Electrophoretic characterization of hepatic alkaline phosphatase released by phosphatidylinositol-specific phospholipase C

A comparison with liver membrane and serum-soluble forms

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Alkaline phosphatase was solubilized from plasma membrane of rat liver with butan-1-ol, bile acids or sodium deoxycholate, and electrophoretically compared with a soluble form in serum which was derived from the liver. The three enzyme preparations from the plasma membrane migrated at the same position on polyacrylamide-gel electrophoresis in the presence of either Triton X-100 or sodium dodecyl sulphate. The mobility of them, however, was distinctly different from that of the serum-soluble form of the liver-derived alkaline phosphatase. On the other hand, phosphatidylinositol-specific phospholipase C isolated from *Bacillus cereus* was used to release alkaline phosphatase from plasma membrane. The released alkaline phosphatase was demonstrated to have the same mobility as the serum-soluble form on polyacrylamide-gel electrophoresis in the presence or absence of detergents. The phospholipase C also converted the butan-1-ol-extracted membrane form into the serum-soluble form. The results suggest that release of alkaline phosphatase from the liver into serum is not simply caused by a detergent effect of bile salts, but involves an enzymic hydrolysis of phosphatidylinositol, with which alkaline phosphatase may strongly interact in the membrane.

The hepatic alkaline phosphatase is a plasma-membrane marker enzyme, especially for the bile canaliculi (Wisher & Evans, 1975), and easily induced in response to a variety of stimuli (Kaplan & Righetti, 1970; Pekarthy *et al.*, 1972; Ikehara *et al.*, 1978*b*). The induced synthesis of alkaline phosphatase in the liver usually results in its remarkable rise in serum. A typical example for this relationship was demonstrated in experimental animals with biliary obstruction (Börnig *et al.*, 1967; Kaplan & Righetti, 1970). A role for bile acids has been strongly suggested in the mechanism by which the membrane-bound enzyme is released into bile, followed by its regurgitation into the bloodstream. Bile acids are shown not only to

induce the synthesis of hepatic alkaline phosphatase (Hatoff & Hardison, 1979), but also to act as a detergent to release the enzyme from membranes *in vitro* (Vyvoda *et al.*, 1977; Billington *et al.*, 1980) and *in vivo* (Barnwell *et al.*, 1983). It is perhaps relevant to note that a remarkable increase of alkaline phosphatase in the regenerating liver, where the bile acid concentration was not increased, did not result in so significant an elevation of the enzyme activity in serum (Schaleger *et al.*, 1982). Thus all the results would be considered to support the hypothesis of a bile-acid-mediated solubilization of the membrane-bound enzyme in cholestatic liver.

On the other hand, it has been shown that alkaline phosphatase and several other enzymes are also released from membranes by PI-PLase C (Slein & Logan, 1965; Ikezawa *et al.*, 1976; Low & Fineán, 1977). The observations suggest that phosphatidylinositol is involved in the attachment of alkaline phosphatase to membranes, although conflicting conclusions have been reported for the

Abbreviations used: Ammediol, 2-amino-2-methylpropane-1,3-diol; PI-PLase C, phosphatidylinositol-specific phospholipase C; SDS, sodium dodecyl sulphate.

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molecular basis for the presumed interaction: non-covalent linkage (Shukla *et al.*, 1980) or covalent linkage (Low & Zilversmit, 1980) between the enzyme and phosphatidylinositol.

In the present study, in order to discover the possible mechanism involved in conversion of the membrane-bound enzyme into a soluble form in serum, we compared, by an electrophoretic technique, two forms of alkaline phosphatase released from the liver plasma membrane by PI-PLase C and by conventional agents, including bile acids and butan-1-ol. Extraction of membrane lipids with butan-1-ol has been widely used as a method for solubilizing, and subsequently purifying, membrane-bound alkaline phosphatase from various tissues (Morton, 1957).

Materials and methods

Materials

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman Biochemicals (Maidstone, Kent, U.K.), and Sephadex G-200 and G-75 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, trypsin and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acrylamide-gel agents, SDS and *p*-nitrophenyl phosphate were from Nakarai Chemicals (Kyoto, Japan) and 5-bromoindol-3-yl phosphate from Wako Pure Chemicals (Tokyo, Japan). α_1 -Proteinase inhibitor (Ikehara *et al.*, 1981) and haptoglobin (Misumi *et al.*, 1983) were purified from rat serum as described by the authors just cited. Bile juice was collected by cannulating the common bile duct of rat and used as a source of bile acids.

