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LECITHINASE ACTIVITIES AT THE EXTERNAL SURFACE OF RUMINANT ERYTHROCYTE MEMBRANES

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SUMMARY

1. Sheep, ox and goat erythrocyte ghosts contain a phospholipase A (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) which preferentially attacks lecithin. In addition, lysophospholipase activity was found in these membranes.

2. With the exception of human and dog red cell ghosts in which no phospholipase A activity could be established, relatively weak activities towards phosphatidylethanolamine were found in various mammalian erythrocyte ghosts (including those from ruminants).

3. Pronase treatment of intact ox and sheep erythrocytes largely inactivated lecithinase activity, suggesting that the enzyme is present at the outside of the membrane. Incubation of serum with sheep red cells in which lecithinase was inactivated by pronase or inhibited by EDTA resulted in a slow net incorporation of lecithin from serum into cells.

4. It is concluded that lecithinase activities may be generally present at the outside of ruminant erythrocytes. It is speculated that this enzyme might play a role (together with other proteins) in maintaining the low lecithin content of the membrane.

INTRODUCTION

In contrast to previous findings [1–5], studies by the group of Polanowski [6, 7] have shown that phospholipase A activities are present in haemolysates of human and rat erythrocytes. In the accompanying paper [8] a phospholipase A_2 in sheep red cell membranes has been described which preferentially attacks lecithin. At present it is well known that these membranes contain very little lecithin [9, 10], in spite of the high lecithin content of serum which is in slow passive equilibrium with the lecithin pool of the membrane [11–14]. Moreover, plasma lyso-lecithin is similarly involved in an exchange equilibrium with red cell lyso-lecithin which can

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be acylated within the membrane to produce the di-acyl phosphoglyceride [1,2,13–17]. These two processes are involved in lipid turnover and renewal pathways, but they do not explain why the red cell membrane phospholipid composition differs among a variety of mammalian species, whereas the plasma phospholipid composition is similar.

The present study deals with phospholipase A_2 activities in a variety of red cell membranes, and with a possible function of this enzyme in ruminant erythrocyte membranes.

MATERIALS AND METHODS

Pronase (Protease Type VI) and trypsin from bovine pancreas (Type III) were both obtained from Sigma. L-α-lecithin from egg yolk was purchased from Koch-Light (grade I). [^{14}C]Phosphatidylcholine (1.8 Ci/mMole, uniformly labelled, from *Chlorella pyrenoidosa*) was obtained from New England Nuclear Company.

Protein was measured according to Lowry et al. [18], using bovine serum albumin as a standard. Erythrocyte ghosts from various mammalian species were isolated by a modification [19] of the method of Dodge et al. [20]. The final sediments were suspended in 15 mosM phosphate buffer (pH 8) at a concentration of approximately 10 mg protein/ml, and stored frozen until use.

Phospholipase assay in erythrocyte ghosts

For routine assays of phospholipase activity, 1 ml of ghost suspension (approx. 10 mg of protein) was mixed with 1 ml of 0.05 M glycyl–glycine buffer (pH 8) containing 4 mg of Triton X-100. To this mixture, 80 μl 0.2 M $CaCl_2$ was added, and incubations were carried out at 37 °C with stirring for periods indicated. Reactions were stopped by addition of 250 μl 0.1 M EDTA. Control experiments for non-enzymatic hydrolysis were performed using 80 μl 10 mM EDTA instead of $CaCl_2$. In some experiments, additional lipids (either egg lecithin or a total lipid extract from human red cell ghosts) were sonicated in desired concentrations in the glycyl–glycine buffer, prior to mixing with the ghost suspensions.

Phospholipid analysis

After incubation the lipids were extracted by the method of Renkonen et al. [21], or Reed et al. [22]. The extracts were taken to dryness under reduced pressure and the residue was dissolved in 0.25 ml of chloroform–methanol (1:1, v/v). The phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse [23], and determined as phosphorus after destruction with 70 % $HClO_4$ at 190 °C by a modification [24] of the procedure of Fiske and SubbaRow. Percentage degradation of glycerophospholipids by membrane-bound phospholipase attack was determined using sphingomyelin (which was not degraded) as an internal standard.

Detection of the plasmalogen content of phosphatidylethanolamine was carried out by allowing the lipids, developed in the first direction, to react with 12 % by weight of HCl in methanol, as described by Singh et al. [25].

