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ADENOSINE TRIPHOSPHATE-INDUCED CONTRACTION OF RAT-LIVER MITOCHONDRIA AND SYNTHESIS OF MITOCHONDRIAL PHOSPHOLIPIDS

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SUMMARY

The incorporation of α-glycero[^{32}P]phosphate into mitochondrial phospholipids was investigated during swelling and contraction of rat-liver mitochondria.

The extent of the incorporation increased considerably during ATP-induced contraction of swollen mitochondria. The chief labelled phospholipid was tentatively identified as phosphatidic acid.

Evidence is presented supporting the view that the increased incorporation of α-glycerophosphate into the mitochondrial phospholipids is in some way related to mitochondrial contraction. This is based on the following results:

1. The time course of α-glycerophosphate incorporation paralled the time course of mitochondrial contraction.

2. There was no increased incorporation, if mitochondria were protected against swelling by serum albumin and if, consequently, no contraction occurred upon the addition of ATP.

3. Inhibitors of mitochondrial contraction usually decreased the extent of α-glycerophosphate incorporation.

4. The incorporation was significantly depressed, when α-glycerophosphate was added to the contracted mitochondria.

The possible role of phospholipids in mitochondrial contraction is discussed.

INTRODUCTION

Mitochondria isolated from a variety of mammalian tissues undergo morphological changes, known as swelling, when placed in isotonic saline or sucrose media. This process, which consists of penetration of water into the mitochondria, can be accelerated by low concentrations of thyroxine, long chain fatty acids, calcium ions, phlorizin, inorganic phosphate and a number of other substances. LEHNINGER AND REMMERT[1] isolated from sonically disrupted liver mitochondria a substance which was a potent swelling-inducing factor. This substance was identified as a mixture of non-esterified long chain fatty acids[2]. It was found[2] that spontaneous swelling of mitochondria, as well as swelling induced by Ca^{2+} and thyroxine, are accompanied

Abbreviations: GP, α-glycerophosphate; PCMB, *p*-chloromercuribenzoate.

by an increase in the content of non-esterified fatty acids, presumably resulting from hydrolytic splitting of mitochondrial lipids.

Swelling of mitochondria can be reversed by the addition of ATP[3,4]; the presence of Mg^{2+} or Mn^{2+} is usually necessary for the maximum effect. This process, which consists of extrusion of water from mitochondria, is known as shrinkage or "contraction". It was reported previously[2], that a considerable part of the non-esterified fatty acids which either accumulated during the swelling of mitochondria or were added to the medium, disappeared during the contraction. A part of the added [^{14}C]-oleic acid was found to be incorporated into mitochondrial phospholipids. Two striking features of this process were observed: (a) the incorporation of [^{14}C]oleic acid into phospholipids was inhibited by sucrose which is known to inhibit the ATP-induced contraction of mitochondria[5]; (b) both the incorporation and the contraction had a similar time course, being highest during the first few minutes following the addition of ATP. This led us to assume that the synthesis of phospholipids might be in some way related to the contraction of the mitochondria.

In the present study the biosynthesis of phospholipids during the swelling and contraction of mitochondria has been further investigated by the use of [^{32}P]GP.

MATERIAL AND METHODS

Rat-liver mitochondria were isolated as described by LEHNINGER *et al.*[3]. Mitochondria derived from one liver (about 5 g wet wt.) were suspended in 3 ml of 0.25 M sucrose. 1 ml of the suspension contained about 40 mg protein.

Swelling and contraction of mitochondria were followed optically[3] at 520 mμ in 18 × 150-mm test tubes, using a Coleman "Junior" Spectrophotometer.

Experiments with [^{14}C]oleic acid, including the extraction and fractionation of the lipids, were carried out as described previously[2].

In experiments with [^{32}P]GP the basic medium was 0.125 M KCl–0.02 M Tris–HCl buffer (pH 7.4) and the swelling agent was usually sodium oleate or sometimes inorganic phosphate (pH 7.4). 0.2 ml of mitochondrial suspension was added per 10 ml of the medium and the incubation was carried out at 20°. After 20 min, 0.1 ml of 0.25 M ATP was added and the incubation was continued for another 15 min. Spectrophotometer readings were taken every 5 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 8 % and the tubes were centrifuged. The precipitate was washed several times with 5 % trichloroacetic acid and then with water.

To the washed precipitate 2 ml of methanol followed by 4 ml of chloroform were added and the samples were left overnight at room temperature. The extract was filtered and the residue was extracted 4 times with chloroform – methanol (2:1, v/v) at 60°. The extracts were combined and the solvent was evaporated *in vacuo* almost to dryness. To the resulting residue a few drops of water were added and the lipids were re-extracted with petroleum ether – chloroform (2:1, v/v). The extract was dehydrated over Na_2SO_4, filtered and made up to a known volume.

Radioactivity of ^{32}P was measured in suitable aliquots of the lipid extract by use of a Geiger–Müller counter with a thin mica end-window and the radioactivity of ^{14}C with a "Micromil" window counter (Nuclear Chicago Co.).