Isolation of liver plasma membrane and serum

Unless otherwise indicated, we used male Wistar rats, weighing 250–350g, which had been treated by bile-duct ligation and colchicine injection (2mg/kg body wt.) 20h before being killed as described previously (Ikehara *et al.*, 1978b; Oda & Ikehara, 1981). These treatments caused a remarkable induction of alkaline phosphatase in liver, resulting in a great increase of the enzyme in serum (Oda & Ikehara, 1981; Kominami *et al.*, 1984). Plasma membranes were isolated by the method of Ray (1970) from livers of rats treated as described above. Rat serum was prepared from blood collected by heart puncture. A serum-soluble form of the liver-derived alkaline phosphatase was isolated by gel filtration of the serum on a column (2.5cm $\times$ 90cm) of Sephadex G-200 as described previously (Kominami *et al.*, 1984).

Treatments of plasma membranes for the release of alkaline phosphatase

Alkaline phosphatase was released from plasma membranes by treatment with butan-1-ol, sodium deoxycholate or bile acids as described previously (Ikehara *et al.*, 1978a). When PI-PLase C was used for release of alkaline phosphatase, the membrane suspension (1–2mg/ml) was incubated in 20mM-Tris/HCl, pH 7.5, with the purified PI-PLase C (finally 0.005unit/ml) at 37°C for 90min. All the treated samples were centrifuged at 105000g for 60min to separate the released alkaline phosphatase from the membranes.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed by using two different systems. For determination of the M_r of purified PI-PLase C, the same system as that of Laemmli (1970) was employed. Marker proteins used to calibrate the gels were: rat serum α_1 -proteinase inhibitor (M_r 54000), rat serum haptoglobin (α , 10000; β , 36000), porcine trypsin (23000) and soya-bean trypsin inhibitor (21000). For separation of alkaline phosphatase, samples in 0.1M-phosphate buffer, pH 7.5, were incubated at 37°C for 60min in the presence of 1% (w/v) SDS but in the absence of a reducing agent, and subjected to electrophoresis on SDS/5.6%-(w/v)-polyacrylamide gels as described by Weber & Osborn (1969). Standard gel electrophoresis in the presence or absence of Triton X-100 (0.1%) was carried out at pH 8.6 with the Tris/glycine buffer system of Davis (1964), except that samples containing 10% (w/v) sucrose were directly loaded on separating gels (7.5%). Localization of alkaline phosphatase after electrophoresis was performed by incubating gels in 50mM-Ammediol buffer, pH 10.5, containing 1mM-5-bromoindol-3-yl phosphate and 5mM-MgCl₂ as described previously (Ikehara *et al.*, 1977).

Enzyme assays

Alkaline phosphatase activity was determined by using *p*-nitrophenyl phosphate as substrate as described previously (Ikehara *et al.*, 1977). PI-PLase C activity was determined by the method of Low (1981), with some modifications. A reaction mixture in 0.5ml containing 2mM-phosphatidylinositol, 0.16% sodium deoxycholate, 20mM-Hepes/NaOH, pH 7.0, and an appropriate amount of the enzyme was incubated at 37°C for 20min. The reaction was terminated by adding 0.5ml of 10% (w/v) trichloroacetic acid, followed by centrifugation at 2000g for 10min. To 0.5ml of the supernatant, 3.0ml of chloroform/methanol/HCl

(500:500:3, by vol.) and 0.8 ml of 1M-HCl were added. The sample was vigorously mixed and centrifuged at 2000g for 10 min. A 0.5 ml portion of upper aqueous phase was used for determination of organic phosphorus by the method of Bartlett (1959). One unit of each enzyme activity was defined as the amount of enzyme causing the hydrolysis of 1 μ mol of substrate/min at 37°C.

