2); acid phosphatase[31] (EC 3.1.3.2, substrate β-glycerophosphate); 5'-nucleotidase[32] (EC 3.1.3.5, substrate 5'-AMP); ATPase[32] (EC 3.6.1.3); (Na^+, K^+) Mg^{2+}-ATPase[32] (EC 3.6.1.4) and glucose-6-phosphatase[33] (EC 3.1.3.9). All spectrophotometric measurements were carried out in a Unicam SP 500 recording spectrophotometer except for monoamine oxidase which was assayed in a Hitachi–Perkin Elmer 356 double-wavelength spectrophotometer at 360–332 nm. Protein was measured by the method of LOWRY *et al.*[34] and phosphorus according to that of CHEN *et al.*[35].

Electron microscopy

Before being processed for electron microscopy, membranes were dialysed against 0.9% NaCl. Aliquots of these dialysed suspensions, containing approx. 0.5 mg of protein, were centrifuged for 5 min at maximal speed in a Beckman Microfuge. After discarding the supernatants, the pellets were covered with 0.5 ml of a 1% OsO_4 solution buffered with veronal–acetate, pH 7.4 and containing $CaCl_2$ (ref. 36). Fixation was continued for 1 h at 4°. The fixed samples were dehydrated in a graded series of

acetone–water mixtures. The pellets were cut to size and embedded in Araldite 512.

White to gray sections were cut on a Reichert OmU2 ultramicrotome with a Dupont de Nemours diamond knife. The sections were picked up on copper grids which were covered with a parlodion film coated with a thin layer of carbon. The sections were stained for 5 min by floating on a drop of saturated uranylacetate followed by staining with lead citrate. The specimens were examined in a Siemens Elmiskop I electron microscope at an accelerating voltage of 60 kV. Micrographs were taken at instrumental magnifications of approx. 4000 and 56000 on Agfa Scientia 23D50 plates. Accurate magnifications were determined according to the method described by ELBERS AND PIETERS[37].

Labeled substrates

2-[^{3}H]-*sn*-Glycero-3-phosphate was prepared from 2-[^{3}H]glycerol and ATP in the presence of glycerokinase and purified according to the method of BUBLITZ AND KENNEDY[38]. The specific activity was 0.5 mC/μmole.

1-Palmitoyl-2-[1-^{14}C]oleoyl-3-*sn*-phosphatidyl-1'-*sn*-glycerol was isolated from *Mycoplasma laidlawii* strain B grown in lipid-poor medium containing 75 μC of [1-^{14}C] oleic acid per l as described by MCELHANEY AND TOURTELOTTE[39]. Analyses of the products after degradation with *Crotalus adamanteus* venom indicated that 80% of the label was present at the 2-position. The specific activity was $1.8 \cdot 10^6$ counts/min per μmole.

1-[9,10-^{3}H]Stearoyl-2-oleoyl-3-*sn*-phosphatidyl-1'-*sn*-glycerol was similarly isolated from Mycoplasma grown in a medium containing 400 μC of [9,10-^{3}H]stearic acid per l. Labeled stearate was 74% at the 1-position. The specific activity was $1.5 \cdot 10^6$ counts/min per μmole.

1-Saturated-2-[1-^{14}C]linoleoyl-3-*sn*-phosphatidylethanolamine was prepared as previously described[21]. Linoleic acid was 97% at the 2-position. The specific activity was $7 \cdot 10^6$ counts/min per μmole.

1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphorylcholine, specific activity 362 counts/min per nmole, was a generous gift of Dr. H. Van den Bosch of this laboratory.

Assay for phosphatidylglycerol biosynthesis by isolated plasma membranes

Plasma membranes were incubated in a medium essentially the same as that of KIYASU *et al.*[26] (see legend to Fig. 3). The radioactive lipid product was determined by a filter paper disc assay as described by GOLDFINE[40]. The filter paper discs were counted in vessels containing 16 ml of scintillation fluid (0.5% PPO and 0.03% POPOP in toluene). Quench corrections were made by the channels ratio method.

Identification of the radioactive product was as follows. After incubation as described for Fig. 3, the total lipids were extracted according to BLIGH AND DYER[41], concentrated to a small volume *in vacuo* and applied to 0.5 mm thick thin-layer plates. Chromatography was carried out in two solvent systems. One consisted of chloroform–methanol–water–25% ammonia (70:30:3:2, by vol.) and was used with plates prepared from silica gel H in 0.4 M sodium borate. The other was chloroform–methanol–acetic acid–water (50:25:7:3, by vol.) and was used with silica gel H plates slurried with 1 mM Na_2CO_3. Reference standards of phosphatidylglycerol were obtained from *Mycoplasma laidlawii* by the method of Shaw *et al.*[42]. Spots were identified by exposure to iodine vapors or by development with periodate–Schiff reagent.

The thin-layer plates were scanned for radioactivity with a Panax TLC scanner (Panax Equipment Ltd., Great Britain) and the single radioactive lipid found was eluted from the silica gel by the method of SKIPSKI *et al.*[43]. The eluted lipid was hydrolyzed with phospholipase D prepared from cabbage as described by LONG[44]. After partitioning as described by BLIGH AND DYER[41], the water-soluble products were applied to Whatman No. 1 filter paper. Descending chromatography was carried out in *n*-propanol–25% ammonia–water (6:3:1, by vol.). Glycerol-3-phosphate and glycerol were used as reference standards. After developing the guide spots with periodate-Schiff reagent, the paper was scanned for radioactivity.