Column chromatography of phospholipids was carried out on silicic acid (Mal-

linckrodt, 100 mesh). Non-phospholipids were removed by chloroform, and phospholipids were fractionated by elution with increasing concentrations of methanol in chloroform.

Paper chromatography of phospholipids was carried out on commercial silicic acid-impregnated paper (Schleicher and Schüll) using the diisobutyl ketone – acetic acid – water (40:30:7, v/v) system of MARINETTI AND STOTZ[6] and the diisobutyl ketone – formic acid – water (40:15:2, v/v) system of THIELE AND WOBER[7]. The chromatograms were analyzed in "Actigraph II" chromatographic strip scanner with a "Micromil" window (Nuclear Chicago Co.) and autoradiography was carried out on usual X-ray photographic plates. Phospholipids were detected by staining with Rhodamine 6G and viewed under an ultraviolet lamp while wet[8].

Mild alkaline hydrolysis of phospholipids was performed according to DAWSON[9]. Products of the hydrolysis were chromatographed on Whatman No. 1 filter paper using the following solvent systems: A, ethanol – acetic acid – water[10] (83:1:16, v/v); B, acetic acid – ethyl acetate – water[11] (3:3:1, v/v); C, phenol saturated with 0.1 % NH_3 in water[9]; D, Cellosolve – methyl ethyl ketone – 3 N NH_3 (7:2:3, v/v) saturated with boric acid[12]; E, butanol – 10 % trichloroacetic acid[9] (62:38, v/v). Descending chromatography was used for phenol–NH_3 and ascending chromatography for all other solvent systems. The paper was washed before use with 1 % EDTA for solvents A and B and with 2 N acetic acid for the other systems. The spots were located by spraying with the acid molybdate reagent of HANES AND ISHERWOOD[13] and irradiation with ultraviolet light[14].

Reagents of analytical grade, and distilled and deionized water were used throughout. The purity of ATP appeared to be of special importance. Among several samples of ATP tested the highest contraction of mitochondria was achieved by using ATP (crystalline disodium salt) from Pabst Laboratories, Milwaukee, Wisc., and only this preparation was used in the present investigation.

[^{32}P]GP was synthesized as described by KENNEDY[15]. It contained 90 % of DL-α-glycerophosphate and only 10 % as the β-form.

Carrier-free [^{32}P]phosphoric acid was obtained from the Institute of Nuclear Research in Warsaw and [1-^{14}C]oleic acid from California Corporation for Biochemical Research, Los Angeles, Calif.

DL-α-Lecithin (synthetic), phosphatidylethanolamine (from brain) and phosphatidyl-L-serine used as reference substances in the chromatography of phospholipids were obtained from Nutritional Biochemicals Co., Cleveland, Ohio.

RESULTS

In the first series of experiments mitochondria were incubated in the presence of [1-^{14}C]oleic acid as the swelling agent and of either α-glycerophosphate, phosphocholine, phosphoethanolamine or phosphoserine. After 20 min of incubation, ATP and $MgCl_2$ were added and the incubation was continued for another 15 min. The reaction was stopped and the incorporation of ^{14}C into mitochondrial phospholipids was measured as described previously[2]. It was found (Table I, Expt. 1) that the incorporation was strongly stimulated by GP but not by the other phosphoric esters tested. However, the presence of GP plus either phosphocholine or phosphoethanolamine resulted in a further increase of the incorporation (Table I, Expt. 2).

These results suggested that GP labelled in the phosphate moiety could be a useful tool in studying the biosynthesis of phospholipids in mitochondria during their swelling and contraction. This was confirmed by experiments in which [^{32}P]GP has been found to be incorporated into phospholipids at a rate greatly exceeding that of the incorporation of either $^{32}P_i$ or [^{32}P]ATP.

TABLE I

EFFECT OF VARIOUS PHOSPHORIC ESTERS ON THE INCORPORATION OF [1-^{14}C]OLEIC ACID INTO MITOCHONDRIAL PHOSPHOLIPIDS

Incubation mixture consisted of 35 ml of 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), 154 mμmoles sodium oleate containing 10^5 counts/min [1-^{14}C]oleic acid, and 0.25 ml of mitochondria suspension; other additions as indicated. The mixture was incubated 20 min at 20°, then ATP and $MgCl_2$ were added to the final concentrations of 0.005 M and 0.003 M respectively, and the incubation was continued for another 15 min.