EDTA extractions of ox and sheep red cell ghosts

EDTA extractions were carried out in a similar way as described by others [26–28]. Ghosts, containing approximately 100 mg of protein, were extracted with 100 ml 1 mM EDTA (pH 8 with Tris) for 15 min at 37 °C. After centrifugation at 50000 × *g* for 30 min, the extracted proteins were precipitated at 77 % $(NH_4)_2SO_4$ saturation. Both the sediment after EDTA extraction and the $(NH_4)_2SO_4$-precipitated proteins from the supernatant were dialysed against 10 mosM phosphate buffer (pH 8) and finally suspended at concentrations of 4–7 mg protein/ml. Both samples were assayed for phospholipase activity as described above.

Proteolytic treatment of ghosts and intact cells

Pronase treatment of ghosts was carried out at pH 7.5 for 3 h at 37 °C, using 1 mg of pronase per 10 mg of membrane protein. For pronase treatment of intact erythrocytes, freshly obtained packed cells (washed three times with isotonic saline) were diluted with an equal volume of isotonic saline (0.2 mM $MgCl_2$, 0.25 mM $CaCl_2$, pH 7.5 with Tris) and incubated for 2 h at 37 °C with 7 mg of pronase per 5 ml of packed cells. After the incubation procedure (which produced no significant haemolysis) the cells were washed three times with isotonic phosphate buffer (pH 7.4) and ghosts were isolated as described above. In each case, identical incubations were carried out without pronase to serve as controls. In some experiments, the cells were washed three times with isotonic saline to remove pronase, and incubated with serum as described below.

Trypsin treatment of human erythrocyte ghosts, prior to the phospholipase assay, was carried out as described by Paysant et al. [7].

Incubations of red cells with serum

Prior to the incubation procedure, freshly obtained serum was heated for 1 h at 56 °C with stirring to destroy lecithin–cholesterol acyl transferase activity [29]. The serum was subsequently centrifuged for 5 min at 1000 × *g* to remove the precipitate formed during the heat-treatment and which contained less than 3 % of the total serum proteins. The supernatant was mixed by sonication for 2–3 min with [^{14}C] lecithin (1.1 nmole per 10 ml of serum) and incubated for various periods with equal volumes of pronase-treated sheep red cells (prepared as described above) at 37 °C with stirring. Incubations with control cells from the pronase experiment were also performed, either with or without 1 mM EDTA in the serum. After the incubation procedure the mixtures were centrifuged for 15 min at 5000 × *g* and serum supernatants were collected. The cells were subsequently washed four times with isotonic saline. Lipid extractions of serum supernatants and washed cells were carried out according to Reed et al. [22]. From each extract duplicate samples were taken for separation of the phospholipids by two-dimensional thin-layer chromatography. One thin-layer plate served for phosphate determination as described above; the other plate was used for measuring the radioactivity in each spot as described in the previous paper [8].

RESULTS

When erythrocyte ghosts from various mammalian species were assayed for phospholipase activity at pH 8 in the presence of Triton X-100 and $CaCl_2$, breakdown of lecithin was found exclusively in sheep, ox and goat red cell ghosts (Table I). It is emphasized that this lecithinase activity of ruminant red cell ghosts was detected on exogenous phosphatidylcholine as substrate, since these ghosts contain very little lecithin [9, 10]. No significant differences in degradation were observed when lecithin was supplied as egg lecithin, as total lipid extract of human erythrocyte ghosts, or as intact human erythrocyte ghosts mixed with an equal amount of the ruminant red cell ghosts. In all cases, incubations in the presence of EDTA did not produce any significant phospholipid breakdown as compared to non-incubated ghosts. With the exception of human and dog, all the mammalian red cell ghosts tested produced

TABLE I

PHOSPHOLIPASE A ACTIVITIES IN VARIOUS MAMMALIAN ERYTHROCYTE GHOSTS

Values indicate percentage degradation of endogenous phospholipids per 6 h incubation at 37 °C. Ruminant red cell ghosts were mixed, prior to incubation, with equal amounts of human red cell ghosts or an equivalent amount of sonicated egg phosphatidylcholine. Degradation of phosphatidylserine is probably absent in all ghosts, but this could not always be established unambiguously (see text).