Assay of plasma membrane phospholipase A activity towards exogenous labeled phospholipids

Labeled phospholipids, after evaporation to dryness under a stream of nitrogen, were resuspended in water at a concentration of 1 μmole/ml and sonicated for 1 min with a Branson sonifier at a setting of 4 A. Appropriate aliquots of the phospholipid dispersions were added to the standard incubation mixture which contained the following or multiples thereof: 10 mM Tris–HCl, (pH 8.0), 10 mM Ca^{2+} and 0.050 mg plasma membrane protein in a total volume of 0.25 ml. The incubations were carried out in a shaking water bath at 37° and the reactions were stopped by the addition of 1 ml of methanol–chloroform (2:1, v/v). Lipid extraction was carried out by the method of BLIGH AND DYER[41], after acidification to pH 4.5 with 0.5 M H_2SO_4.

The lipid extract was chromatographed on silica gel G thin-layer plates in two solvent systems: first in chloroform–light petroleum–acetic acid (65:33:2, by vol.) and subsequently in chloroform–methanol–water (65:35:4, by vol.) in the same direction. Unlabeled compounds were co-chromatographed in order to facilitate identification. Following visualization by iodine vapor, the appropriate spots were scraped off into vials containing 15 ml of scintillation fluid. For ^{14}C-labeled compounds, the scintillation medium consisted of 0.7% PPO, 0.03% dimethyl-POPOP and 10% naphthalene per l of dioxane diluted 5 parts to 1 of water (v/v). For tritiated samples the scintillation medium consisted of 0.5% PPO and 0.03% dimethyl-POPOP per l of toluene diluted with Triton X-100 and water in a ratio of 2:1:0.2 (by vol.). A Packard TriCarb liquid scintillation counter was employed for the measurements.

Assay of plasma membrane phospholipase A activity towards endogenous labeled phospholipid

Two rats were injected intraperitoneally with 25 μC of [1,2-^{14}C]ethanolamine–HCl. After 3 h the animals were sacrificed and the plasma membranes isolated. 1.35 mg plasma membrane proteins were incubated at 37° in a medium containing 0.5 mM EGTA, 2 mM Ca^{2+}, 66 mM KCl, 53 mM Tris–HCl buffer, pH 7.4, in a total volume of 0.5 ml. The reaction was started by addition of Ca^{2+}. At the indicated times, the reaction was stopped by addition of excess EGTA (final concentration, 5 mM) followed by 2 ml of chloroform–methanol (1:2, v/v). The lipids were extracted as described by BLIGH AND DYER[41] and then chromatographed on silica gel G thin-layer plates and lysophosphatidylethanolamine spots were identified by means of reference standards and scraped directly into scintillation vials.

Lysophospholipase assay

The assay for lysophospholipase was carried out as described in detail by VAN DEN BOSCH *et al.*[45].

Chemicals

2-[^{3}H]glycerol and labeled fatty acids were purchased from the Radiochemical Centre, Amersham (G.B.). [1.2-^{14}C]Ethanolamine–HCl was obtained from New England Nuclear (U.S.A.). CDP-choline and CDP-diglyceride were obtained from Tracerlab (U.S.A.) and Koch–Light (G.B.), respectively. ATP, kynuramine-di-HBr and glucose-6-phosphate were bought from Sigma (U.S.A.), AMP from Merck (Germany), cytochrome *c* and *sn*-glycero-2-phosphate from Fluka (Switzerland), NADH, NADPH, coenzyme A, and glycerokinase (EC 2.1.1.3) from Boehringer (Germany), rotenone from Aldrich (U.S.A.), and DNA from Koch–Light (G.B.).

RESULTS

Sedimentation properties of plasma membranes

A typical sedimentation profile of plasma membranes is shown in Fig. 1. The average banding density for five runs was 41.2% sucrose (w/w) (d = 1.182 g/ml).

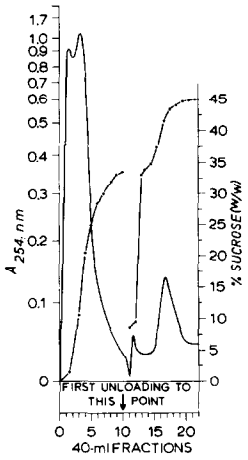


Fig. 1. Rate-isopycnic zonal separation of plasma membranes in the Ti14 rotor. Fractions 16, 17, and 18 represent mainly plasma membranes.

Distribution of marker enzymes in cell fractions

Table I shows the specific activities of enzymes present in the different cell fractions studied. The activities of mitochondrial, microsomal, and lysosomal enzymes are drastically reduced in the plasma membranes. Conversely, there is an enrichment in those enzymes believed to be associated with the plasma membrane[32] (5'-nucleotidase and (Na^+,K^+) Mg^{2+}ATPase). On the basis of studies with rotenone-insensitive NADPH:cytochrome *c* reductase, glucose-6-phosphatase, succinate:cytochrome *c* reductase, monoamine oxidase and acid phosphatase, the maximal possible contamination with microsomes, intact mitochondria, mitochondrial outer membrane fragments

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF RAT LIVER

The results are given as nmoles substrate or product transformed per min per mg protein, except for monoamine oxidase which is expressed in arbitrary absorbance units/min per mg protein. All values are the means of triplicate determinations. In all cases the dispersion was less than 5%. For plasma membranes, the assays were carried out in pooled samples from at least five zonal runs. —, Not determined.