	Additions	*Total radioactivity of the phospholipid fraction (counts/min)*
Expt. 1	None	8 000
	DL-α-Glycerophosphate (10 μmoles)	13 350
	Phosphocholine (10 μmoles)	6 500
	Phosphoethanolamine (10 μmoles)	8 000
	Phosphoserine (10 μmoles)	6 950
Expt. 2	None	4 050
	DL-α-Glycerophosphate (10 μmoles)	8 100
	DL-α-Glycerophosphate (10 μmoles) + phosphocholine (10 μmoles)	11 200
	DL-α-Glycerophosphate (10 μmoles) + phosphoethanolamine (10 μmoles)	11 600
	DL-α-Glycerophosphate (10 μmoles) + phosphoserine (10 μmoles)	5 300

Incorporation of [^{32}P]GP into mitochondrial phospholipids

The effect of ATP on the incorporation of glycerophosphate into mitochondrial phospholipids is shown in Table II, and the effect of CoA and oleate in Table III. It is evident that the addition of ATP to the swollen mitochondria greatly increased the incorporation (see also Fig. 1). A stimulatory effect of CoA and of oleate was also observed (Table III, Expt. 2). However, it should be noted that in some cases the addition of oleate did not affect the incorporation. This fact might be due to the presence of a somewhat larger amount of endogenous non-esterified fatty acids in some samples of mitochondria. Oleate at higher concentrations was even inhibitory (Table III, Expt. 1).

As can be seen in Table III, the mitochondrial phospholipids contained only a very small part of the [^{32}P]GP present in the medium. Therefore, it appeared necessary to use [^{32}P]GP of a high specific activity (10^7 counts/min/μmole) in subsequent experiments.

Fig. 1 shows the time course of the swelling and contraction of mitochondria as well as of [^{32}P]GP incorporation into mitochondrial phospholipids. It is evident that the swelling of mitochondria (as visualized by the fall in light absorbancy) was accompanied by only a small incorporation of label into the phospholipids. The addition of ATP induced mitochondria to contract (as shown by the increase in light ab-

TABLE II

EFFECT OF ATP ON THE INCORPORATION OF GLYCERO[^{32}P]PHOSPHATE INTO MITOCHONDRIAL PHOSPHOLIPIDS

Each test tube contained 10 ml of 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), 30 μmoles $MgCl_2$, about 0.1 μmole [^{32}P]GP (600000 counts/min), the swelling agent(s) as indicated and 0.2 ml of mitochondria suspension. After 20 min of incubation, 25 μmoles ATP were added and the reaction was stopped 15 min thereafter. Control samples were incubated 35 min with no ATP added.

Expt. No.	*Swelling agent(s)*	*Total radioactivity of phospholipids (counts/min)*		*Per cent increase*
		Control	*ATP added*	
1.	Oleate (0.1 μmole)	6 300	13 000	206
2.	Oleate (0.1 μmole)	8 700	15 150	174
3.	Oleate (0.1 μmole)	5 100	11 400	224
4.	Orthophosphate (10 μmoles) + oleate (0.01 μmole)	7 000	26 400	377
5.	Orthophosphate (10 μmoles) + oleate (0.01 μmole)	14 850	39 000	263
6.	Orthophosphate (10 μmoles) + oleate (0.01 μmole)	7 200	13 700	190

TABLE III

EFFECT OF CoA AND OLEATE ON THE INCORPORATION OF [^{32}P]GP INTO MITOCHONDRIAL PHOSPHOLIPIDS

Each test tube contained 5.0 ml of 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), 15 μmoles $MgCl_2$, 4 μmoles [^{32}P]GP (about 600000 counts/min) and 0.2 ml of the mitochondria suspension; other additions as indicated; 25 μmoles ATP were added after 20 min incubation which was continued for another 15 min.

	Additions	*Total radioactivity of phospholipids (counts/min)*
Expt. 1	None	8200
	Oleate (50 mμmoles)	8850
	Oleate (150 mμmoles)	7500
	Oleate (300 mμmoles)	2800
	Oleate (500 mμmoles)	1800
	Oleate (1000 mμmoles)	1060
Expt. 2	None	640
	Oleate (200 mμmoles)	2300
	Oleate (200 mμmoles) +CoA (500 mμmoles)	5500
Expt. 3	Oleate (200 mμmoles)	4600
	Oleate (200 mμmoles) + CoA (500 mμmoles)	6200

sorbancy in Fig. 1) and brought about a marked increase in the rate of [^{32}P]GP incorporation (see also Table II). However, this high rate of incorporation persisted a few minutes only, *i.e.* during the actual shrinking of the mitochondria. When the contraction was completed, the rate of incorporation fell to a low level comparable to that observed before the addition of ATP. This decrease in the rate of GP incorporation could not be accounted for by the exhaustion of ATP since only a small part of ATP added was found to undergo hydrolysis under these conditions. These observations led us to assume that the increased incorporation of [^{32}P]GP (which will be referred to as the "extra" incorporation) as well as the increased incorporation

of [^{14}C]oleic acid[2] into mitochondrial phospholipids might be in some way related to the process of mitochondrial contraction. The aim of further experiments was to check this hypothesis.