	Sheep	Ox	Goat	Rabbit	Human	Pig	Dog	Rat
Phosphatidylcholine	90–95	60–80	70–75	—	—	—	—	—
Phosphatidylethanolamine	30–45	5–15	5–20	10–20	—	5–20	—	35–50

breakdown of phosphatidylethanolamine, although to a lower extent than the lecithin breakdown with ruminant erythrocyte ghosts. Degradation of lecithin or phosphatidylethanolamine was always paralleled by the formation of the corresponding lyso-derivatives. In some case phosphatidylserine was reduced relative to sphingomyelin, but no lyso-phosphatidylserine was produced. Moreover, this occurrence appeared to be completely arbitrary and uncontrollable, and is probably due to a lower extractability of phosphatidylserine from ghosts incubated in the presence of Ca, in spite of adding EDTA prior to lipid extraction. It has been suggested by Paysant et al. [7] that lysates of human erythroycytes exhibit a weak phospholipase A activity which is enhanced by trypsin treatment. Under our experimental conditions, however, pretreatment of human ghosts with trypsin, as described by Paysant et al. [7], did not produce any detectable phospholipid degradation.

As stated above, degradation of lecithin and phosphatidylethanolamine was accompanied by the production of their lyso-derivatives. This is shown for sheep erythrocyte membranes in Fig. 1. Prolonged incubation periods produced a decrease in the lyso-compounds, probably due to lysophospholipase activity which is known to be present in other red cell membranes [3, 5, 30]. At present it is unclear why this lysophospholipase activity seems to be enhanced in a later stage of the incubation. Although lyso-lecithin was nearly completely degraded, a residual amount of lysophos-

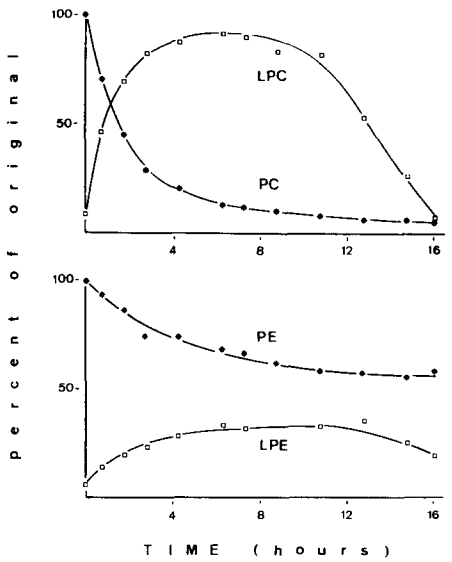

Fig. 1. Degradation of glycerophospholipids by sheep red cell membrane phospholipase A. Abbreviations: PC, lecithin; LPC, lyso-lecithin; PE, phosphatidylethanolamine; LPE, lyso-phosphatidylethanolamine. Experimental details are given in the text.

phatidylethanolamine was always observed. The plasmalogen content of phosphatidylethanolamine appeared to be approximately 25 % by weight. Since this content was not changed in the phosphatidylethanolamine fraction after degradation, it is likely that the residual lyso-phosphatidylethanolamine is formed from the original alk-1-enyl compound and hence is not susceptible to lysophospholipase activity.

In further experiments, attention was focused on the degradation of lecithin by phospholipase A of ruminant erythrocyte membranes. In order to establish whether this activity is caused by a membrane-bound enzyme, both ox and sheep erythrocyte ghosts were extracted with 1 mM EDTA. In both cases, 15–20 % of the membrane proteins were extracted but no significant lecithinase activity could be detected in this fraction. On the other hand, the sediment after EDTA extraction showed a marked lecithinase activity, although approximately 20 % inactivation (as compared to the original ghosts) was observed.

Treatment of ox or sheep erythrocyte ghosts with pronase almost completely prevented degradation of lecithin (Fig. 2). Similar results were obtained when intact cells were treated with pronase, suggesting that the majority of the lecithinase activity is located at the outside of the cell.

In order to detect whether this enzyme might play a role in lecithin turnover and renewal in intact ruminant erythrocytes, both pronase-treated and control cells (derived from sheep) were incubated with equal volumes of heat-treated serum to which [^{14}C]lecithin was added. As shown in Fig. 3A, pronase-treated cells incorporated approximately three times as much radioactive lecithin as control cells. In the case of control cells the incorporation was probably due to exchange between cold lecithin in cells and radioactive lecithin in serum, since the lecithin content of the membranes was not altered as compared to freshly isolated ghosts (0.7–0.9 % of the total phos-