Marker enzyme	*Plasma membranes*	*Total homogenate*	*Mitochondria (containing lysosomes)*	*Microsomes*
Succinate:cytochrome *c* reductase	0.5	22.4	60.8	—
Monoamine oxidase	0.002	0.003	0.024	—
(Rotenone-insensitive) NADH:cytochrome *c* reductase	31.3	151.0	74.6	700.0
(Rotenone-insensitive) NADPH:cytochrome *c* reductase	5.0	9.2	—	64.7
Acid deoxyribonuclease	0.6	3.4	24.4	—
5′-Nucleotidase	594.0	63.0	—	91.0
Acid phosphatase	3.0	12.9	79.0	—
Mg^{2+}-ATPase	1760.0	246.0	—	—
(Na^+, K^+) Mg^{2+}-ATPase	320.0	27.0	—	—
Glucose-6-phosphatase	35.0	—	—	350.0
Cholinephosphotransferase	0.044	—	—	0.720

and lysosomes was judged to be less than 10%, 1%, 1% and 1%, respectively. For the calculation of lysosomal contamination, a purposely high figure of 20% was assumed for the amount of lysosomes present in the classical mitochondrial pellet[46]. The outer membrane calculation is based on a mitochondrial content of 10% outer membrane on a protein basis[47–49].

The amount of cholinephosphotransferase in plasma membranes relative to that in microsomes does not exceed the corresponding value for glucose-6-phosphatase and rotenone-insensitive-NADPH:cytochrome *c* reductase, indicating that the *de novo* synthesis of lecithin is localized almost exclusively in the endoplasmic reticulum[15,25].

Electron microscopy of isolated plasma membranes

Electron micrographs of the membrane preparations showed that they were essentially free from whole mitochondria, lysosomes and nuclei. Microsomal and mitochondrial outer membrane contamination is somewhat harder to assess morphologically, since in thin sections vesicles derived from outer membrane fragments and smooth microsomes cannot be discriminated from small plasma membrane vesicles. The presence, however, of microsomes derived from rough endoplasmic reticulum can, almost certainly, be excluded. Although rough-surfaced membranes were occasionally observed, it was clear from their irregular size and shape at high magnification that the particles responsible for this rough appearance were not ribosomal in origin but rather were due to nonspecific stain deposits.

Most of the membrane material was present as nearly spherical vesicles ranging in diameter from approx. 0.1 to 2 μm (Fig. 2A). Often many small vesicles were contained within larger ones. Occasionally, junctional structures were encountered. They usually appeared as sheets rather than vesicles. A high-resolution micrograph of such a structure is shown in Fig. 2B. At high magnification, the membranes clearly reveal the familiar trilaminar appearance as is shown in Fig. 2C.

