The Adenosine-Triphosphatase System Responsible for Cation Transport in Electric Organ: Exclusion of Phospholipids as Intermediates

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1. Subcellular fractions were prepared from the electric organs of *Electrophorus* and *Torpedo* and assayed for adenosine-triphosphatase activity. 2. Treatment of the 'low-speed' fraction from *Torpedo* with m-urea gave an adenosine-triphosphatase preparation that was almost completely (98%) inhibited by ouabain (0.1 mg./ml.) and dependent on the simultaneous presence of Na⁺ and K⁺. 3. The adenosine-triphosphatase preparations were exposed to [γ -³²P]ATP for 30 sec. in the presence of (i) Na⁺, (ii) K⁺, (iii) Na⁺ + K⁺ and (iv) Na⁺ + K⁺ + ouabain. No significant labelling of phosphatidic acid, triphosphoinositide or any other phospholipid was observed. 4. The results suggest that phospholipids do not act as phosphorylated intermediates in the 'transport adenosine-triphosphatase' system of electric organ.

There is now good evidence that the transport of Na⁺ and K⁺ across cell membranes is closely associated with the hydrolysis of ATP (Skou, 1957; Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Hoffman, 1960; Glynn, 1962; Whittam, 1962; Bonting, Caravaggio & Hawkins, 1962). This hydrolysis characteristically occurs only when K⁺ is present at the outside surface of the membrane and Na⁺, Mg²⁺ and ATP are present at the inside surface; it is inhibited by cardiac glycosides at low concentrations. The demonstration by Skou (1960) that the 'transport ATPase'‡ can catalyse an ATP-ADP exchange reaction suggests that a phosphorylated intermediate is involved. There may, of course, be more than one intermediate.

Hokin & Hokin (1960) showed that, when the secretion of sodium chloride by slices of albatross salt gland was stimulated by acetylcholine, the rate of incorporation of orthophosphate into phosphatidic acid was greatly increased. These observations were extended in studies of erythrocyte 'ghosts' and brain microsomal preparations,

and evidence was obtained that phosphatidyl-inositol might also play some part in salt transport (Hokin & Hokin, 1963). A third compound that has been suggested as a possible intermediate in 'transport ATPase' activity is triphosphoinositide (Ellis & Hawthorne, 1962; Thompson & Dawson, 1962). In brain tissue this compound is very rapidly synthesized from ATP, and dephosphorylated (Ellis & Hawthorne, 1962; Wagner, Lissau, Hölzl & Horhammer, 1961; Brockerhoff & Ballou, 1962*a,b*; Thompson & Dawson, 1964; Dawson & Thompson, 1964). On the other hand, Järnefelt (1962) calculated that ³²P incorporation from [³²P]ATP into the total phospholipid fraction of brain microsomes was too slow to represent the formation of an intermediate in the ATPase reaction.

The experiments reported below were designed to test whether any of the phospholipids mentioned above, or any other phospholipids, were in fact phosphorylated intermediates in the hydrolysis of ATP associated with ion transport. Preparations of 'transport ATPase' from the electric organs of electric eels and torpedoes were exposed briefly to [γ -³²P]ATP of high specific activity. As little is known of the mechanism of hydrolysis of ATP by these preparations, it was not possible to predict the conditions that might favour the accumulation of

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‡ Abbreviation: ATPase, adenosine triphosphatase.

any phosphorylated intermediate. The preparations were therefore exposed (i) under conditions that allowed hydrolysis to take place, (ii) in the presence of a cardiac glycoside, (iii) in the absence of Na^+ and (iv) in the absence of K^+ . It was thought that conditions (ii), (iii) and (iv) might interfere with the reaction mechanism at different points, so that the chances of trapping an intermediate would be increased. After the exposure, the phospholipids were extracted from the preparations and examined for incorporated radioactivity. The results provide no evidence that phospholipids are intermediates in the 'transport ATPase' system.

MATERIALS

ATP (disodium salt). This was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. ATP (tris salt) was prepared according to the method of Dunham & Glynn (1961).

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This was prepared by the method of Glynn & Chappell (1964). The specific activity was between 10 and 80 mc/m-mole.