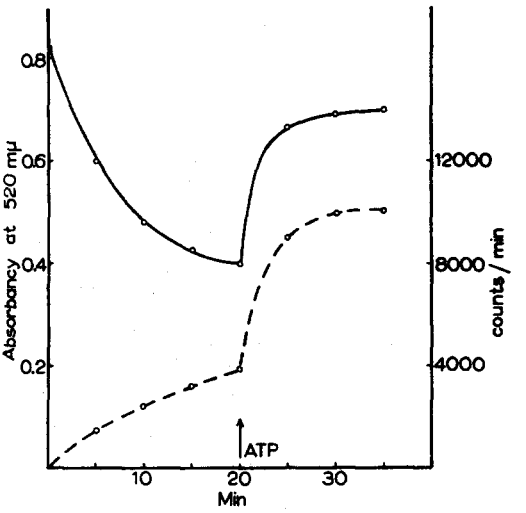

Fig. 1. Time course of swelling and contraction of rat-liver mitochondria (O—O) and of incorporation of α-glycero[^{32}P]phosphate into mitochondrial phospholipids (O- - -O). Incubation medium was 10 ml of 0.125 M KCl–0.02 M Tris–HCl (pH 7.4) containing 30 μmoles $MgCl_2$, 0.1 μmole sodium oleate and 0.1 μmole [^{32}P]GP (about 600000 counts/min). After 20 min of incubation, 25 μmoles ATP were added, as indicated by the arrow.

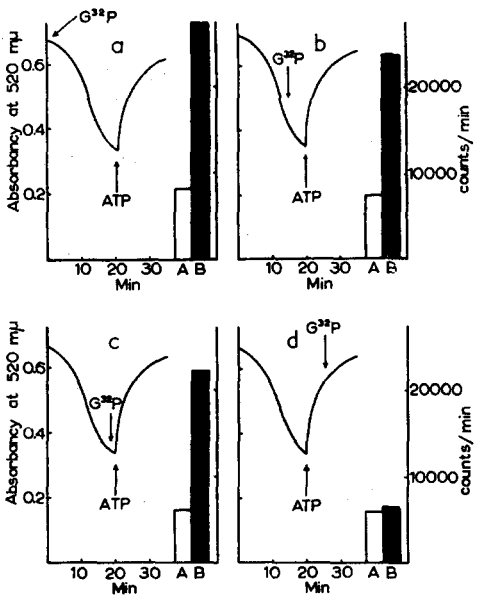

Fig. 2. Incorporation of α-glycero[^{32}P]phosphate into mitochondrial phospholipids. [^{32}P]GP was added at various times before and after the addition of ATP, as indicated by the arrows. The curves show the swelling and contraction of mitochondria. The columns represent the radioactivity of mitochondrial phospholipids: A, in the controls (ATP omitted); B, in the presence of ATP. Experimental conditions as in Fig. 1.

In the following experiments, [^{32}P]GP was added to the incubation mixture at various times before and after the addition of ATP. In the controls ATP was omitted. The results are shown in Fig. 2. It is evident that in all cases when [^{32}P]GP was added before ATP, there was a highly significant "extra" incorporation. This was observed even if the addition of [^{32}P]GP preceded that of ATP by as little as 30 sec. On the other hand, the incorporation of [^{32}P]GP into the phospholipids did not appreciably exceed that in the control when [^{32}P]GP was added 5 min after ATP, *i.e.* to the contracted mitochondria. In this case the presence of ATP had no effect on the incorporation.

Contraction of mitochondria induced by ATP has been reported to be inhibited by sucrose[5], glucose and other saccharides[16], by azide[5], by some ions[17], and by glutathione[4].

These substances were tested for their ability to affect the extent of [^{32}P]GP incorporation into mitochondrial phospholipids. Table IV shows that the "extra" incorporation of [^{32}P]GP was completely inhibited by sucrose and glucose, but not by glycerol and ethanol, which did not inhibit the contraction.

From Table V it can be seen that the addition of KCNS, PCMB or glutathione brought about a remarkable decrease in the "extra" incorporation. However, sodium azide which is also a potent inhibitor of the contraction was without any effect on the incorporation.

It is also evident from Table V that damage to the mitochondrial structure caused

TABLE IV

EFFECT OF SOME HYDROXYLIC COMPOUNDS ON THE INCORPORATION OF [^{32}P]GP INTO MITOCHONDRIAL PHOSPHOLIPIDS

Experimental conditions as in Fig. 1; hydroxylic compounds added as indicated.

Hydroxylic compound added (final concentration)	*Contraction*	*"Extra" incorporation (relative values)*
None	+	100
Sucrose (0.25 M)	—	0
Glucose (0.20 M)	—	0
Glycerol (0.20 M)	+	61
Ethanol (0.25 M)	+	114

TABLE V

EFFECT OF INHIBITORS OF MITOCHONDRIAL CONTRACTION AND OF DAMAGE TO MITOCHONDRIAL STRUCTURE ON THE INCORPORATION OF [^{32}P]GP INTO PHOSPHOLIPIDS

Experimental conditions as in Fig. 1.

Additions (final concentrations)	*Contraction*	*"Extra" incorporation (relative values)*
None	+	100
PCMB (1 mM)	—	0
Glutathione (15 mM)	—	20
KCNS (0.125 M) substituted for KCl in the medium	—	22
NaN_3 (2 mM)	—	85
None, mitochondria frozen and thawed	—	15

by repeated freezing and thawing markedly decreased the incorporation. Such mitochondria showed a very slight if any swelling and contraction as measured optically.