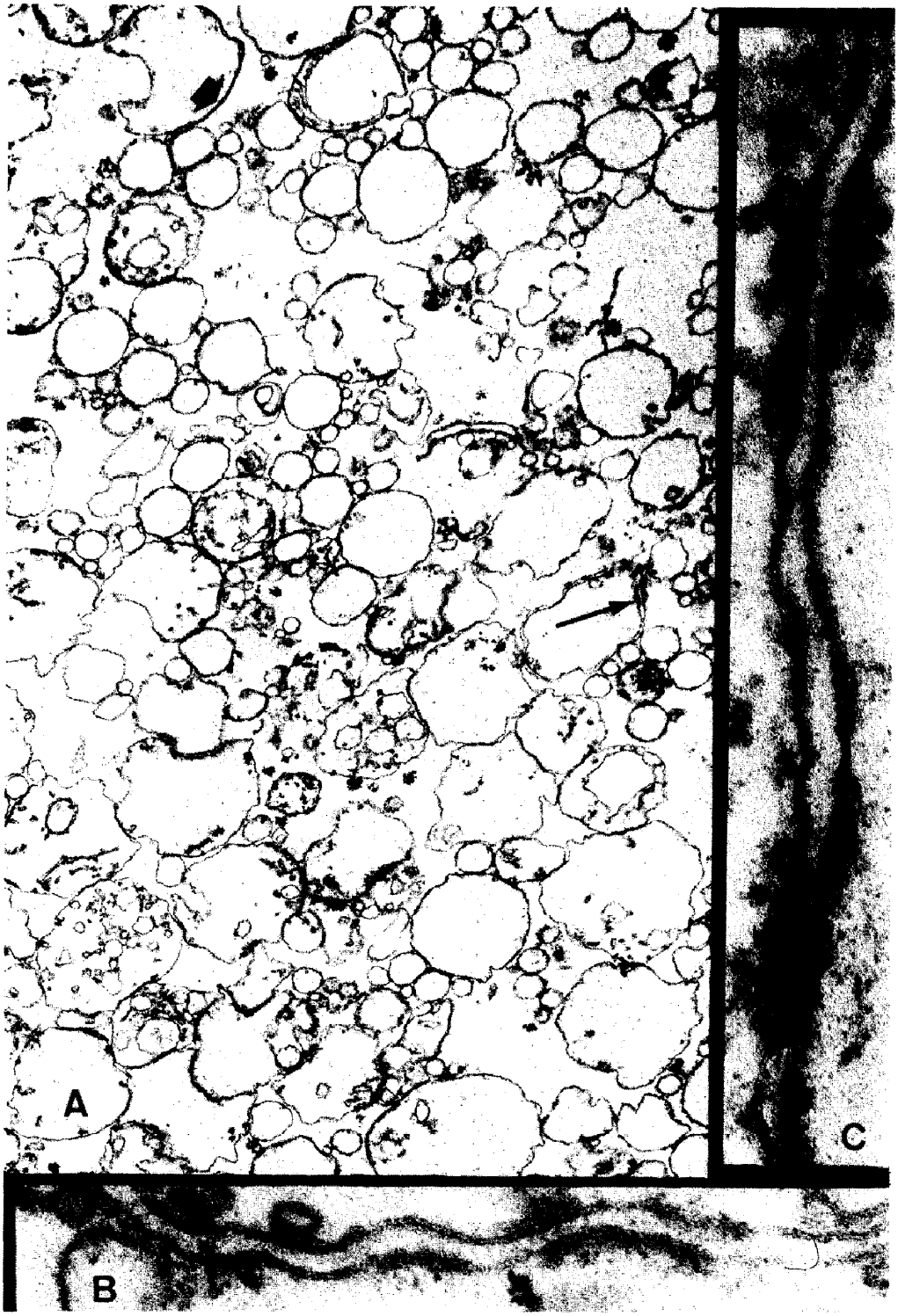


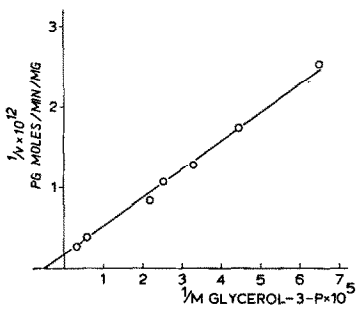
Fig. 2. Electron micrographs of thin sections of plasma membrane preparations. A. Low-magnification survey micrograph. The thick arrow points to an apparent double-membrane structure which, upon higher magnification, resolves into two triple-layered membranes in close proximity (see Fig. 2B). The thin arrow indicates an area containing junctional structures as shown in Fig. 2C. Magnification, 19 000×. B. High resolution picture of an area as indicated by the thick arrow in Fig. 2A. Magnification, 170 000×. C. High resolution picture of a junctional complex. Magnification, 170 000×.

Biosynthesis of phosphatidylglycerol in plasma membranes

Phosphatidylglycerol was the only detectable product of *sn*-glycerol-3-phosphate esterification by plasma membranes in the presence of CDP-diglyceride. The product was identified as follows: Thin-layer chromatography of the total lipid extract in chloroform–methanol–water–25% ammonia (70:30:3:2, by vol.) and in chloroform–methanol–acetic acid–water (50:25:7:3, by vol.) gave a single radioactive spot which co-chromatographed with phosphatidylglycerol isolated and purified from *Mycoplasma laidlawaii*. There was a single radioactive spot on the paper chromatogram of the water-soluble products produced by the action of phospholipase D on the radioactive lipid product. The spot had an R_F of 0.69. This value was identical to that obtained with glycerol, whereas glycerol-3-phosphate had an R_F value of 0.21 in this solvent system. These results establish that the radioactive lipid product synthesized by plasma membranes is phosphatidylglycerol.

Phosphatidylglycerol synthesis reaction characteristics

The velocity of the reaction was measured by the rate of appearance of radioactive lipid product. Under the assay conditions, the rate of synthesis was directly proportional to the protein concentration and length of incubation. The reaction had a broad pH optimum with a maximum at 7.5. The relationship between *sn*-glycerol-3-phosphate concentration and phosphatidylglycerol synthesized is shown in Fig. 3. The apparent K_m was $1.9 \cdot 10^{-5}$M. Fig. 4 relates the velocity of the reaction to CDP-diglyceride concentration. The apparent K_m value for the reaction was $3.4 \cdot 10^{-5}$M.



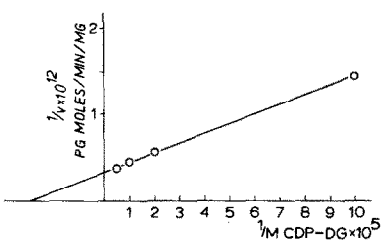


Fig. 3. Double-reciprocal plot of the formation of phosphatidylglycerol (PG) as a function of *sn*-glycerol-3-phosphate concentration. The incubation mixture contained 50 mM Tris–HCl, pH 7.5, 16 mM β-mercaptoethanol, 0.1 mM CDP-diglyceride, 0.088 mg plasma membrane protein and the varying concentrations of *sn*-glycerol-3-phosphate in a total volume of 0.5 ml. Incubations were carried out at 37° for 60 min.

Fig. 4. Double-reciprocal plot of the formation of phosphatidylglycerol (PG) as a function of CDP-diglyceride (CDP-DG) concentration. The incubation mixture contained 0.038 mM *sn*-glycerol-3-phosphate and varying concentrations of CDP-diglyceride (CDP-DG) but was otherwise the same as given for Fig. 3.