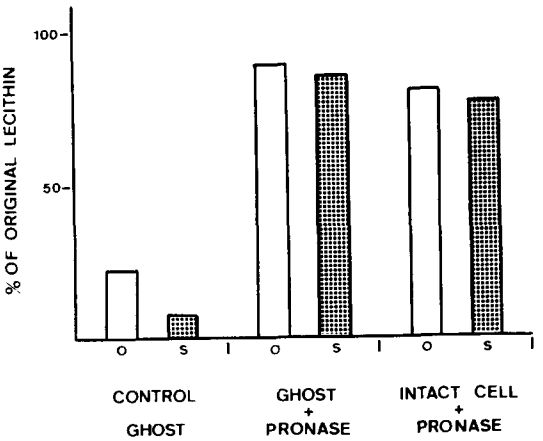

Fig. 2. Degradation of lecithin by ox (o) and sheep (S) erythrocyte ghosts (16 h at 37 °C) after treatment of ghosts or intact cells with pronase.

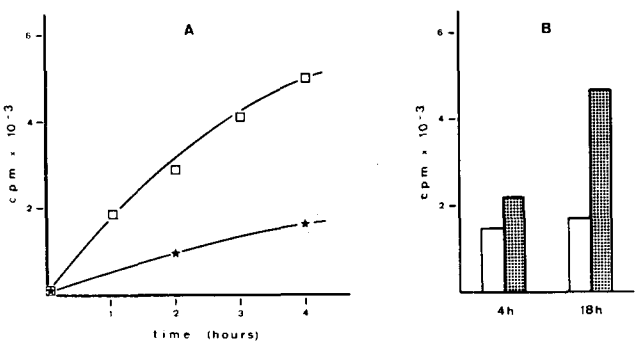

Fig. 3. Incorporation of radioactive lecithin from serum into sheep red cells. Ordinate indicates counts/min in lecithin per mg of total membrane phospholipids. A. Cells treated with pronase prior to incubation procedure (□ —□); control cells (× — ×). B. Cells incubated with serum in the presence of EDTA (shaded bars) versus control cells (open bars).

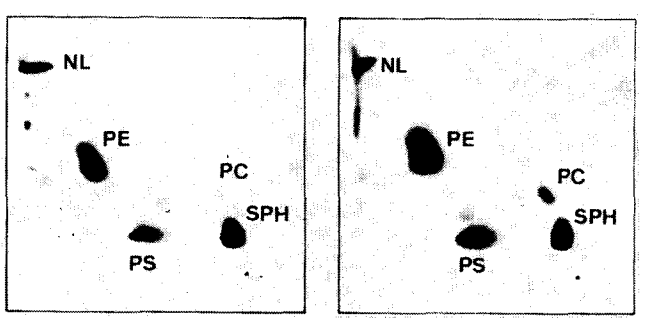

Fig. 4. Two-dimensional thin-layer plates of sheep erythrocyte membrane lipids using the procedure of Broekhuyse [23] before (left) and after (right) incubation of pronase-treated intact cells with serum for 4 h at 37 °C. Abbreviations: PC, lecithin; SPH, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; NL, neutral lipids.

pholipids). On the other hand, the lecithin content of pronase-treated cells incubated with serum was definitely increased (2.5–3.0 % of the total phospholipids, after 4 h incubation), suggesting a net incorporation of lecithin into the cells. Thin-layer plates of phospholipids derived from both experiments clearly show an increase of lecithin after treatment of proteolysed cells with serum (Fig. 4). Unfortunately, prolonged incubations with pronase-treated cells could not be carried out since this usually resulted in lysis after 5–6 h. Until now, conclusive data from lipid analysis of the corresponding serum supernatants could not be obtained, mainly due to the presence of a phospholipase A activity in the sheep serum itself which seriously complicated the final results.

Treatment of red cells with an equal volume of serum containing 10 mM EDTA also resulted in a net incorporation of lecithin, although the incorporation rate was much lower as in the case of pronase-treated cells (Fig. 3B). Incubation of red cells with EDTA-containing serum for 18 h produced similar results as incubation of pronase-treated cells with serum for 4 h. In both cases, no significant alterations in the levels of the other membrane phospholipids could be detected.

DISCUSSION

Investigations of Kramer et al. [8] have shown that sheep erythrocyte membranes contain a phospholipase A which preferentially attacks lecithin. The present results clearly demonstrate that this activity is not only found in sheep red cells but is also present in ox and goat erythrocyte ghosts, and could therefore be a more common phenomenon of ruminant red cell membranes. A variety of mammalian erythrocyte ghosts (including the ruminants) exhibited relatively weak phospholipase A activities towards phosphatidylethanolamine. At present it is not clear whether in ruminant red cells this breakdown should be attributed to the same enzyme which attacks the lecithin, or that a separate enzyme specific for phosphatidylethanolamine is involved. Although identical pH optima (pH 8) of both activities were observed, this does not necessarily imply that the first possibility is correct.