It has been shown previously[2], that serum albumin, which is known to bind free fatty acids, strongly inhibits swelling of mitochondria caused by oleate, thyroxine and several other agents, but it does not interfere with phosphate-induced swelling. It seemed interesting therefore to investigate the effect of serum albumin on the incorporation of [^{32}P]GP into the phospholipids. The results of these experiments are shown in Fig. 3.

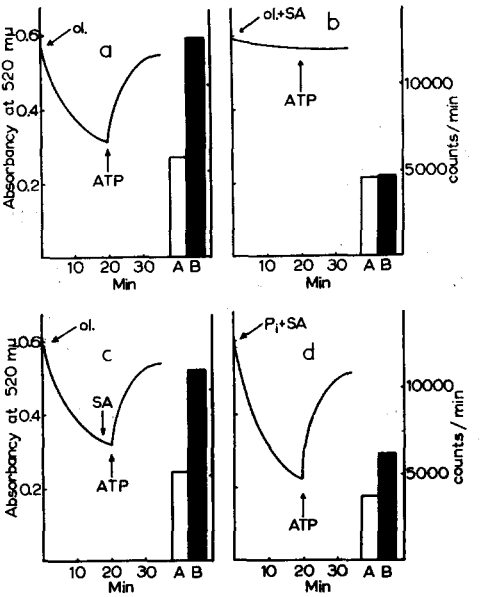

Fig. 3. Effect of serum albumin on mitochondrial swelling and on the incorporation of [^{32}P]GP into phospholipids. The curves represent swelling and contraction, and the columns the radioactivity of mitochondrial phospholipids. Experimental conditions for Figs. 3a, 3b and 3c are the same as for Fig. 1; Fig. 3d, 10 μmoles of inorganic phosphate were used as swelling agent instead of oleate. Bovine serum albumin (20 mg) and ATP (25 μmoles) added as indicated. SA = serum albumin; ol. = oleate.

When 20 mg of bovine-serum albumin were added to the oleate-containing incubation mixture prior to the addition of mitochondria, no swelling and consequently no contraction upon the addition of ATP were observed. At the same time, no increase in the incorporation of [^{32}P]GP into the phospholipids was brought about by ATP (Fig. 3b). However, when the same amount of serum albumin was added to mitochondria already swollen in the presence of oleate, just before or together with ATP, contraction of the mitochondria did occur and an appreciable "extra" labelling of mitochondrial phospholipids was observed (Fig. 3c).

In another set of experiments serum albumin was added to the incubation medium which contained inorganic phosphate as the only swelling agent. In this case swelling did occur and it was reversed by the addition of ATP. A significant "extra" incorporation of [^{32}P]GP was evident (Fig. 3d). However, this incorporation was less pronounced than in the presence of oleate.

These results may be interpreted in the following way: When mitochondria were

protected against swelling by serum albumin and consequently ATP did not cause any contraction, the incorporation of [^{32}P]GP was equal to that observed in the absence of ATP. Hence the addition of ATP, if not followed by contraction of mitochondria, did not bring about any "extra" incorporation of [^{32}P]GP into the phospholipids. On the other hand, serum albumin did not affect the extent of incorporation if added to the swollen mitochondria, *i.e.* when ATP produced a normal contraction. The less pronounced "extra" incorporation found in mitochondria which underwent phosphate-induced swelling might be due to lack of fatty acids necessary for the synthesis of phospholipids.

In view of the observation that the biosynthesis of phospholipids was stimulated by the contraction of mitochondria, it seemed interesting to investigate whether substances known to be involved in phospholipid synthesis would affect mitochondrial contraction, *e.g.* GP and oleic acid. We were not able to demonstrate any effect of GP on mitochondrial contraction; we found, however, that small amounts of oleic acid did, in fact, increase the initial rate of the contraction. In these experiments swelling was produced by 2 mM P_i which does not cause any endogenous release of fatty acids[2]. After 20 min of incubation, ATP was added either alone or together with a minute quantity of sodium oleate. It was observed that the initial rate, but usually not the final extent, of the contraction was substantially increased by oleate (Fig. 4). A similar effect was observed when oleate was added at the beginning of the incubation.

Another experiment is illustrated in Fig. 5. Here, ATP was added at the beginning of the incubation. In this case oleate did not produce any swelling and even a slight contraction was sometimes observed (Curve A), while in the presence of P_i the swelling proceeded for 5–10 min and was followed by a spontaneous contraction (Curve B).

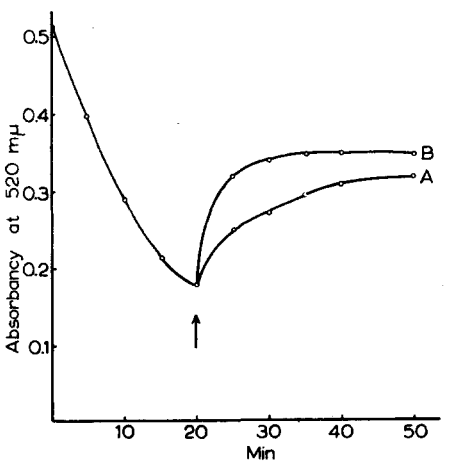

Fig. 4. Effect of oleate on ATP-induced contraction of mitochondria. The medium was 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), containing 0.002 M inorganic phosphate. At time shown by the arrow, the following additions were made: A, 0.002 M ATP and 0.0012 M $MgCl_2$; B, 0.002 M ATP, 0.0012 M $MgCl_2$, $6.7 \cdot 10^{-7}$ M sodium oleate (concentrations given are those in the complete medium).