Deacylation of exogenous labeled phospholipids by plasma membrane phospholipase

The phospholipase A activity of plasma membrane was measured by the appearence of labeled products, either lysophospholipids or fatty acids. For all substrates, the reaction was measured under conditions of linear dependence on protein concentration and length of incubation.

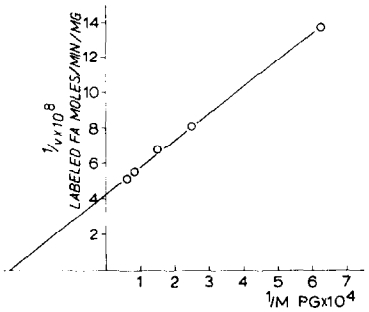


Fig. 5. Plasma membrane phospholipase A activity. Kinetics of labeled fatty acid (FA) release as a function of 1-palmitoyl-[2-^{14}C]oleoyl-3-*sn*-phosphatidyl-1′-*sn*-glycerol (PG) concentration. The incubation volume of 0.25 ml contained 0.050 mg plasma membrane protein, 10 mM Ca^{2+}, 70 mM Tris–maleate, pH 8.0, and PG. The reaction was stopped after 15 min at 37°.

When 1-palmitoyl-2-[1-^{14}C]oleoyl-3-*sn*-phosphatidyl-1′-*sn*-glycerol was incubated with plasma membranes, fatty acid was the main labeled product. The effect of substrate concentration on fatty acid release is shown in Fig. 5. An apparent K_m value of $3.6 \cdot 10^{-5}$ M was obtained and v_{max} was 2.3 nmoles/min per mg protein. Using 1-saturated-2-[1-^{14}C]linoleoyl-phosphatidylethanolamine as a substrate, an apparent K_m value of $5 \cdot 10^{-4}$ M was obtained, whereas v_{max} was found to be 6.6 nmoles ^{14}C-labeled fatty acid released per min per mg protein. In addition to indicating a phospholipase A activity in plasma membranes, the results suggest that the action may be directed to the 2-position of the phospholipid molecule mainly.

TABLE II

POSITIONAL SPECIFICITY OF PHOSPHOLIPASE A FROM RAT LIVER PLASMA MEMBRANES

Incubations were carried out as described in MATERIALS AND METHODS. The monoacyl phosphoglyceride and fatty acids formed were assayed for radioactivity. The ratio of radioactivity in the fatty acids to that in monoacylphosphoglycerides is shown.

Substrate	*Isotopic distribution in substrate (C-1/C-2)*	*Released fatty acid radioactivity/released monoacylphosphoglyceride radioactivity*			
		Microsomes	*Plasma membranes*		
			*1**	*2***	*3****
[^{3}H]Phosphatidylglycerol	74/26	76/24	27/73	—	—
[^{14}C]Phosphatidylglycerol	20/80	23/77	85/15	—	—
[^{14}C]Phosphatidylethanolamine	3/97	13/87	78/22	70/30	76/24

* Membranes prepared as described in this paper.
** Membranes provided by Prof. Emmelot.
*** Membranes (from beef liver) provided by Prof. Fleischer.

In order to confirm the latter point, a series of experiments was carried out with various phospholipids labeled at the 1- or 2-positions. As the results in Table II indicate, while microsomes are phospholipase A_1-specific[21,22], phospholipase A_2 specificity predominates in plasma membranes. In agreement with findings on mitochondria and microsomes[19,21], phosphatidylcholine was not significantly hydrolyzed. The results in Table II are consistent with the presence of only phospholipase A_2 activity in the experiments where phosphatidylglycerol was the substrate. However, with phosphatidylethanolamine as a substrate, the results suggest the presence of both phospholipase A_2 and A_1 activities, the former being predominant.

In order to obtain an independent check on the phospholipase A_2 specificity and, indirectly, on the purity of our plasma membrane preparations, phospholipase assays were carried out with membranes generously donated by Prof. Dr. P. Emmelot (Department of Biochemistry, Netherlands Cancer Institute, Amsterdam) and Prof. Dr. S. Fleischer (Department of Molecular Biology, Vanderbilt University, Nashville). The results obtained were similar to those obtained using our own membranes and confirm that plasma membrane phospholipase A activity is primarily specific for the 2-position.

A partial characterization of the enzyme was undertaken. The pH optimum for

TABLE III

ENZYMATIC PROPERTIES OF PLASMA MEMBRANE PHOSPHOLIPASE A

The standard enzymic assay contained 0.060 mg plasma membrane protein, 80 μM 1-palmitoyl-[2-^{14}C]oleoyl-3-*sn*-phosphatidyl-1′-*sn*-glycerol, 10 mM Ca^{2+}, and 70 mM Tris–maleate, pH 8.0, in a total volume of 0.25 ml. The incubation was carried out at 37° for 15 min. When used, the following reagents were present in these concentrations or amounts: 10 mM Mg^{2+}, 2 mM *N*-ethylmaleimide, 50 mM EDTA and 0.1 mg deoxycholate.