Under the experimental conditions employed (Triton, Ca^{2+}, pH 8), no detectable phospholipase A activity could be found in human erythrocyte ghosts, this being in agreement with observations of various other investigators [1–5]. However, Paysant et al. [7] described a phospholipase A in human red cells, which could be activated with trypsin. It should be mentioned that these experiments have been carried out with haemolysates and not with ghosts which have been washed several times to remove most of the haemoglobin. These washing procedures are known to result in a concomitant loss of enzymes from the membrane [31] and may remove originally present phospholipase activities as well. Mulder and van Deenen [4] were unable to detect any appreciable phospholipase A activity in red cell ghosts of different animals, although it is not directly clear which animal species have been tested. Although no experimental details were given, it is likely that detergents and optimal Ca concentrations were not used in their assay systems, the absence of which results in hardly measurable activities [8].

It has been shown that phospholipase A activity is present in human [32–34] and rat [35] serum as well as in some commercially available albumin preparations [36]. In this respect it is of interest to mention that lecithinase activity of ox and sheep

red cell ghosts could not be extracted with 1 mM EDTA, suggesting that the enzyme is firmly attached to the membrane and is certainly not a contamination from the serum.

Pronase treatment of ox and sheep ghosts largely inactivated lecithinase, although relatively large amounts (1 mg per 10 mg ghost protein) and prolonged incubation times (3 h) were necessary. Proteolysis of intact cells produced a similar inactivation of the lecithinase, indicating that the enzyme is localized at the exterior of the membrane.

Although ruminant erythrocytes differ strongly from those of other mammals in that they contain very little lecithin, no such differences in lipid composition are known to exist between various mammalian plasmas [9]. We observed that the phospholipid composition of ox, sheep and human plasma is virtually identical, containing approximately 68 % of lecithin, 21 % of sphingomyelin, 7 % of lysolecithin and 4 % of phosphatidylethanolamine. Lecithin and to a lesser extent also sphingomyelin are exchangeable between serum and red cells (see for recent review: ref. 14), but in spite of this ruminant red cells maintain a very low lecithin level. However, incubations of serum with sheep cells which have been pretreated with pronase resulted in a net incorporation of lecithin from serum into the cells. Moreover, incubation of control cells with serum in the presence of EDTA caused a similar lecithin incorporation into the cells, though at a much lower rate. The actual amounts of lecithin involved in this transport process, however, are quite small but of the same order of magnitude as the lecithin turnover in human red cells [12]. Preliminary experiments suggest that incubation of sheep erythrocytes with serum in the absence of EDTA produces degradation of lecithin in serum, though not altering the low lecithin content of the cells. These results, however, are complicated by the observation that incubation of sheep serum alone also results in lecithin breakdown, although to a less extent than serum incubated with sheep erythrocytes. This might suggest that sheep serum also contains a phospholipase A similar to serum from other mammals [32–36].

Although alternative explanations are possible, it is speculated that the inhibitory effect of pronase and EDTA on lecithinase activity may contribute to the net incorporation of lecithin into the cells. It is obvious that pronase treatment of intact cells not only inactivates lecithinase but also hydrolyses other membrane proteins at the cell exterior. These proteins may also play a role in regulating the membrane lipid composition, or have a direct effect on lipid exchange; for example, Kramer et al. [37] have shown that sheep red cell membranes contain a protein which preferentially binds sphingomyelin, Since the majority of the sphingomyelin is located at the outside of the membrane [38, 39], such a protein may also be located at the cell exterior and therefore by hydrolysed during proteolysis of intact cells. It may be possible that both lecithinase and sphingomyelin-binding protein play a role (among other proteins) in maintaining low levels of lecithin in sheep cells and, by extension, in other ruminant erythrocytes. In this respect it is of interest to mention that Robertson and Lands [1] in 1964 considered the possibility that sheep red cell membranes contain a phospholipase A to maintain a low lecithin level in spite of an active acylation of lysolecithin, but they have not tested these membranes for phospholipase activity. The present observation that not only sheep red cells but also ox and goat erythrocytes contain a phospholipase A which preferentially attacks lecithin suggest that lecithinase activity is a more general phenomenon of ruminant red cell membranes. At present, however, its possible relation to the low lecithin content of

these membranes (in spite of high lecithin levels in plasma) cannot be more than just a hypothesis.