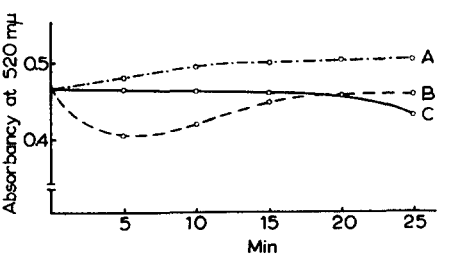

Fig. 5. Effect of oleate and inorganic phosphate on mitochondria suspended in ATP-containing medium. Incubation medium was 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), containing 0.0012 M $MgCl_2$ and 0.002 M ATP. Swelling agents: A, $6.7 \cdot 10^{-7}$ M sodium oleate; B, 0.002 M P_i; C, 0.002 M P_i and $6.7 \cdot 10^{-7}$ M sodium oleate.

There was no swelling, however, if both P_i and oleate were present in the medium (Curve C).

This observation may be interpreted as follows: ATP prevents swelling only if a small quantity of free fatty acid is already present. The spontaneous contraction of mitochondria swollen in the presence of P_i might be due to a release of endogenous fatty acids during the ageing of mitochondria.

It might be expected that PP_i would interfere with biosynthesis of phospholipids by shifting to the left the equilibrium of acyl-CoA formation according to the equation:

$$\text{fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acyl-CoA} + \text{AMP} + PP_i.$$

Thus 0.01 M of sodium pyrophosphate was added to the otherwise unchanged incubation medium. It is evident from Fig. 6 that a significant decrease in the rate of contraction did, in fact, occur (1 mM NaF which was present in the medium in order to inhibit mitochondrial pyrophosphatase was found to have no effect on the contraction).

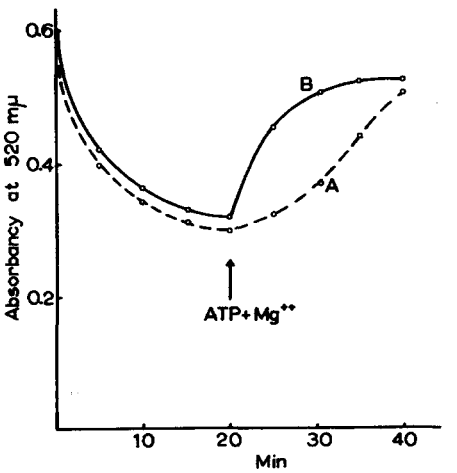

Fig. 6. Effect of PP_i on ATP-induced contraction of rat-liver mitochondria. Incubation mixture: A, 0.125 M KCl–0.02 M Tris–HCl (pH 7.4), containing $2 \cdot 10^{-6}$ M sodium oleate, 0.001 M NaF and 0.01 M sodium pyrophosphate; B, the same components minus pyrophosphate. At time shown, ATP and $MgCl_2$ were added to final concentrations of 0.005 M and 0.003 M respectively.

Identification of [^{32}P]phospholipids

Mitochondria derived from 5 g of rat liver were added to 100 ml of the usual KCl–Tris medium which contained 1 μmole sodium oleate, 300 μmoles $MgCl_2$ and 1 μmole [^{32}P]GP (about $2.2 \cdot 10^8$ counts/min). The swelling proceeded for 20 min at 20°, then 250 μmoles ATP were added to produce the contraction, and 15 min thereafter the reaction was stopped by the addition of 20 ml of 40 % trichloroacetic acid. Lipids were extracted as described under METHODS and analyzed by column and paper chromatography.

The extracted lipids dissolved in 5 ml of chloroform were loaded on a column of 1 g silicic acid. After passage of 10 ml chloroform, which removed the non-phospholipids, phospholipids were eluted with increasing concentrations of methanol in chloroform. The effluent was collected in 1-ml portions and the radioactivity was

measured in each portion after the evaporation of the solvent. Fig. 7 presents the distribution of radioactivity in the fractions.

A very sharp peak appeared almost immediately, eluted by 4% methanol in chloroform, and it contained practically all the radioactivity of the lipid applied to the column. No other radioactive peaks could be detected by increasing the concentration of methanol in chloroform. This pointed to phosphatidic acid and/or cardiolipin as the only labelled lipid(s) as only these phospholipids have been found to be eluted by 4% methanol in chloroform[18,19].

