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Further Evidence for the Concept of Bovine Plasma Arylesterase as a Lipoprotein

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Purified preparations of bovine plasma arylesterase were obtained by isoelectric focusing of enzyme prepared by $(NH_4)_2SO_4$ fractionation of plasma and chromatography on DEAE-cellulose and Sephadex G-200. Although the high-density-lipoprotein fraction (HDL_2) of serum provides an alternative source of enzyme, the enzymic activity of preparations made from it is much less stable. The purified arylesterase preparation has a molecular weight of 440000 and a partial specific volume of 0.91 ml/g, properties indistinguishable from those of the less highly purified enzyme. Extraction with acetone and ether removes neutral lipids from the enzyme, but the resulting lipid-depleted preparation retains most of the phospholipid present initially. A partial specific volume of 0.81 ml/g and a minimum molecular weight of approx. 100000 were determined for the lipid-depleted preparations of arylesterase. The present results support the concept of bovine plasma arylesterase as a lipoprotein in its own right, rather than as an enzymic polypeptide that is loosely associated with the HDL_2 fraction of serum.

A previous investigation of the effects of lipid removal on the molecular size and kinetic properties of bovine plasma arylesterase (Kitchen *et al.*, 1973) led to the conclusion that the arylesterase preparation purified by the method of Choi & Forster (1967*a*,*b*) is either a lipoprotein or an enzyme–lipoprotein complex with properties very similar to those of the α_1-lipoprotein or high-density lipoprotein (HDL_2) fraction of serum. As a possible means of distinguishing between these alternative concepts of bovine plasma arylesterase as a lipoprotein or an enzyme–lipoprotein complex, we have attempted further purification of the arylesterase activity from the Choi & Forster (1967*a*,*b*) and HDL_2 preparations. The aims of the present paper are (i) to show by isoelectric focusing that arylesterase activity is associated with a relatively minor protein fraction of either preparation, and (ii) to report studies of the molecular size of the more highly purified arylesterase.

Materials and Methods

Isolation of bovine plasma arylesterase and HDL_2 fraction

Blood (7 litres) was collected from an individual animal at the time of slaughter. Half of the blood was allowed to clot and the serum so obtained used for the purification of HDL_2 fraction by the flotation technique of Scanu & Granda (1966). The other half was treated with heparin (300000 USP units) and centrifuged at 3000*g* for 20min at 5°C. Arylesterase was then prepared from the clear plasma by the Kitchen *et al.* (1973) modification of the Choi & Forster (1967*a*,*b*) procedure.

The plasma arylesterase preparation had a specific activity of 40 μmol/min per mg of protein, as determined with phenyl acetate as substrate by the method described previously (Kitchen *et al.*, 1973). Although this specific activity is about three times that of the earlier preparations (Kitchen *et al.*, 1973), it represents no improvement in the purification factor (50-fold), since the plasma enzyme activity was similarly higher; the origin of differences between enzymic activities of plasma samples has not been traced. Arylesterase activity of the HDL_2 fraction amounted to 20 μmol/min per mg of protein.

Neutral lipid and phospholipid determinations

The neutral lipid content of the bovine plasma arylesterase was measured with the aid of the appropriate 'Biochemica Test Combination' kit supplied by Boehringer Mannheim G.m.b.H., Mannheim, Germany. Phospholipid content was determined by the measurement of P_i (Fiske & SubbaRow, 1925) released by digestion with H_2SO_4 and H_2O_2 of a chloroform–methanol extract of the enzyme preparation (Youngburg & Youngburg, 1930), and the conversion of the phosphate concentration into approximate phospholipid concentration (Varley, 1969). On the basis of estimates of protein concentration from absorbance measurements at 280nm and 260nm (Warburg & Christian, 1942), the plasma arylesterase contained 26mg of triacylglycerols and 35mg of phospholipid per 100mg of protein.

Solvent extraction

Lipid was removed from bovine plasma arylesterase and HDL_2 preparations by two extractions at −10°C with acetone and one with ether, also at −10°C (Kitchen *et al.*, 1973). Each resulting protein precipitate was dissolved in a minimum volume of 10mM-Tris–HCl buffer (pH8.0) containing $CaCl_2$ (1mM) and EDTA (5μM), and dialysed against the same buffer in readiness for further studies on the protein fraction.