Purification of phosphatidylinositol-specific phospholipase C

PI-PLase C was purified from *Bacillus cereus* T by the procedure described by Ikezawa & Taguchi (1981) with some modifications. The culture medium (12 litres) was inoculated with 1% (v/v) of the preculture of *Bacillus cereus* T and incubated at 37°C for 6 h. The cells were removed by centrifugation at 12000g for 20 min. The enzyme contained in the supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation. The resulting precipitates were collected by centrifugation at 12000g for 20 min, dissolved in distilled water, and dialysed against 0.01M-Tris/HCl buffer, pH 8.5. The non-diffusible material was applied on to a DEAE-cellulose column (3.8 cm $\times$ 30 cm) previously equilibrated with 0.01M-Tris/HCl buffer, pH 8.5. After the column was washed with the above buffer, the protein was eluted with a linear gradient from 0 to 0.3M-NaCl in the same buffer. Fractions containing PI-PLase C activity were pooled and dialysed against 0.01M-acetate buffer, pH 5.5. The non-diffusible material was then applied on to a column (3.0 cm $\times$ 30 cm) of CM-cellulose. The column was washed with the above buffer and eluted with a linear gradient from 0 to 0.4M-NaCl in the same buffer. The active fractions were pooled and concentrated in an Amicon ultrafiltration cell with a YM-2 membrane. The concentrate was dialysed against 0.01M-Tris/HCl buffer, pH 7.5, containing 0.05M-NaCl and applied to a column (2.5 cm $\times$ 90 cm) of Sephadex G-75 equilibrated with the same buffer. The active fractions were pooled and stored at -20°C until use. The results of all purification steps are summarized in Table 1.

Results and discussion

Comparison of liver membrane and serum-soluble forms of alkaline phosphatase

Previously we reported that simultaneous bile-duct ligation and colchicine injection in rats caused a remarkable induction of alkaline phosphatase in liver (Ikehara *et al.*, 1978b; Oda & Ikehara, 1981), resulting in a great elevation of the enzyme in serum (Kominami *et al.*, 1984). The serum alkaline phosphatase derived from the liver was fractionated into two activity peaks by gel filtration on a Sephadex G-200 column, a sedimentable form in the void volume and a soluble form corresponding to an M_r of about 170000 (Kominami *et al.*, 1984). The serum-soluble form in the major peak was not identical, on electrophoresis, with the enzyme extracted from the liver plasma membrane with butan-1-ol.

When the liver plasma membrane was treated with bile acids (bile juice), sodium deoxycholate or butan-1-ol, all the preparations of released alkaline phosphatase showed an identical mobility on SDS/polyacrylamide-gel electrophoresis (Fig. 1a, gels 1-3) or Triton X-100 (Fig. 1b, gels 1-3). The serum-soluble form, however, migrated at a position distinctly different from that of the liver membrane form (Figs. 1a and 1b, gel 4). In addition, when the three preparations of alkaline phosphatase extracted from the plasma membrane were extensively dialysed in the absence of detergents, the enzyme did not migrate into, but stuck on top of, gels when subjected to standard electrophoresis (the same system as shown in Fig. 1b, except for no addition of Triton X-100), indicating that the enzyme assumed an aggregated form during dialysis (results not shown). The serum-soluble form, however, showed no change in electrophoretic behaviour before and after such treatment. It has been confirmed that the serum-soluble form used here was derived from the liver (Kominami *et al.*, 1984). Thus these results suggest that there is some difference in molecular structure between the serum-soluble form and the membrane-extracted one, in spite of the fact that the enzymes share the same origin.

Table 1. *Purification of phosphatidylinositol-specific phospholipase C of Bacillus cereus* T

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery (%)
Culture medium	66500	3880	0.058	1	100
80%-satd.-(NH ₄) ₂ SO ₄ precipitation	3200	3240	1.013	17	84
DEAE-cellulose	355	2940	8.282	143	76
CM-cellulose	1.65	645	398.2	6865	17
Sephadex G-75	0.44	192	436.4	7524	5

Fig. 1. Electrophoretic comparison of liver membrane and serum-soluble forms of alkaline phosphatase

Alkaline phosphatase was prepared from the liver plasma membrane by treatment with butan-1-ol (gel 1), bile acids (gel 2) and sodium deoxycholate (gel 3), followed by ultracentrifugation. The serum-soluble form (gel 4) derived from the liver was prepared by gel filtration of the serum on a Sephadex G-200 column. Electrophoresis was performed on disc gels (5.6%, w/v, acrylamide) in the presence of SDS (a) or disc gels (7.5% acrylamide) in the presence of Triton X-100 (b) as described in the Materials and methods section. Gels were stained for alkaline phosphatase activity with 5-bromoindol-3-yl phosphate as substrate.