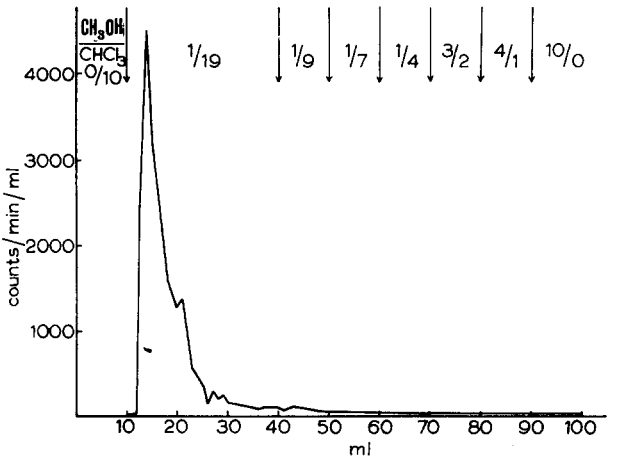

Fig. 7. Silicic acid chromatography of mitochondrial phospholipids. Distribution of radioactivity in the fractions.

In order to get more information on the nature of the radioactive phospholipid, paper chromatography was carried out and typical chromatograms and autoradiograms are shown in Fig. 8 and Fig. 9. Lecithin, phosphatidylethanolamine and phosphatidylserine were used as reference compounds. Each of these compounds gave more than one spot when revealed with Rhodamine 6G and viewed under an ultraviolet lamp.

In the solvent system of THIELE AND WOBER[7] (Fig. 8), the bulk of the radioactivity was found in a spot which did not correspond to either lecithin, phosphatidylethanolamine or phosphatidylserine. The R_F value of this spot provided no information about the nature of the labelled material. In the solvent system of MARINETTI AND STOTZ[6] (Fig. 9) the predominant radioactivity was found at R_F 0.75 which according to the above authors, corresponds to phosphatidic acid. The radioactive spot could not be visualized by Rhodamine 6G, which indicated that the amount of labelled material was too small to be revealed by the staining method used.

A portion of the lipids was subjected to mild alkaline hydrolysis. The aqueous phase was evaporated in vacuo and the products of hydrolysis were chromatographed. Hydrolysis products of lecithin, phosphatidylethanolamine, phosphatidylserine and α- and β-glycerophosphates were run in the same manner. In all the five systems used, the resulting chromatograms showed the predominant radioactivity at the same R_F value, as given by the authentic α-glycerophosphate. No radioactivity was found at those positions which corresponded to β-glycerophosphate or to the hydrolysis

products of lecithin, phosphatidylethanolamine and phosphatidylserine. If the radioactive products of hydrolysis were mixed with authentic α-glycerophosphate and submitted to chromatography, the total activity was found in the stained spot of α-glycerophosphate. The radioactive hydrolysis product is therefore considered to be identical with α-glycerophosphate. A typical chromatogram obtained with the Cellosolve–methyl ethyl ketone–NH_3 solvent system is shown in Fig. 10. Since phosphatidic acid is the only known phospholipid which yields glycerophosphate on mild alkaline hydrolysis by the method of DAWSON[9], these data suggest that the radioactive lipid formed in the mitochondria was phosphatidic acid.

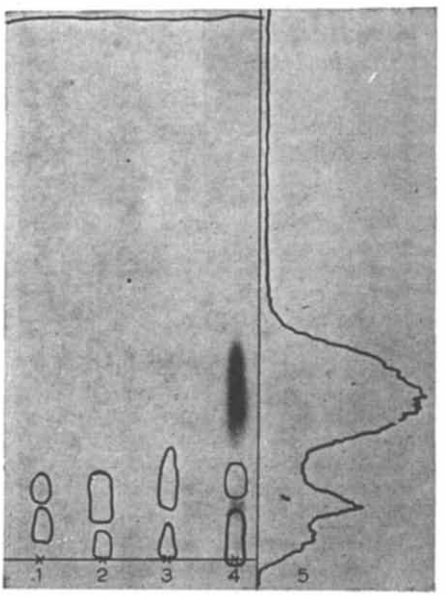

Fig. 8. Paper chromatogram and the superimposed autoradiogram of mitochondrial phospholipids. The solvent system was diisobutyl ketone–formic acid–water (40:15:2, v/v). 1,2,3, standards of phosphatidylethanolamine, lecithin and phosphatidylserine respectively; 4, mitochondrial phospholipids; 5, activity determined on the chromatogram 4 by the chromatographic strip scanner.

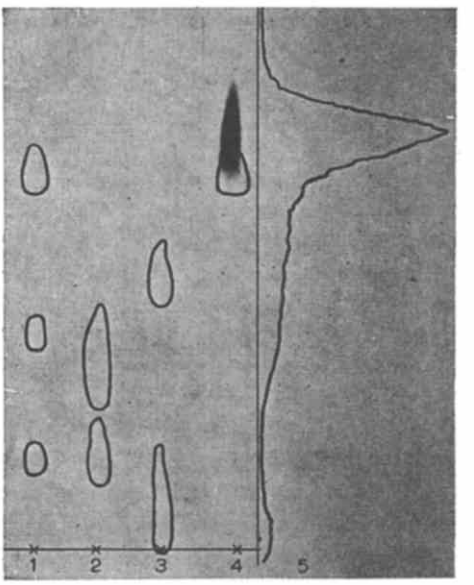

Fig. 9. Paper chromatogram and the superimposed autoradiogram of mitochondrial phospholipids. The solvent system was diisobutyl ketone–acetic acid–water (40:30:7, v/v). Indications as in Fig. 8.