In other experiments on bovine plasma arylesterase the acetone–ether treatment was replaced by extraction either with chloroform–methanol (2:1, v/v) by the method of Folch *et al.* (1957) or with di-isopropyl ether–butanol (2:1, v/v); in the latter case 2 vol. of solvent was added to 1 vol. of enzyme solution and the mixture shaken for 2h at room temperature (B. E. Cham, personal communication).

Thin-layer chromatography

Native and solvent-extracted samples of bovine plasma arylesterase and fraction HDL_2 were analysed by t.l.c. on silica-gel plates prepared by the method of Gloster & Fletcher (1966). Neutral lipids were separated with light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (90:10:1, by vol.). Phospholipids were separated by the method of Skipski & Barclay (1969), which entails pre-washing loaded plates with acetone–light petroleum (b.p. 60–80°C) (1:3, v/v) to remove neutral lipids, and then running the air-dried plates in chloroform–methanol–acetic acid–water (25:15:4:2, by vol.). Lipids were detected on t.l.c. plates either by spraying with aq. 50% (v/v) H_2SO_4 and heating to 160°C for 30 min, or by exposure to iodine vapour.

Arrhenius plots

In studies of the effect of temperature on arylesterase activity towards phenyl acetate, the cell holder of a Unicam SP.800 recording spectrophotometer was thermostatically controlled at several temperatures in the range 7–30°C. All components of the assay system (Kitchen *et al.*, 1973) were equilibrated at the required temperatures before commencement of the enzymic reaction. Reaction mixtures contained either 4.5μg of native plasma arylesterase or 3.0μg of esterase preparation that had been subjected to acetone–ether extraction.

Isoelectric focusing

The preparations of HDL_2 and bovine plasma arylesterase were fractionated separately on an LKB 8100-10 isoelectric-focusing column (110ml capacity). Procedures for the generation of a pH gradient over the pH range 4–6 were those recommended by the manufacturer of the equipment (LKB-Produkter, Uppsala, Sweden). Samples for fractionation, containing 10mg of protein (Warburg & Christian, 1942), were dialysed against 1% glycine solution before insertion in the apparatus, and the column was subjected to a potential difference of 900V for 65h. Column temperature was maintained at 5°C.

On completion of the run the gradient was divided into 2ml fractions, which were assayed for arylesterase activity. Absorbances at 280nm and 465nm of each fraction, and also the pH, were determined. Fractions with the highest enzymic activity were pooled and dialysed exhaustively against 10mM-Tris–HCl buffer containing Ca^{2+} (1mM) and EDTA (5μm) to remove the sucrose and ampholytes (Vesterberg *et al.*, 1967); the Ca^{2+} and EDTA were included to stabilize arylesterase activity (Choi & Forster, 1967*a*,*b*).

Estimation of molecular weights

Before ultracentrifugation all preparations were dialysed for 16h at 4°C against 10mM-Tris–HCl buffer (pH8.0) containing Ca^{2+} (1mM), EDTA (5μM) and KCl (100mM). A protein concentration of approx. 3mg/ml was used, and each sample subjected to sedimentation at 20000rev./min and 20°C in a Spinco model E ultracentrifuge. As noted previously (Kitchen *et al.*, 1973), equilibrium was attained within 16h, the patterns being of the Yphantis (1964) type. Schlieren patterns were measured on a Nikon two-dimensional comparator and the results analysed by the Lamm (1929) procedure.

Partial specific volumes ($\bar{v}$) of the native and acetone–ether-extracted preparations of bovine plasma arylesterase were determined by velocity sedimentation of 0.5mg/ml solutions of the appropriate material in which the density was varied either by including KCl (0.25–1.25M) or by substituting deuterium oxide for water in the buffer. Through lack of information about the likely extent of deuterium–hydrogen exchange in the lipoproteins, the *k* factor of Martin *et al.* (1959) was taken as unity. Values of 0.91 and 0.81ml/g were obtained for $\bar{v}$ of the native and solvent-extracted preparations respectively.