Salts. NaCl and KCl were obtained 'spectroscopically pure' from Johnson, Matthey and Co. Ltd., Wembley, Middlesex. MgCl_2 was made from 'spectroscopically pure' magnesium rod and analytical-reagent grade HCl. Tris was the Trizma grade supplied by the Sigma Chemical Co. EDTA (tris salt) was made from the free acid prepared by acidifying analytical-reagent grade EDTA (tetrasodium salt).

Ouabain. Ouabain (British Drug Houses Ltd., Poole, Dorset) was dissolved in ethanol and the solution diluted with water.

METHODS

Preparation of the electric-organ ATPase. Electric eels (*Electrophorus electricus*) were obtained from the Paramount Aquarium Inc., Ardsley, N.Y., U.S.A.; torpedoes (*Torpedo torpedo* and *Torpedo marmorata*) were obtained from Captain L. Fusco, of Naples, Italy. The electric eels were killed by decapitation, the torpedoes by section across the base of the brain followed by pithing. With both electric eel and torpedo, the electric organ was dissected out and washed quickly with 174 mM-NaCl (eel) or with elasmobranch Ringer solution (Brock & Eccles, 1958) (torpedo). Weighed portions of tissue were then homogenized in 10 vol. of a solution containing sucrose (250 mM), EDTA (2 mM) and tris (30 mM) adjusted to pH 7.8 with HCl. Homogenization for 30 sec. in an MSE homogenizer running at nearly full speed was followed by further treatment for about 15 sec. in a Dounce-type Perspex-in-glass homogenizer with a fairly loose pestle driven at 900 rev./min. The homogenate was filtered through glass wool and separated by differential centrifuging into 'low-speed' (3000–5000 g for 15–20 min.), 'intermediate-speed' (10000 g for 20 min.) and 'high-speed' (35000 g for 120 min.) fractions. All centrifuging was done at 0–8° in an MSE H.S.17 centrifuge. The 'low-speed' and 'intermediate-speed' fractions were washed by resuspending the precipitates in more of the same medium and recentrifuging. The washed precipitates were then resuspended in convenient volumes of the same medium

(usually 1–2 times the original volume of the electric organ) and stored at 4°. From 2 to 19 days before the enzyme preparation was to be used, 10 M-urea was added to portions of the preparation to give a final concentration of 1 M. The day before an experiment was to be carried out, most of this urea was removed by centrifuging the preparation (15 000 g for 40 min.) and resuspending the precipitate in the sucrose–EDTA–tris medium described above. Usually the washing procedure was repeated once, but no attempt was made to get rid of the urea completely, as low concentrations did not appear to interfere with the ATPase activity; in the living torpedo, urea is normally present at quite high concentration (0.33 M).

Assay of ATPase. The enzyme suspension was diluted with the sucrose–EDTA–tris medium so that 0.1 ml. of the diluted suspension in 3 ml. of test medium hydrolysed a convenient amount of ATP in 20 min. at 25°. Incubation was carried out in 5 ml. graduated tubes fitted with glass stoppers. The reaction was started by the addition of ATP in a volume of fluid equal to one-tenth of the total volume, so that the cooling effect was small. At the end of the incubation period the tubes were transferred to an ice bath and ice-cold 55% (w/v) trichloroacetic acid was added to give a final concentration of 5% (w/v). Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925). Activities have been expressed as the amount of phosphate liberated/g. of original electric organ in 20 min., and not converted into a rate/min., as with the *Torpedo* preparation the reaction rate decrease with time. This probably came about mainly because of inhibition by ADP formed during the reaction, though a small decrease in activity was observed when the enzyme was incubated in the absence of substrate. In one experiment with *Electrophorus*, the protein content of the different fractions was estimated (Lowry, Rosebrough, Farr & Randall, 1951) so that activity could be expressed/unit weight of protein.

Exposure of enzyme preparations to radioactive ATP. Portions of enzyme preparation equivalent to about 20 g. of original tissue were suspended in salt solutions in Erlenmeyer flasks and placed in a water bath at 25°. After 5 min. equilibration, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added as a 20 mM solution of the tris salt in approx. 195 mM-tris adjusted to pH 7.2 with HCl. The final concentration of the ATP was 2.7 mM, and the volume of the reaction mixture was chosen so that, when the conditions allowed hydrolysis, between 10 and 20% of the ATP would be hydrolysed in 30 sec. After 30 sec. the reaction was stopped with 10 vol. of ice-cold chloroform–methanol (1:1, v/v), and the resulting mixture stored at 0° until it was convenient to extract the phospholipids.