Enzymic assay	*Fatty acid released (nmoles/min per mg protein)*
Control	1.75
No Ca^{2+}	0.73
No Ca^{2+}, *plus* EDTA	0.29
EDTA	0.33
No Ca^{2+} *plus* Mg^{2+}	0.60
N-Ethylmaleimide	1.77
10 min at 70°	0.76
Deoxycholate	1.73

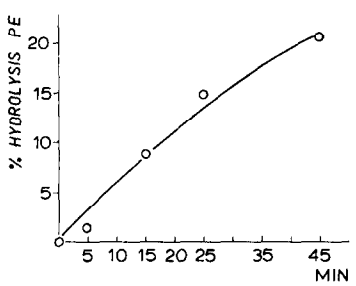


Fig. 6. Deacylation of endogenously labeled phosphatidylethanolamine (PE) by plasma membrane phospholipase. For details, see MATERIALS AND METHODS.

phospholipase hydrolysis was 8.0. Table III shows the effect of various modifications of the incubation medium on phospholipase activity. The enzyme had a requirement for calcium. The presence of *N*-ethylmaleimide or deoxycholate had no effect. In agreement with data on other phospholipases[50], the enzyme was rather heat stable.

As Fig. 6 indicates, a significant amount of endogenous phosphatidylethanolamine hydrolysis can occur due to phospholipase A activity. The rate of reaction was linear with time over the range studied.

Lysophospholipase activity in plasma membranes

Previous work from this laboratory has shown that a lysophospholipase is pre-

sent in rat liver and that this enzyme is localized predominantly in the 100000 × *g* supernatant fraction[45]. Table IV shows that no detectable amount of lysophospholipase was found in rat liver plasma membranes when compared to the activity of this enzyme in the 100000 × *g* supernatant.

TABLE IV

HYDROLYSIS OF 1-[^{14}C]PALMITOYL-*sn*-GLYCERO-3-PHOSPHORYLCHOLINE BY 100 000 × *g* SUPERNATANT AND PLASMA MEMBRANES FROM RAT LIVER

20 nmoles of 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine were incubated for 15 min with the indicated amount of proteins in a final volume of 1 ml. The amount of ^{14}C-labeled fatty acids released was measured as described in detail in a previous work from this laboratory[45].

Amount of protein added (μg)	*nmoles [^{14}C]palmitate released*	
	Rat liver 100 000 × g supernatant	*Rat liver plasma membranes*
50	2.0	<0.05
100	5.1	<0.05
200	7.7	<0.05

DISCUSSION

Several reports have appeared on the isolation of liver plasma membranes by zonal centrifugation[3,51–53]. However, in all cases the isolation procedure has involved A-type or size 15 zonal rotors. The plasma membrane fraction obtained by zonal centrifugation in the size 14 rotor shows very similar sedimentation behavior to that obtained in the larger size 15 rotor as described by ANDERSON[3]. The yield of our preparations is more comparable to that obtained by flotation methods[54]. This probably stems from the lower capacity of the rotor we employed.

The results of the marker enzyme tests clearly indicate that the only significant contamination in our plasma membrane preparation is endoplasmic reticulum (max. 10%). This probably originates at the very beginning of the procedure when the cell is disrupted. Mitochondria and lysosomes are not present in significant amounts (less than 1%) in the isolated plasma membranes as judged by marker enzyme tests and electron microscopy.

The absence of mitochondrial outer membranes was ascertained by the very low level of monoamine oxidase activity in the plasma membranes. Moreover, isolated outer membranes show a banding density of 28.5% sucrose (w/w)[55], sufficiently far from that of plasma membranes (41.2%, w/w) so as not to constitute a significant source of contamination.

The presence of phosphatidylglycerol in plasma membranes has recently been reported by RAY *et al.*[8]. In addition, cardiolipin, which is known to be synthesized from phosphatidylglycerol[56,57], has also been detected in rat-liver plasma membranes[9]. KIYASU *et al.*[26] have shown that the biosynthesis of phosphatidylglycerol takes place predominantly in the mitochondria, whereas the endoplasmic reticulum is the main locus for the synthesis of nitrogen-containing phospholipids. The present paper demonstrates that the synthesis of phosphatidylglycerol from CDP-diglyceride and glycero-3-phosphate occurs also in the plasma membrane of the rat liver cell. The specific activity of the enzyme catalyzing this synthesis in plasma membranes is about 30% of that in mitochondria. Therefore, the finding of phosphatidylglycerol

biosynthesis cannot be accounted for by the mitochondrial contamination of the plasma membrane preparation which was shown to be less than 1%. Finally, a series of incubations were carried out with and without penicillin G (200 units/ml) and streptomycin (200 μg/ml). The antibiotics were found to have no effect on the rate of phosphatidylglycerol synthesis.

Choline phosphotransferase was not found in significant amounts in plasma membranes. This finding and earlier observations with mitochondria[25,15] corroborate the idea that the *de novo* synthesis of lecithin is localized exclusively in the endoplasmic reticulum. On the other hand, both mitochondria[13–15] and plasma membranes[11] have been shown to possess the acyltransferases catalyzing the conversion of lysolecithin into lecithin. In a very recent paper, however, KAULEN *et al.*[58] describe that the acyltransferases reported to be present in plasma membranes[11] are due to microsomal contamination of the plasma membrane.