Paucity of sample precluded use of the same technique for estimating the partial specific volume of the more highly purified bovine plasma arylesterase that resulted from isoelectric focusing of the Choi & Forster (1967*a*,*b*) preparation. Accordingly samples (approx. 0.4mg) of the Choi & Forster (1967*a*,*b*) material and the purified arylesterase were subjected to isopycnic density-gradient centrifugation in separate buckets of an SW-50 rotor, which was spun for 18h at 50000rev./min in a Beckman model L2-65B ultracentrifuge. After the run the tubes were pierced and the contents divided into 0.1ml fractions, which were assayed for arylesterase

activity. Each fraction was also analysed refractometrically to determine the CsCl concentration and hence density, which varied between 1.05 and 1.30 g/ml. Hydrated densities of 1.09 and 1.11 were obtained for the Choi & Forster (1967*a*,*b*) and purified arylesterase preparations respectively. The results are consistent with a partial specific volume of 0.91 ± 0.01 ml/g for either preparation.

Results

Comparison of bovine plasma arylesterase and fraction HDL_2

In the course of this investigation several qualitative differences between HDL_2 and the Choi & Forster (1967*a*,*b*) preparations were noted. From the viewpoint of specific activity the arylesterase preparation was twice as active as HDL_2. Although these specific activities refer explicitly to a protein basis, the fact that the two preparations have similar partial specific volumes (Kitchen *et al.*, 1973) implies a twofold difference in activities expressed per g of preparation as well as per g of protein. Extraction of lipid with acetone and ether has no effect on the specific activity of the plasma arylesterase preparation (40 μmol/min per mg of protein), but causes a 50% increase (from 20 to 30 μmol/min per mg of protein) in the activity of fraction HDL_2.

Analyses of the lipid contents of arylesterase and HDL_2 preparations showed that although both materials contained the same spectrum of lipids, differences in relative amounts of lipid classes were detectable by qualitative inspection of t.l.c. plates. In particular a higher phospholipid content of fraction HDL_2 was indicated by the presence of a heavy precipitate at the origin of t.l.c. plates that were developed in chromatographic solvent for neutral lipids; no such precipitation was observed on plates loaded with an equivalent amount of bovine plasma arylesterase protein.

Lipid content of bovine plasma arylesterase

A more detailed examination of the lipid contents of the plasma arylesterase preparation and its lipid-depleted derivative obtained by extraction with acetone and ether is shown in Fig. 1. From t.l.c. plates run in light petroleum–diethyl ether–acetic acid (90:10:1, by vol.) it is evident that the acetone and ether extractions have removed effectively all of the neutral lipids from the plasma arylesterase (Fig. 1*a*). However, Fig. 1(*b*) indicates the existence of considerable amounts of phospholipid in the acetone–ether-extracted preparation. Some phospholipid, notably phosphatidylethanolamine and phosphatidylserine, has been removed by solvent extraction, but about 60–80% of the phospholipid remains bound to protein. Di-isopropyl ether–butanol (2:1, v/v) is approximately four times as effective as acetone and ether in removing phospholipids from the Choi & Forster (1967*a*,*b*) preparation, but again significant amounts of phosphatidylserine and phosphatidylinositol are not

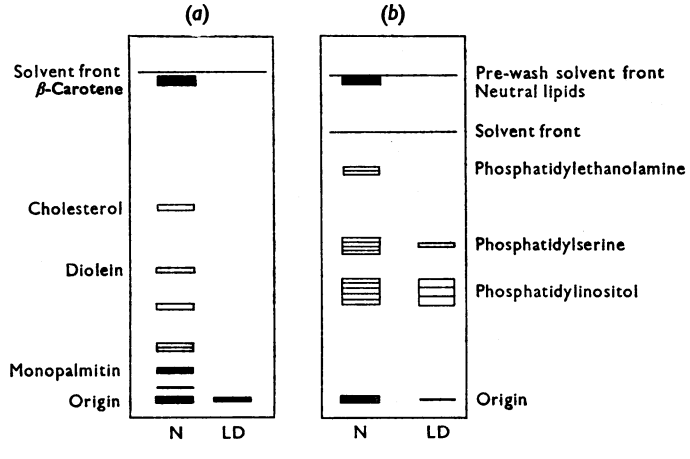

Fig. 1. *Thin-layer chromatography of native (N) and lipid-depleted (LD) preparations of bovine plasma arylesterase*

(*a*) Separation of neutral lipids by development of silica-gel plates with light petroleum–diethyl ether–acetic acid (90:10:1, by vol.). (*b*) Separation of phospholipids: silica-gel plates were pre-washed with acetone–light petroleum (1:3, v/v) to remove neutral lipids, after which chloroform–methanol–acetic acid–water (25:15:4:2, by vol.) was used as the developing solvent. Black areas, intense staining; hatched areas, moderate staining; open areas, weak staining.