Extraction of the phospholipids. The mixture was centrifuged and the supernatant decanted through a sintered-glass funnel. The residue was washed with 5 vol. of chloroform–methanol (1:1, v/v) and the supernatant again decanted through sintered glass.

(a) Phospholipids soluble in chloroform–methanol. The filtered supernatants were combined and 0.5 vol. of chloroform was added. The resulting 2:1 (v/v) chloroform–methanol solution was treated with 0.2 vol. of N-HCl and, after shaking, the lower phase was collected and washed twice with the 'theoretical upper phase' of Folch, Lees & Sloane-Stanley (1957) (chloroform–methanol–0.9% NaCl, 3:48:47, by vol.). In the *Electrophorus* experiment, an additional wash with 0.1 M- H_3PO_4 was carried out after the

N-HCl wash. The washed lower phase was evaporated to dryness *in vacuo*, and the phospholipids were hydrolysed and the products separated by the procedure of Dawson Hemington & Davenport (1962).

(b) Triphosphoinositide. The residue remaining after chloroform-methanol extraction was extracted twice for 20 min. at 37° with 0.25 vol. (relative to the volume of the original extract) of chloroform-methanol (2:1, v/v) acidified with 0.25% (v/v) of conc. HCl. The successive extracts were centrifuged and the supernatants filtered through sintered glass. The combined filtrates were shaken with N-HCl, which is known to release any combined triphosphoinositide into the lower phase (J. Eichberg & R. M. C. Dawson, unpublished work). The lower phase was washed twice with 'theoretical upper phase' (chloroform-methanol-N-HCl, 3:48:47, by vol.). The washed lower phase was evaporated to dryness *in vacuo*. After the addition of a small amount of marker triphosphoinositide, the residue was hydrolysed for 10 min. with 5 N-HCl at 100° and the hydrolysis products were separated by paper ionophoresis and chromatography (Dawson & Dittmer, 1961).

Measurement of radioactivity. The paper chromatograms were sprayed to detect P-containing compounds (Hanes & Isherwood, 1949). The developed paper chromatograms were radioautographed with Gevaert (Belgium) X-ray film for 18-120 hr. to locate radioactive areas. All P-containing spots were cut out, digested with perchloric acid and assayed for ³¹P and ³²P (Dawson, 1954, 1955).

RESULTS

Distribution of the ATPase in centrifuged fractions of electric organ. In a previous paper on electric-

organ ATPase in *Electrophorus electricus* (Glynn, 1963), attention was restricted to the fraction of homogenate that sedimented after prolonged centrifuging at high speed (35 000 g for 180 min.). As shown in Table 1, activity is not limited to that fraction, but occurs to a similar extent in the 'low-speed' and 'intermediate-speed' fractions. With *Torpedo* a different result has been obtained; nearly all the activity came down at low speeds. The reason for the difference in behaviour between *Electrophorus* and *Torpedo* is not known, but it probably has to do with the way the electroplates break up on homogenization and does not reflect a different distribution of the enzyme in the cells. M. N. Sheridan & V. P. Whittaker (unpublished work) have shown that, when *Torpedo* electric organ is homogenized in an iso-osmotic solution, centrifuging at low speed brings down particles that consist almost entirely of broken-up pieces of the non-innervated faces of the electroplates. It is likely that our 'low-speed' fraction from *Torpedo* had a similar composition, and for this reason we have preferred to use non-committal names for the different fractions rather than the usual terms 'nuclear', 'mitochondrial' and 'microsomal' fractions. In any case the metabolism of electric organs is largely anaerobic (Nachmansohn, Coates, Rothenberg & Brown, 1946; Chance, Lee & Oshino, 1964; Aubert, Chance & Keynes, 1964) and they are very poor in mitochondria (Sheridan, 1964).

Table 1. *Distribution of ouabain-sensitive ATPase in centrifuged fractions of the electric organs of Torpedo and Electrophorus*

The incubation medium contained: NaCl, 100 mM; KCl, 20 mM; MgCl₂, 3.2 mM; ATP (sodium salt), 3 mM; EDTA (tris salt), 0.13 mM; sucrose, 16 mM; ethanol, 0.5%; ouabain, 0 or 0.1 mg./ml.; tris, 30 mM (adjusted to pH 7.8 with HCl). Incubation was at 25° for 20 min.