Purification of PI-PLase C and its activity in releasing alkaline phosphatase

It has been shown that alkaline phosphatase and several other membrane-bound enzymes are released from membranes by bacterial PI-PLase C (Ikezawa *et al.*, 1976; Low & Finean, 1977). These observations suggest that phosphatidylinositol may be responsible for the attachment of these enzymes to membranes, although the molecular details have not been clarified. It would be of interest to know how alkaline phosphatase released from the liver plasma membrane by PI-PLase C compares with the serum-soluble and liver membrane forms described above.

For the purpose of the present study, it is most important to use a purified PI-PLase C that is completely separated from other phospholipases C and hydrolytic enzymes such as proteinases and glycosidases. We have purified PI-PLase C from *Bacillus cereus* as summarized in Table 1. The purified enzyme was found to be a single protein on SDS/polyacrylamide-gel electrophoresis and its M_r was estimated to be 33000 with standard marker proteins (Fig. 2). The value is comparable with that of the enzyme purified from *Clostridium novyi* (M_r

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the purified PI-PLase C

Electrophoresis was carried out on a slab gel (15%, w/v, acrylamide) in the presence of SDS as described in the Materials and methods section. The gel was stained for protein with Coomassie Brilliant Blue. Lane 1, mixture of marker proteins, which are, from top to bottom: α_1 -proteinase inhibitor (M_r 54000); β -chain of haptoglobin (M_r 36000); porcine trypsin (M_r 23000); soya-bean trypsin inhibitor (M_r 21000); and α -chain of haptoglobin (M_r 10000). Lane 2 contained purified PI-PLase C (10 μ g).

30000) (Taguchi & Ikezawa, 1978), and is a little higher than those of other bacterial enzymes (M_r 22000–28000) (Ikezawa *et al.*, 1978, 1983). The protein thus purified exerted a phospholipase C activity specific for phosphatidylinositol, but not for other phospholipids, including phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and sphingomyelin (Table 2). Furthermore, the preparation did not, so far as tested, contain any proteolytic activity nor glycosidic activity (results not shown).

When the liver plasma membrane was incubated with the purified PI-PLase C, alkaline phos-

Table 2. *Substrate specificity of the purified PI-PLase C*
Reaction mixtures containing 2mM-substrate, 0.16% sodium deoxycholate, 20mM-Hepes/NaOH, pH 7.0, and an appropriate amount of the purified enzyme in volumes of 0.5 ml were incubated at 37°C for 20 min. The enzyme activity was determined as described in the Materials and methods section. Values are expressed as percentages of the activity obtained with phosphatidylinositol as a substrate, and represent the mean for three experiments.

Substrate	Relative activity (%)
Phosphatidylinositol	100
Phosphatidylcholine	0
Phosphatidylserine	1.6
Phosphatidylethanolamine	3.2
Sphingomyelin	0

Fig. 3. *Release of alkaline phosphatase from liver plasma membrane by PI-PLase C*

Plasma-membrane suspension (2mg/ml) in 20mM-Tris/HCl buffer, pH 7.5, was incubated at 37°C with (○) or without (●) the purified PI-PLase C (0.005 unit/ml) with occasional shaking. At the indicated times, aliquots were taken and centrifuged at 105000g for 60 min. Values are expressed as percentage release of alkaline phosphatase by measuring the enzyme activity of samples before centrifugation and that of supernatants after centrifugation, and each point represents the mean for three experiments.

phatase was almost completely released from the membrane, as shown in Fig. 3.

Comparison of PI-PLase C-released alkaline phosphatase with liver membrane and serum-soluble forms

Alkaline phosphatase released from the membrane by the purified PI-PLase C was electrophor-

etically compared with the liver membrane form extracted with butan-1-ol and the serum-soluble form (Fig. 4). When no detergent was added to the gels and running buffer (Fig. 4a), the butan-1-ol-extracted liver enzyme did not completely penetrate into the gel, whereas the PI-PLase C-released alkaline phosphatase migrated normally into the gel like the serum-soluble form. Under all conditions used, in the absence (Fig. 4a) or presence of Triton X-100 (Fig. 4b) or SDS (Fig. 4c), the PI-PLase C-released alkaline phosphatase (gel 2 in each panel) migrated to the same position as the serum-soluble form (gel 3) and had a mobility distinctly different from that of the liver membrane form extracted with butan-1-ol (gel 1). The results, which were very reproducible, clearly demonstrate that the PI-PLase C-released alkaline phosphatase is electrophoretically identical with the serum-soluble form, in contrast with the difference observed with the membrane form extracted with butan-1-ol and other detergents (Fig. 1).