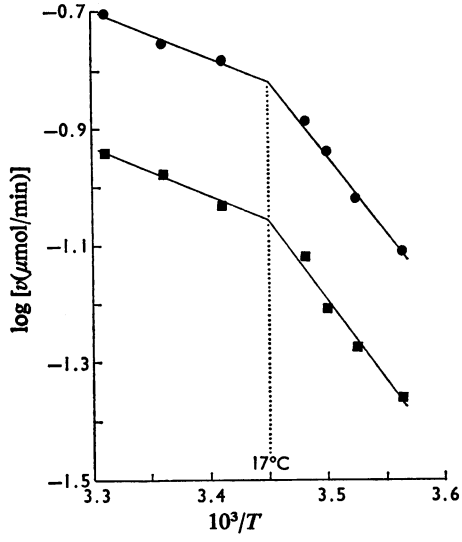

Fig. 2. *Arrhenius plots for native and lipid-depleted preparations of bovine plasma arylesterase*

Initial velocities were measured for enzymic reaction mixtures with 2.7ml of 10mM-Tris–HCl buffer (pH8.0) containing Ca^{2+} (1mM), EDTA (5μm) and eserine sulphate (40μM), 0.3ml of phenyl acetate (10mM) and either 4.5μg of native bovine plasma arylesterase (●) or 3.0μg of plasma arylesterase preparation that had been subjected to acetone–ether extraction (■).

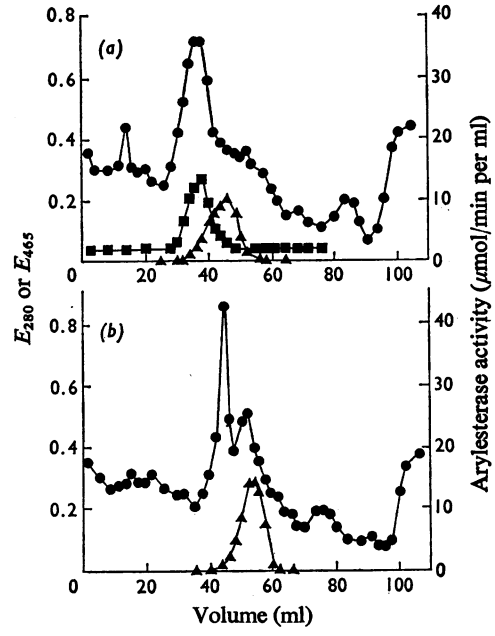

Fig. 3. *Isoelectric focusing of (a) native and (b) lipid-depleted preparations of fraction HDL_2 from bovine serum*

Samples (10mg of protein) of native and acetone–ether-extracted preparations of fraction HDL_2 were subjected to isoelectric focusing in a pH gradient extending over the range 4–6. At the end of the run the contents of the 110ml column were analysed in terms of E_{280} (●), E_{465} (■) and arylesterase activity (▲).

extracted. With chloroform–methanol (2;1, v/v) extraction of lipid is virtually complete, but all enzymic activity is destroyed.

Additional evidence for the existence of considerable amounts of lipid in the acetone–ether-extracted preparation is provided by the magnitude of $\bar{v}$, 0.81 ml/g, for this material (see the Materials and Methods section). Further, the Arrhenius plot of the activity of either native or lipid-depleted arylesterase (Fig. 2) exhibits a discontinuity in the region of 17°C, a phenomenon that is commonly observed with enzymes in tight association with lipid (Lyons & Raison, 1970; Raison *et al.*, 1971; Kumamoto *et al.*, 1971).