Fraction	Total activity (μmoles of phosphate liberated/g. fresh wt. of tissue/20 min.)	Activity in the presence of ouabain (μmoles of phosphate liberated/g. fresh wt. of tissue/20 min.)	Inhibition by ouabain (%)	Proportion of total ouabain-sensitive activity in fraction (%)
<i>Torpedo</i> (1)*				
'Low-speed' (3000 g for 15 min.)	49.0	5.7	88.5	66.8
'Intermediate-speed' (10 000 g for 20 min.)	20.8	7.6	63.5	20.4
'High-speed' (35 000 g for 120 min.)	18.7	10.4	44.5	12.8
Supernatant	0	0	—	0
<i>Electrophorus</i> (7)*				
'Low-speed' (3000 g for 20 min.)	25.6	2.7	89.5	35.4
'Intermediate-speed' (10 000 g for 20 min.)	22.0	1.3	94.0	31.9
'High-speed' (35 000 g for 120 min.)	21.1	2.0	90.5	29.5
Supernatant	2.1	0	100.0	3.2

* The numbers given in parentheses in this and subsequent Tables refer to the code numbers used for individual fish.

As we were interested in the glycoside-sensitive portion of the ATPase, in subsequent experiments on *Torpedo* we restricted our attention to the 'low-speed' fraction, where the ratio of ouabain-sensitive to ouabain-insensitive activity was most favourable. In the experiment on *Electrophorus* this ratio was much the same in the different centrifuged fractions. The 'intermediate-speed' fraction had a slightly higher activity/weight of protein (20.4 μ moles of phosphate/mg. of protein/20 min. compared with 10.4 μ moles of phosphate/mg. of protein/20 min. for the 'low-speed' fraction and 12.9 μ moles of phos-

phate/mg. of protein/20 min. for the 'high-speed' fraction).

Effect of urea on the ouabain-sensitivity of the ATPase preparations. In experiments on the ATPase activity of a microsomal preparation from guinea-pig kidney, Kinsolving, Post & Beaver (1963) showed that the proportion of activity inhibited by ouabain could be increased by allowing the preparation to age, or by treating it with mixtures of urea and ammonium sulphate. In preliminary experiments on the *Torpedo* electric-organ ATPase, aging had somewhat unpredictable effects and ammonium sulphate was ineffective, but urea led to a marked increase in the proportion of activity inhibited by ouabain. The results of a more systematic examination of the effects of urea are shown in Tables 2 and 3. After exposure of the preparation for 48 hr. to 2M-urea the activity could be almost completely inhibited by ouabain, but the absolute amount of activity was greatly decreased (Table 2). Urea concentrations between 0.6 and 1.4M gave almost complete loss of ouabain-insensitive activity with little change in total activity. Increasing the duration of exposure to M-urea to 8 days did not affect the result (Table 3), but in an experiment in which the preparation was exposed for 19 days activity declined by about 60%; it remained almost entirely sensitive to ouabain.

The effects of urea on the *Electrophorus* ATPase have not been examined systematically, but are similar to those found with the *Torpedo* preparation.

Dependence of the ouabain-sensitive ATPase on Na⁺ and K⁺. In experiments with a broken-cell preparation it was, of course, impossible to demonstrate that different ions were required at the inner and outer surfaces of the cell membrane. However, Table 4 shows that activity of the glycoside-sensitive ATPase did require the simultaneous

Table 2. *Effect of different concentrations of urea on the ouabain-sensitivity of Torpedo electric-organ ATPase*

The enzyme was suspended in a solution containing sucrose (250 mM), EDTA (tris salt) (2 mM) and tris (30 mM) adjusted to pH 7.8 with HCl, and urea was added to give the final concentrations shown. After 48 hr. at 4°, the activity of the preparation was tested under conditions similar to those described in Table 1, except that urea was present at a concentration 30-fold less than that shown in the Table.

Concn. of urea (M)	Total activity (μ moles of phosphate liberated/g. fresh wt. of tissue/20 min.)	Inhibition by ouabain (0.1 mg./ml.) (%)
0	47.5	93.0
0.6	50.8	97.5
0.8	53.6	97.6
1.0	51.2	98.4
1.2	50.4	99.0
1.4	49.5	100
1.6	43.5	100
2.0	3.7	100
4.0	0	—

Table 3. *Stability of the Torpedo electric-organ ATPase in M-urea*

The procedure was the same as that described in Table 2, except that the duration of exposure to urea was varied.