Furthermore, when the butan-1-ol-extracted liver enzyme was treated with PI-PLase C, the conversion of the membrane form into the serum-soluble form was also observed, as shown in Fig. 5. Thus it seems that PI-PLase C can exert the same effect on the conversion into the serum-soluble form from the liver enzyme under both conditions, not only when it is attached to the membrane but also when it is free of the membrane. Taken together, these results suggest that the liver alkaline phosphatase, even after being extracted from the membrane with butan-1-ol and other commonly used detergents, still preserves some component(s), possibly phosphatidylinositol, which makes the enzyme electrophoretically different from the serum-soluble form, and that treatment with PI-PLase C may remove this component from the liver enzyme, resulting in its conversion into a form corresponding to the serum-soluble form. The direct proof for such a conversion mechanism will require analysis of the membrane form for phosphatidylinositol and demonstration that the phosphatidylinositol content decreases or disappears on incubation with PI-PLase C; this has not been demonstrated in the present study, because it is quite difficult to obtain sufficient of the purified membrane form of alkaline phosphatase for analysis.

Low & Zilversmit (1980) reported the difference, in recombination with phospholipid vesicles, of two preparations of alkaline phosphatase from pig kidney microsomes. Alkaline phosphatase released from microsomes by PI-PLase C did not bind to phospholipid vesicles, whereas the enzyme solubilized by butan-1-ol extraction was able to bind to phospholipid vesicles. The ability of butan-

Fig. 4. Electrophoretic comparison of PI-PLase C-released alkaline phosphatase with butan-1-ol-extracted and serum-soluble forms

Electrophoresis was performed under three different conditions: standard gel electrophoresis at pH 8.6 in the absence (a) or presence (b) of Triton X-100 (0.1%) in gels and running buffer; (c) SDS/polyacrylamide-gel electrophoresis. Gels were stained for alkaline phosphatase activity. Gel 1, butan-1-ol extract of liver plasma membrane; gel 2, PI-PLase C-released samples of liver plasma membrane; gel 3, serum-soluble form.

Fig. 5. Conversion of the butan-1-ol-extracted membrane form to the serum-soluble form by treatment with PI-PLase C

The liver membrane form extracted with butan-1-ol (about 4 units/ml) was incubated at 37°C for 90 min in the presence or absence of the purified PI-PLase C (0.005 unit/ml) as described in the Materials and methods section. Samples thus treated in the absence (gel 1) or presence (gel 2) of PI-PLase C were subjected to polyacrylamide-gel electrophoresis in the presence of Triton X-100 (a) or SDS (b), and gels were stained for alkaline phosphatase activity. Arrows indicate the position of the serum-soluble form.

1-ol-extracted alkaline phosphatase to bind phospholipid vesicles was destroyed by added PI-PLase C. On the basis of these results, it was concluded that alkaline phosphatase is attached to membranes by a strong interaction with phosphatidylinositol (Low & Zilversmit, 1980). The present results strongly support their conclusion and further suggest the possibility that PI-PLase C is involved in the appearance of hepatic alkaline phosphatase in serum.

However, all the preparations of PI-PLase so far tested for release of alkaline phosphatase are those isolated from bacteria (Ikezawa *et al.*, 1976; Low & Finean, 1977; Low & Zilversmit, 1980; Shukla *et al.*, 1980). To determine the involvement of PI-PLase C in release of the membrane-bound enzyme, it is essential to identify an endogenous activity of PI-PLase C in tissues. It has been reported that there exist in mammalian tissues several different types of PI-PLase C, including lysosomal, cytosolic and membrane-bound types, which are also classified by requirement of Ca^{2+} for their activation (Michell, 1975; Irvine *et al.*, 1978; Matsuzawa & Hostetler, 1980). None of them, however, has been tested as candidate responsible for the release of alkaline phosphatase. Low & Zilversmit (1980) suggested that pig kidney microsomes contain an endogenous PI-PLase C that is activated with butan-1-ol. In a preliminary experiment we have indeed observed that an alkaline phosphatase electrophoretically identical with the serum-soluble one was released from the liver plasma membrane when the membrane was incubated in the presence of butan-1-ol at low pH (pH 4.5–6.5), although no conversion took place

when the pH of the medium was increased above pH 7.5, as shown in the present study.