Isoelectric focusing of bovine plasma arylesterase and fraction HDL_2

Fig. 3 presents patterns typical of isoelectric-focusing experiments on native and acetone–ether-extracted preparations of fraction HDL_2. Clearly the peaks of absorbance at 280nm do not coincide with those of arylesterase activity; indeed, enzymic activity seems to be associated with protein responsible for a relatively small shoulder on the absorbance peak. On the basis of the pH gradient the isoionic pH (pI) of the native protein peak is 5.4, whereas that of the native enzyme peak is 5.0. Qualitatively similar results were obtained with native and acetone–ether-extracted preparations of bovine plasma arylesterase, as is evident from Table 1, which summarizes the pI values for protein (E_{280}), pigment (E_{465}) and enyzme that were obtained with plasma arylesterase as well as HDL_2 preparations. Several points in relation to Table 1 merit comment. (i) The yellow coloration of native preparations due to β-carotene is associated with the major protein fraction and not the enzyme. (ii) Extraction of lipid material with acetone and ether causes a shift of pI values 0.2–0.4 unit towards neutral pH. (iii) Although differences exist between the isoionic points of native HDL_2 and plasma arylesterase preparations, the lipid-depleted samples of both materials have the same pI. (iv) In all cases considerable losses of arylesterase activity attended isoelectric-focusing experiments, presumably because of the absence of the enzyme-stabilizing factors Ca^{2+} and EDTA (Choi & Forster, 1967*a*,*b*). These activity losses, which were more

Table 1. *Comparison of isoionic points of protein, pigment and enzymic materials in Choi & Forster (1967a,b) arylesterase and HDL_2 preparations*

Results are obtained from profiles of isoelectric-focusing experiments performed on native preparations and on lipid-depleted samples obtained by extraction with acetone and ether. Values reported are the means of duplicate experimental determinations, which differed by less than 0.05 pH unit.

Material	Isoionic pH			
	Arylesterase		Fraction HDL_2	
	Native	Lipid-depleted	Native	Lipid-depleted
Protein (E_{280})	5.0	4.8	5.4	5.0
Pigment (E_{465})	5.0	—	5.4	—
Enzyme activity	4.8	4.6	5.0	4.6

extensive for native and lipid-depleted HDL_2 preparations than for their Choi & Forster (1967*a*,*b*) counterparts, precluded specific-activity measurements for the more highly purified arylesterase preparations obtained by isoelectric focusing. However, from the shapes of the protein profiles in the isoelectric-focusing experiments at least a 10-fold purification of the arylesterase was achieved.

Molecular weight of bovine plasma arylesterase

The demonstration that bovine plasma arylesterase constitutes only a minor fraction of either fraction HDL_2 or Choi & Forster (1967*a*,*b*) preparations (Fig. 3 and Table 1) has necessitated redetermination of its molecular weight. Lamm (1929) plots of the results from high-speed sedimentation-equilibrium runs on native and lipid-depleted samples of the purified plasma arylesterase are shown in Fig. 4, together with runs on the corresponding Choi & Forster (1967*a*,*b*) preparations. The following points are noted. (i) The results for native unfractionated arylesterase are in excellent agreement with those reported previously (Kitchen *et al.*, 1973), and hence the molecular weight of 440000 for the Choi & Forster (1967*a*,*b*) preparation is confirmed. (ii) Isolation of only a small fraction of the Choi & Forster (1967*a*,*b*) plasma arylesterase has led to no significant change in sedimentation-equilibrium behaviour, since the results may clearly be described by a line with the same slope as that for the unfractionated material (Fig. 4*a*). (iii) The previous estimate of approximately 70000 for the minimum molecular weight of lipid-depleted Choi & Forster (1967*a*,*b*) arylesterase is in error, since the assumed value of 0.73 for $\bar{v}$ was based on the absence of lipid from preparations extracted with acetone and ether. Combination of the present equilibrium data, which are also in excellent agreement with the earlier result (Fig. 5*b* of Kitchen *et al.*, 1973), with the measured $\bar{v}$ of 0.81 ml/g yields a minimum molecular weight of approx. 100000 for lipid-depleted preparations of unfractionated plasma arylesterase. (iv) Good correspondence is also observed between the sedimentation behaviour of lipid-depleted samples of purified and Choi & Forster (1967*a*,*b*) preparations of arylesterase (Fig. 4*b*). Provided that the same value of $\bar{v}$, i.e. 0.81, applies to the fractionated material, a minimum molecular weight of approx. 100000 is also indicated.