Phospholipases have been reported to occur in rat liver in mitochondria[19,20], lysosomes[22,23], and microsomes[21,22]. The mitochondrial phospholipase was found to be located predominantly in the outer membrane fraction[59,60] and was shown to act preferentially at the 2-position of phospholipids in contrast to microsomal phospholipase A which attacks primarily the 1-position[21,22]. The very recent results of TORQUEBIAU *et al.*[24] demonstrating the presence of the phospholipase A in rat liver plasma membranes with exogenous phospholipids as substrates did not include data on the positional specificity of this enzyme. The results in this paper show the presence of a phospholipase A acting both on exogenous phosphatidylethanolamine and phosphatidylglycerol and on endogenous phosphatidylethanolamine. The enzymic activity was shown to be mainly phospholipase A_2 although the presence of activity towards the 1-position of phosphatidylethanolamine cannot be explained completely by the microsomal contamination in our plasma membrane preparation. At the time that this paper was being submitted, an article by NEWKIRK AND WAITE[61] appeared describing the presence of plasma membrane phospholipase activity which was predominantly A_1. Although it is difficult to calculate from the data given by these authors (the specific activities of the microsomal marker enzymes in the microsomal fraction are not given), this discrepancy may be due, in fact, to a higher degree of microsomal contamination in the preparation of NEWKIRK AND WAITE.

Our results indicate that the biosynthesis capacities of plasma membranes are not limited to the synthesis of phosphatidic acid which was observed by STEIN *et al.*[11]. Synthesis of phosphatidylglycerol can also take place at a significant rate in the plasma membrane. However, the plasma membrane apparently does not possess the enzyme(s) required for the *de novo* synthesis of nitrogen-containing phospholipids such as phosphatidylcholine. For the supply of these phospholipids, the plasma membrane may be dependent on the endoplasmic reticulum. In such processes an exchange protein as found in the cytoplasm by WIRTZ AND ZILVERSMIT[62] might play an important role.

ACKNOWLEDGMENTS

The present investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). E.J.V.

is a Postdoctoral Fellow of the American Cancer Society and K.Y.H. is a U.S. Public Health Service Postdoctoral Fellow in Medicine (Grant No.AM-1005).

The authors wish to thank Prof. Dr. P. EMMELOT (Netherlands Cancer Institute, Department of Biochemistry, Amsterdam) and Prof. Dr. S. Fleischer (Vanderbilt University, Department of Molecular Biology, Nashville) for kindly providing preparations of plasma membranes. The authors are much indebted to Miss A. H. Niemeyer for excellent technical assistance.

REFERENCES

1 E. L. BENEDETTI AND P. EMMELOT, in A. J. DALTON AND F. HAGUENAU, *The Membranes*, Academic Press, New York, 1968, p. 33.
2 D. M. NEVILLE, Jr., *J. Biophys. Biochem. Cytol.*, 8 (1960) 413.
3 N. G. ANDERSON, A. I. LANSING, I. LIEBERMAN, C. T. RANKIN Jr. AND H. ELROD, *Wistar Inst. Monogr.*, 8 (1968) 23.
4 N. TAKEUCHI AND H. TERAYAMA, *Exp. Cell Res.*, 40 (1965) 32.
5 B. J. DOD AND G. M. GRAY, *Biochim. Biophys. Acta*, 150 (1968) 397.
6 R. C. PFLEGER, N. G. ANDERSON AND F. SNYDER, *Biochemistry*, 7 (1968) 2826.
7 W. L. STAHL AND E. G. TRAMS, *Biochim. Biophys. Acta*, 163 (1968) 459.
8 T. K. RAY, V. P. SKIPSKI, M. BARCLAY, E. ESSNER AND F. M. ARCHIBALD, *J. Biol. Chem.*, 244 (1969) 5528.
9 R. WOOD, *Arch. Biochem. Biophys.*, 141 (1970) 174.
10 R. HENNING, H. D. KAULEN AND W. STOFFEL, *Z. Physiol. Chem.*, 351 (1970) 1191.
11 Y. STEIN, C. WIDNELL AND O. STEIN, *J. Cell Biol.*, 39 (1968) 185.
12 W. E. M. LANDS, *J. Biol. Chem.*, 235 (1960) 2233.
13 G. L. SCHERPHOF AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 113 (1966) 417.
14 W. STOFFEL AND H. G. SCHIEFER, *Z. Physiol. Chem.*, 349 (1968) 1017.
15 M. G. SARZALA, L. M. G. VAN GOLDE, B. DE KRUYFF AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 202 (1970) 106.
16 W. E. M. LANDS AND I. MERKL, *J. Biol. Chem.*, 238 (1963) 898.
17 L. L. M. VAN DEENEN, H. VAN DEN BOSCH, L. M. G. VAN GOLDE, G. L. SCHERPHOF AND B. M. WAITE, *Cellular Compartmentalization and Control of Fatty Acid Metabolism*, Universitets forlaget, Oslo, 1968, p. 89.
18 H. VAN DEN BOSCH, L. M. G. VAN GOLDE, A. J. SLOTBOOM AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 152 (1968) 694.
19 G. L. SCHERPHOF AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 98 (1965) 204.
20 C. R. ROSSI, L. SARTORELLI, L. TATO, L. BARETTA AND N. SILIPRANDI, *Biochim. Biophys. Acta*, 98 (1965) 207.
21 G. L. SCHERPHOF, B. M. WAITE AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 125 (1966) 406.
22 P. J. BJÖRNSTAD, *Biochim. Biophys. Acta*, 116 (1966) 500.
23 W. STOFFEL AND U. TRABERT, *Z. Physiol. Chem.*, 350 (1969) 836.
24 O. TORQUEBIAU-COLARD, M. PAYSANT, R. WALD AND J. POLONOVSKI, *Bull. Soc. Chem. Biol.*, 52 (1970) 1061.
25 G. F. WILGRAM AND E. P. KENNEDY, *J. Biol. Chem.*, 238 (1963) 2615.
26 J. Y. KIYASU, R. A. PIERINGER, H. PAULUS AND E. P. KENNEDY, *J. Biol. Chem.*, 238 (1963) 2293.
27 H. D. TISDALE, in R. W. ESTABROOK AND M. E. PALLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 213.
28 H. WEISSBACH, T. E. SMITH, J. W. DALY, B. WITKOP AND S. UDENFRIEND, *J. Biol. Chem.*, 235 (1960) 1160.
29 G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER AND A. BERGSTRAND, *J. Cell Biol.*, 32 (1967) 415.
30 M. G. BURDON, R. M. S. SMELLIE AND J. N. DAVIDSON, *Biochim. Biophys. Acta*, 91 (1964) 46.
31 R. WATTIAUX AND C. DE DUVE, *Biochem. J.*, 63 (1956) 606.
32 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 120 (1966) 369.
33 S. M. DUTTERA, W. L. BYRNE AND M. C. GANOZA, *J. Biol. Chem.*, 243 (1968) 2216.
34 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
35 P. S. CHEN, T. Y. TORIBORA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
36 E. KELLENBERGER, A. RYTER AND J. SÉCHAUD, *J. Biophys. Biochem. Cytol.*, 4 (1958) 671.