Duration of exposure (days)	Total activity (μ moles of phosphate liberated/g. fresh wt. of tissue/20 min.)		Inhibition by ouabain (%)	
	<div>ControlUrea present</div>		<div>ControlUrea present</div>	
	Control	Urea present	Control	Urea present
<i>Torpedo</i> (1)				
0	48.8	—	88.5	—
2	47.5	51.2	93.0	98.4
5	50.4	52.8	92.7	98.1
8	46.8	49.6	91.2	96.0
<i>Torpedo</i> (8 and 9)				
4	—	27.4	—	95.4
19	—	10.8	—	99.0

Table 4. *Dependence of the ouabain-sensitive ATPase activity on Na⁺ and K⁺*

The enzyme activity was tested under conditions similar to those described in Table 1. The activity is less than that shown in Table 1 because much material was lost during the two washes to remove urea. In later experiments this loss was avoided by centrifuging at higher speeds.

Preparation	Activity (μ moles of phosphate liberated/g. fresh wt. of tissue/20 min.)			
	Complete medium	Complete medium + ouabain	Na ⁺ absent	K ⁺ absent
<i>Torpedo</i> (2 and 3) (‘low-speed’ fraction)	31.4	0.9	0.6	0.7
<i>Torpedo</i> (4 and 5) (‘low-speed’ fraction)	21.4	0.3	0.2	0.4
<i>Torpedo</i> (8 and 9) (‘low-speed’ fraction)	27.4	1.3	0.4	1.5
<i>Electrophorus</i> (7) (‘intermediate-speed’ fraction)	14.8	0.0	0.1	0.0
<i>Electrophorus</i> (8) (‘intermediate-speed’ fraction)	18.0	0.7	0.1	0.4

presence of Na⁺ and K⁺ in the suspending medium. It follows that nearly all the ATPase activity of these preparations possessed the characteristic features of ‘transport ATPase’.

Phospholipid composition of the electric organs. In both *Electrophorus* and *Torpedo* electric organs the predominant phospholipids were lecithin, phosphatidylethanolamine and phosphatidylserine, and there were small quantities of phosphatidic acid, phosphatidylinositol, triphosphoinositide and cardiolipin. Phosphatidylinositol was not detected in the ‘high-speed’ fraction of *Electrophorus*. Phosphatidylserine accounted for about 19% of the alkali-labile phospholipid in the *Electrophorus* electric organ, and 27–35% in the ‘low-speed’ fraction of *Torpedo*, which is one of the richest sources of phosphatidylserine yet reported.

Exposure of the ATPase preparations to [γ -³²P]-ATP. Table 5 summarizes the results of several experiments in which electric-organ ATPase preparations were incubated for 30 sec. with [γ -³²P]ATP. The exposure was carried out under four different conditions: (1) with both Na⁺ and K⁺ present; (2) with Na⁺, K⁺ and sufficient ouabain to inhibit the ouabain-sensitive activity completely; (3) without Na⁺; (4) without K⁺. Only the first set of conditions allowed hydrolysis of ATP by the glycoside-sensitive ATPase, and the concentration of enzyme was chosen so that between 10 and 20% of the ATP would be split during the exposure. The incorporation of ³²P into individual phospholipids is expressed in two ways: (1) The actual amount of phosphorus incorporated into the lipid from the terminal phosphate of ATP. This is calculated from the total activity of the phospholipid and the specific activity of the ATP. It would provide a

direct measure of the new lipid formed from the ATP if there were no breakdown of the newly synthesized lipid during the 30 sec. incubation period. (2) The ratio of the specific activity of the individual phospholipid to the specific activity of the terminal phosphate of the ATP.