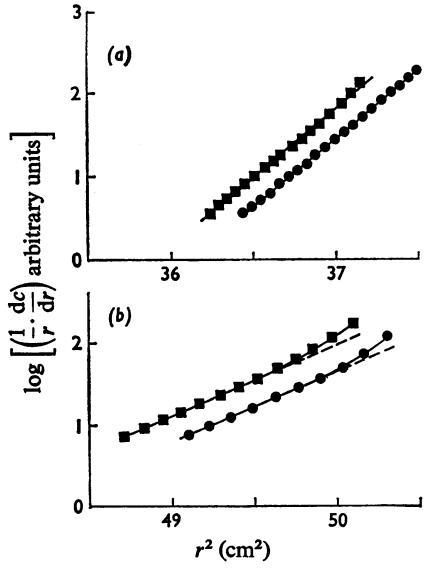

Fig. 4. *Lamm plots of equilibrium-sedimentation data for native and lipid-depleted preparations of bovine plasma arylesterase*

Samples of native (*a*) and lipid-depleted (*b*) preparations of bovine plasma arylesterase were subjected to equilibrium sedimentation at 20000rev./min after dialysis of the solutions (approx. 3mg/ml) against 10mM-Tris–HCl buffer (pH8.0) containing Ca^{2+} (1mM), EDTA (5μM) and KCl (100mM): ●, enzyme prepared by the Choi & Forster (1967*a*,*b*) procedure; ■, arylesterase purified by isoelectric focusing of the Choi & Forster (1967*a*,*b*) preparation.

Discussion

The initial impetus for this investigation was the conclusion (Kitchen *et al.*, 1973) that the Choi & Forster (1967*a*,*b*) preparation of bovine plasma arylesterase is either a lipoprotein or an enzyme–lipoprotein complex with properties very similar to the HDL_2 fraction of serum. Further, it was suggested that the relatively low purification of enzymic activity achieved made the second alternative more likely. This suggestion has been confirmed by the present isoelectric-focusing experiments, which show that arylesterase activity is indeed associated with only a minor fraction of the Choi & Forster (1967*a*,*b*) preparation. However, the concept of the enzyme as a lipoprotein still survives the present study, since complete removal of lipid is attended by complete destruction of enzymic activity. Of interest is the observation that the molecular weight reported previously (Kitchen *et al.*, 1973) for the Choi & Forster (1967*a*,*b*) preparation also applies to the more highly purified arylesterase, the lipoprotein nature of which is established unequivocally by measurement of its partial specific volume. We must therefore take the view that bovine plasma arylesterase is an enzymic lipoprotein that exists *in vivo* in relatively loose association with other lipid and lipoprotein components of plasma by virtue of hydrophobic interactions.

The previous suggestion (Kitchen *et al.*, 1973) that the HDL_2 fraction of serum may provide a more convenient source of arylesterase for further purification is not borne out by the present investigation. Although fraction HDL_2 provides a ready source of arylesterase, the enzymic activity is much less stable than that of the Choi & Forster (1967*a*,*b*) preparation. This greater loss of arylesterase activity from fraction HDL_2 suggests the presence of a stabilizing factor in blood which is retained in the Choi & Forster (1967*a*,*b*) preparation of arylesterase from plasma, but which is absent from the HDL_2 fraction of serum. Its removal could occur either during clotting or in the flotation procedure used for HDL_2 preparation (Scanu & Granda, 1966).

The effect of lipid removal on the molecular size of purified plasma arylesterase parallels that reported previously for the Choi & Forster (1967*a*,*b*) preparation (Kitchen *et al.*, 1973) inasmuch as dissociation into smaller units occurs. The curvilinearity of the equilibrium-sedimentation plots (Fig. 4*b*) indicates either incomplete dissociation or complete dissociation and an array of subunit sizes. An apparent z-average molecular weight of 135000 is obtained for arylesterase extracted with acetone and ether, and a value of 100000 daltons (approximately) for the minimum subunit size. On the basis of the magnitude of $\bar{v}$, 0.81 ml/g, the molecular weight of the polypeptide component of such a subunit would be 70000–80000, and thus not so very different from that found for many other mammalian esterases (Kingsbury & Masters, 1970).

Finally, in relation to a previous suggestion, (Kitchen, 1971) that the physiological function of this enzyme may involve a transport role, it is noteworthy that phosphatidylinositol remains tightly associated with the bovine arylesterase after repeated lipid extraction. There is a rapidly expanding body of evidence which implicates this phospholipid in cellular transport processes also (Hokin & Huebner, 1967; Scott *et al.*, 1968; Dawson & Clarke, 1972).

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