37 P. F. ELBERS AND J. PIETERS, *J. Ultrastruct. Res.*, 11 (1964) 25.
38 C. BUBLITZ AND E. P. KENNEDY, *J. Biol. Chem.*, 211 (1954) 951.
39 R. N. MCELHANEY AND M. E. TOURTELOTTE, *J. Bact.*, 101 (1970) 72.
40 H. GOLDFINE, *J. Lipid Res.*, 7 (1966) 146.
41 E. G. BLIGH AND W. J. DYER, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
42 N. SHAW, P. F SMITH AND W. L. KOOSTRA, *Biochem. J.*, 107 (1968) 329.
43 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.
44 C. LONG, R. ODAVIC AND E. J. SARGENT, *Biochem. J.*, 102 (1967) 216.
45 H. VAN DEN BOSCH, A. J. AARSMAN, A. J. SLOTBOOM AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 164 (1968) 215.
46 W. SCHNEIDER AND E. L. HUFF, in G. H. BOURNE, *Cytology and Cell Physiology*, Academic Press, New York, 1964, p. 19.
47 D. F. PARSONS, G. R. WILLIAMS, W. THOMPSON, D. WILSON AND B. CHANCE, in E. QUAGLIARELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Mitochondrial Structure and Compartmentalization*, Adriatrica Editrice, Bari, 1967, p. 29.
48 G. L. SOTTOCASA, L. ERNSTER, B. KUYLENSTIERNA AND A. BERGSTRAND, in E. QUAGLIARELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Mitochondrial Structure and Compartmentalization*, Adriatrica Editrice, Bari, 1967, p. 74.
49 J. W. GREENAWALT AND C. SCHNAITMAN, *J. Cell Biol.*, 46 (1970) 173.
50 L. L. M. VAN DEENEN AND G. H. DE HAAS, *Annu. Rev. Biochem.*, 35 (1966) 157.
51 A. A. EL-AASER, J. T. R. FITZIMONS, R. H. HINTON, E. REID, E. KLUCIS AND P. ALEXANDER, *Biochim. Biophys. Acta*, 127 (1966) 553.
52 R. A. WEAVER AND W. BOYLE, *Biochim. Biophys. Acta*, 173 (1969) 377.
53 W. H. EVANS, *Biochem. J.*, 166 (1970) 833.
54 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND PH. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
55 D. F. PARSONS, G. R. WILLIAMS AND B. CHANCE, *Ann. N.Y. Acad. Sci.*, 137 (1966) 643.
56 N. Z. STANACEV, Y. Y. CHANG AND E. P. KENNEDY, *J. Biol. Chem.*, 242 (1967) 3018.
57 K. Y. HOSTETLER, H. VAN DEN BOSCH AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 239 (1971) 113.
58 H. D. KAULEN, R. HENNING AND W. STOFFEL, *Z. Physiol. Chem.*, 351 (1970) 1555.
59 M. WAITE, *Biochemistry*, 8 (1969) 2536.
60 P. M. VIGNAIS AND J. NACHBAUER, *Bull. Soc. Chem. Biol.*, 50 (1968) 1473.
61 J. D. NEWKIRK AND M. WAITE, *Biochim. Biophys. Acta*, 225 (1971) 224.
62 K. W. A. WIRTZ AND D. B. ZILVERSMIT, *J. Biol. Chem.*, 243 (1968) 3596.