Most of the phospholipids studied showed virtually no incorporation of ³²P. A small amount of radioactivity was found in phosphatidic acid in the *Electrophorus* preparation, but the amount was not significantly affected by the presence of ouabain or the absence of Na⁺ or K⁺. In the *Torpedo* preparations no labelling at all was observed in the phosphatidic acid; there was sufficient phosphatidic acid in the preparations to give a well-defined spot on the chromatogram. The inositol triphosphate spot arising from triphosphoinositide was also unlabelled. The inositol diphosphate spot on the same chromatograms was slightly radioactive, but the immediate proximity of a heavily labelled inorganic phosphate spot suggests that the radioactivity should be attributed to incomplete separation rather than to genuine incorporation into diphosphoinositide. In one experiment with *Torpedo* there was slight labelling of the cardiolipin, but no labelling was observed in a second preparation. There is no obvious explanation of this discrepancy; the radioautograph in the first experiment showed a well-defined radioactive polyglycerol phosphate spot that was completely separated from a much less radioactive inorganic phosphate spot.

Although the phosphatidic acid in the *Torpedo* preparation did not become labelled in any experiment, in the absence of Na⁺ its concentration in the tissue was observed to increase eightfold. It is possible that this increase may be related to the

Table 5. *Lack of incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phospholipids of electric-organ ATPase preparations*

The composition of the incubation medium in the *Torpedo* experiments was: NaCl, 0 or 100 mM; MgCl_2 , 2 mM; ATP (tris salt), 2.7 mM; EDTA (tris salt), 0.24 mM; sucrose, 30 mM; ethanol, 0.5%; ouabain, 0 or 0.1 mg./ml.; tris, 30 mM (adjusted to pH 7.8 with HCl). Incubation was at 25° for 30 sec. The activities of the enzyme preparations under the different conditions are shown in Table 4. The composition of the incubation medium in the *Electrophorus* experiment was: NaCl, 0 or 64 mM; KCl, 0 or 16 mM; MgCl_2 , 3 mM; ATP (tris salt), 3 mM; EDTA (tris salt), 0.5 mM; sucrose, 65 mM; ethanol, 0.5%; ouabain, 0 or 0.1 mg./ml.; tris, 30 mM (adjusted to pH 7.8 with HCl). Incubation was at 37° for 30 sec. This experiment was carried out before the effect of urea on the enzyme activity was known, and the preparation had not been treated with urea. Preliminary testing of the preparation showed that the ATPase activity was decreased by 81% when K^+ was omitted from the medium.

Preparation	Wt. of original tissue (g.)	Estimated inorganic P formed in complete medium ($\mu\text{g.}$)	Phospholipid	Incorporation of ^{32}P							
				Complete medium + ouabain				Na ⁺ absent			
				Complete medium		P incorporated ($\mu\text{g.}$)		Sp. activity relative to ATP (%)		P incorporated ($\mu\text{g.}$)	
				P incorporated ($\mu\text{g.}$)	Sp. activity relative to ATP (%)	P incorporated ($\mu\text{g.}$)	Sp. activity relative to ATP (%)	P incorporated ($\mu\text{g.}$)	Sp. activity relative to ATP (%)	P incorporated ($\mu\text{g.}$)	Sp. activity relative to ATP (%)
<i>Torpedo</i> (2 and 3) ('low-speed' fraction)	18.7	455	Triphosphoinositide	0.04	—	0.00	—	0.05	—	0.16	—
<i>Torpedo</i> (4 and 5) ('low-speed' fraction)	20.6	341	Lecithin	0.04	0.00	0.08	0.02	—	—	—	—
			Phosphatidylethanolamine	0.20	0.01	0.00	0.00	—	—	—	—
			Phosphatidylserine	0.01	0.00	0.40	0.01	—	—	—	—
			Phosphatidylinositol	0.30	0.01	0.25	0.02	—	—	—	—
			Phosphatidic acid	0.08	0.00	0.08	0.01	—	—	—	—
			Cardiolipin	2.10	0.69	4.26	1.24	—	—	—	—
<i>Torpedo</i> (8 and 9) ('low-speed' fraction)	17.0	380	Triphosphoinositide	0.01	—	0.03	—	0.09	—	0.02	—
			Lecithin	<0.14	0.00	<0.07	0.00	<0.11	0.00	<0.04	0.00
			Phosphatidylethanolamine	<0.07	0.00	<0.07	0.00	<0.07	0.00	<0.07	0.00
			Phosphatidylserine	<0.07	0.00	<0.11	0.00	<0.11	0.00	<0.07	0.00
			Phosphatidylinositol	<0.04	0.00	<0.04	0.00	<0.04	0.00	<0.04	0.00
			Phosphatidic acid	<0.04	0.00	<0.04	0.00	<0.04	0.00	<0.04	0.00
			Cardiolipin	<0.04	<0.01	<0.04	0.00	<0.04	0.00	<0.04	0.01
			Alkali-stable lipids	0.00	—	0.00	—	0.00	—	0.01	—
<i>Electrophorus</i> (3) ('high-speed' fraction)	9.8	230	Lecithin	0.02	0.00	*	—	*	—	*	—
			Phosphatidylethanolamine	0.02	0.00	*	—	*	—	*	—
			Phosphatidylserine	0.02	0.00	*	—	*	—	*	—
			Phosphatidic acid	1.36	0.11	1.3	0.13	1.52	0.15	1.0	<0.13
			Cardiolipin	0.04	0.01	0.01	0.01	0.06	0.04	0.04	0.00
			Alkali-stable lipids	0.20	—	0.18	—	0.20	—	0.17	—