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References

- Barnwell, S. G., Godfrey, P. P., Lowe, P. J. & Coleman, R. (1983) *Biochem. J.* **210**, 549–557
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Billington, D., Evans, C. E., Godfrey, P. P. & Coleman, R. (1980) *Biochem. J.* **188**, 321–327
- Börnig, V. H., Stepan, J., Horn, A., Giertler, R., Thiele, G. & Vecerek, B. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1311–1318
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Hatoff, D. E. & Hardison, W. G. M. (1979) *Gastroenterology* **77**, 1062–1067
- Ikehara, Y., Takahashi, K., Mansho, K., Eto, S. & Kato, K. (1977) *Biochim. Biophys. Acta* **470**, 202–211
- Ikehara, Y., Mansho, K., Takahashi, K. & Kato, K. (1978a) *J. Biochem. (Tokyo)* **83**, 1471–1483
- Ikehara, Y., Mansho, K. & Kato, K. (1978b) *J. Biochem. (Tokyo)* **84**, 1335–1338
- Ikehara, Y., Miyasato, M., Ogata, S. & Oda, K. (1981) *Eur. J. Biochem.* **115**, 253–260
- Ikezawa, H. & Taguchi, R. (1981) *Methods Enzymol.* **71**, 731–741
- Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. & Ohyabu, Y. (1976) *Biochim. Biophys. Acta* **450**, 154–164
- Ikezawa, H., Mori, M., Ohyabu, T. & Taguchi, R. (1978) *Biochim. Biophys. Acta* **528**, 247–256
- Ikezawa, H., Nakabayashi, T., Suzuki, K., Nakajima, M., Taguchi, T. & Taguchi, R. (1983) *J. Biochem. (Tokyo)* **93**, 1717–1719
- Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1978) *Biochem. J.* **176**, 475–484
- Kaplan, M. M. & Righetti, A. (1970) *J. Clin. Invest.* **49**, 508–516
- Kominami, T., Oda, K. & Ikehara, Y. (1984) *J. Biochem. (Tokyo)* **96**, 901–911
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Low, M. G. (1981) *Methods Enzymol.* **71**, 741–746
- Low, M. G. & Finean, J. B. (1977) *Biochem. J.* **167**, 281–284
- Low, M. G. & Zilversmit, D. B. (1980) *Biochemistry* **19**, 3913–3918
- Matsuzawa, Y. & Hostetler, K. Y. (1980) *J. Biol. Chem.* **255**, 646–652
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–145
- Misumi, Y., Tanaka, Y. & Ikehara, Y. (1983) *Biochem. Biophys. Res. Commun.* **114**, 729–736
- Morton, R. K. (1957) *Biochem. J.* **65**, 674–682
- Oda, K. & Ikehara, Y. (1981) *Biochim. Biophys. Acta* **640**, 398–408
- Pekarthy, J. M., Short, J., Lansing, A. I. & Lieberman, I. (1972) *J. Biol. Chem.* **247**, 1767–1774
- Ray, T. K. (1970) *Biochim. Biophys. Acta* **196**, 1–9
- Schaleger, R., Haux, P. & Kattermann, R. (1982) *Enzyme* **28**, 3–13
- Shukla, S. D., Coleman, R., Finean, J. B. & Michell, R. H. (1980) *Biochem. J.* **187**, 277–280
- Slein, M. W. & Logan, G. F. (1965) *J. Bacteriol.* **90**, 69–81
- Taguchi, R. & Ikezawa, H. (1978) *Arch. Biochem. Biophys.* **186**, 196–201
- Vyvoda, O. S., Coleman, R. & Holdsworth, G. (1977) *Biochim. Biophys. Acta* **465**, 68–76
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wisher, M. H. & Evans, W. H. (1975) *Biochem. J.* **146**, 375–388