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REFERENCES

1 Robertson, A. F. and Lands, W. E. M. (1964) J. Lipid Res. 5, 88–93
2 Oliveira, M. M. and Vaughan, M. (1964) J. Lipid Res. 5, 156–162
3 Mulder, E., van den Berg, J. W. O. and van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 118–127
4 Mulder, E. and van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 348–356
5 Munder, P. G., Ferber, E. and Fischer, H. (1965) Z. Naturforschg. 20b, 1048–1061
6 Paysant, M., Delbauffe, D., Wald, R. and Polonovski, J. (1967) Bull. Soc. Chim. Biol. 49, 169–176
7 Paysant, M., Bitran, M., Wald, R. and Polonovski, J. (1970) Bull. Soc. Chim. Biol. 52, 1257–1269
8 Kramer, R., Jungi, B., and Zahler, P. (1974) Biochim. Biophys. Acta 373, 404–415
9 Van Deenen, L. L. M. and de Gier, J. (1964) The Red Blood Cell (Bishop, C. and Surgenor, D. M., eds), Chapter VII, pp. 243–307, Academic Press, New York
10 Nelson, G. J. (1967) Biochim. Biophys. Acta 144, 221–232
11 Sakagami, T., Minari, O. and Orii, T. (1965) Biochim. Biophys. Acta 98, 111–116
12 Reed, C. F. (1968) J. Clin. Invest. 47, 749–760
13 Shohet, S. B. (1970) J. Clin. Invest. 49, 1668–1678
14 Shohet, S. B. (1972) New Engl. J. Med. 286, 577–583
15 Mulder, E. and van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 106–117
16 Donabedian, R. K. and Karmen, A. (1967) J. Clin. Invest. 46, 1017–1027
17 Shohet, S. B., Nathan, D. G. and Karnovsky, M. L. (1968) J. Clin. Invest. 47, 1096–1108
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Zahler, P. and Weibel, E. R. (1970) Biochim. Biophys. Acta 219, 320–338
20 Dodge, C. T., Mitchell, C. D. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
21 Renkonen, O., Kosunen, T. U. and Renkonen, O. V. (1963) Ann. Med. Exp. Biol. Fenniae (Helsinki) 41, 375–381
22 Reed, C. F., Swisher, S. N., Marinetti, G. V. and Ede, E. G. (1960) J. Lab. Clin. Med. 56, 281–289
23 Broekhuyse, R. M. (1969) Clin. Chim. Acta 23, 457–461
24 Böttcher, C. J. F., van Gent, C. M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204
25 Singh, H., Spritz, N. and Geyer, B. (1971) J. Lipid Res. 12, 473–481
26 Hoogeveen, J. T., Juliano, R. L., Coleman, J. and Rothstein, A. (1970) J. Memb. Biol. 3, 156–172
27 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack. T. W. (1970) Biochemistry 9, 50–57
28 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2616
29 Murphy, J. R. (1965) J. Lab. Clin. Med. 65, 756–774
30 Ferber, E., Munder, P. G., Kohlschütter, A. and Fischer, H. (1968) Eur. J. Biochem. 5, 395–401
31 Burger, S. P., Fujii, T. and Hanahan, D. J. (1968) Biochemistry 7, 3682–3700
32 Vogel, W. C. and Zieve, L. (1964) J. Lipid. Res. 5, 177–183
33 Vogel, W. C. and Bierman, E. L. (1967) J. Lipid Res. 8, 46–53
34 Etienne, J., Paysant, M., Grüber, A. and Polonovski, J. (1969) Bull. Soc. Chim. Biol. 51, 709–716
35 Paysant, M., Bitran, M., Etienne, J. and Polonovski, J. (1969) Bull. Soc. Chim. Biol. 51, 863–873
36 Elsbach, P. and Pettis, P. (1973) Biochim. Biophys. Acta 296, 89–93
37 Kramer, R., Schlatter, Ch. and Zahler, P. (1972) Biochim. Biophys. Acta 282, 146–156
38 Bretscher, M. (1972) Nat. New Biol. 236, 11–12
39 Zwaal, R. F. A., Roelofsen, B. and Colley, C. M. (1973) Biochim. Biophys. Acta 300, 159–182