* None detected by radioautography.

Na⁺ stimulation of phosphatidic acid phosphatase observed in erythrocyte 'ghosts' by Hokin, Hokin & Mathison (1963).

DISCUSSION

None of the phospholipids in the ATPase preparations showed a significant incorporation of ³²P during the short incubation period under any of the experimental conditions. Before concluding that no phospholipid serves as a phosphorylated intermediate in the 'transport ATPase' system, it is necessary to consider two questions. Were the enzyme preparations exposed to [γ -³²P]ATP long enough for the hypothetical intermediate to become labelled? And was the estimated total quantity of intermediate sufficient for the labelling to be detectable?

The ATPase preparations used in the experiments had sufficient activity to hydrolyse 10–20% of the ATP during the 30sec. incubation period. The time necessary for the labelling of an intermediate must therefore be much shorter than 30sec. There is evidence that in an erythrocyte the number of sites on the membrane at which active transport of Na⁺ and K⁺ occurs is not more than about 1000 (Glynn, 1957). If the splitting of each molecule of ATP by the 'transport ATPase' is associated with the extrusion of 3Na⁺ ions (Glynn, 1962; Sen & Post, 1964), a minimum value for the rate of turnover at each site can be calculated from the known efflux of Na⁺, and is of the order of 1000/min. The same value can be calculated from the rate of hydrolysis of ATP by the glycoside-sensitive ATPase of erythrocyte 'ghosts' (Dunham & Glynn, 1961), without making any assumptions about the ratio of Na⁺ transported to ATP hydrolysed. These considerations leave little doubt that the preparations were exposed to the radioactive ATP long enough for the hypothetical intermediate to become labelled.

If the turnover rate at the transport sites in electric organ were fast enough, the number of sites could be so small that, even if all become labelled, the amount of radioactivity incorporated would not be detectable. In this case experiments like those that have been performed could not exclude the possibility that phospholipids take part as intermediates, but would provide an upper limit for the amount of phospholipid that might be involved. For phosphatidic acid, for example, this amount would be 0.1% of the total quantity present in the 'low-speed' fraction. The required turnover rate, however, would be enormously greater than the estimated turnover rate at the transport sites in erythrocytes. If the turnover rate in electric organ is the same as in erythrocytes, and there are, say, 1000 transport sites on the

erythrocyte membrane, the number of sites in electric organ can be calculated from the relative ATPase activities of the two tissues. The calculation suggests that the 'low-speed' fraction from 1g. of *Torpedo* electric organ contains about 1.5×10^{15} sites. It follows that if all the sites were labelled with phosphate containing 10mc of ³²P/m-mole, the 'low-speed' fraction from 20g. of electric organ should contain about 0.5 μ C of incorporated radioactivity or 1.55 μ g. of incorporated P. The greatest amount of radioactivity actually found in any phospholipid from the *Torpedo* preparations was 10⁶-fold less than this. In the experiment with *Electrophorus* electric organ, there was about half as much ATPase preparation in each flask, but the specific activity of the [γ -³²P]ATP was eightfold higher; the sensitivity of the method was therefore about four times as great. These calculations show that the total quantity of intermediate was probably sufficient for the labelling to be detectable. Since virtually no labelling was detected, it is unlikely that any phospholipid serves as a phosphorylated intermediate in the 'transport ATPase' system.

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