DISCUSSION

Previous observations[2] have shown that [1-^{14}C]oleic acid is incorporated into mitochondrial phospholipids upon the addition of ATP and that this incorporation parallels the mitochondrial contraction. In experiments reported here the incorporation of [^{32}P]GP into mitochondrial phospholipids was observed under similar conditions. Both these findings seem to indicate a synthesis *de novo* of phospholipids from GP and oleic acid.

It has been shown by KORNBERG AND PRICER[20] that a particulate fraction of liver homogenate is able to synthesize phospholipids from fatty acids and α-glycero-

phosphate and that ATP is necessary in this process as an energy donor for fatty acid activation. Thus, it was to be expected that in our experiments the addition of ATP to the mitochondria would stimulate the synthesis of phospholipids. Consequently, the significant "extra" incorporation of [^{32}P]GP which followed the addition of ATP might be solely attributed to the known role of ATP in phospholipid synthesis and might bear no relation to mitochondrial contraction, both processes occurring simultaneously but being independent of each other.

However, the results reported here seem to indicate that a close relationship exists between the synthesis of mitochondrial phospholipids and the mitochondrial contraction and that ATP, if not followed by contraction, does not usually bring about any "extra" incorporation.

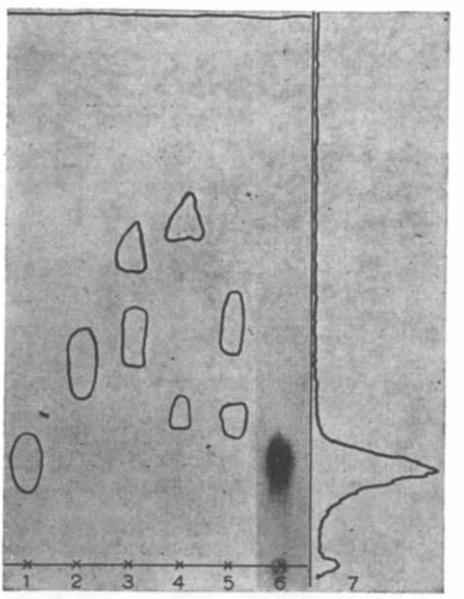

Fig. 10. Paper chromatogram and the superimposed autoradiogram of the hydrolysis products of mitochondrial phospholipids. The solvent system was Cellosolve – methyl ethyl ketone – 3 N NH_3 (7:2:3, v/v) saturated with boric acid. 1, α-glycerophosphate; 2, β-glycerophosphate; 3,4,5, hydrolysis products of phosphatidylethanolamine, lecithin and phosphatidylserine respectively; 6, hydrolysis products of mitochondrial phospholipids; 7, activity determined on Chromatogram 6 by the chromatographic strip scanner.

Evidence in favour of this assumption is provided firstly by the close resemblance of the time course of [^{32}P]GP incorporation into mitochondrial phospholipids to the time course of mitochondrial contraction. Next, it was found that the rate of [^{32}P]GP incorporation was significantly lower in spite of the presence of ATP, if [^{32}P]GP was added to the contracted mitochondria. A further proof is given by the absence of "extra" incorporation, if the mitochondria were protected against swelling by serum albumin and if, consequently, ATP did not produce any contraction. The possibility that fatty acids bound to albumin would not be available for phospholipid synthesis is to be rejected, as serum albumin did not inhibit the "extra" incorporation when added to the swollen mitochondria.

Other evidence suggesting the coupling of phospholipid synthesis with mitochondrial contraction is provided by the fact that substances which inhibit contraction depress the extent of [^{32}P]GP incorporation. (The only exception was azide which did not affect [^{32}P]GP incorporation although it strongly inhibited the contraction. No explanation for this can be given as yet.) Furthermore, substances which might be expected to interfere with the mechanism of phospholipid synthesis, such as PCMB and PP_i, were found to decrease or even completely to inhibit the contraction of mitochondria. On the other hand, small amounts of oleate increased the rate of contraction of mitochondria swollen in the presence of P_i, presumably by supplying the fatty acid moiety for the synthesis of the phospholipid molecule. An increase in the rate of mitochondrial contraction brought about by thyroxine has also been observed by LEHNINGER[21]. It seems reasonable to attribute this effect of thyroxine likewise to the action of fatty acids, as thyroxine has been found to stimulate the endogenous release of free fatty acids from mitochondria[2].

Thus, one may conclude that the biosynthesis of some mitochondrial phospholipids is an obligatory prerequisite for mitochondrial contraction, even if it is not solely responsible for it.

On the basis of column and paper chromatography and analysis of the hydrolysis products, there is good reason to assume that phosphatidic acid accounts for almost all the radioactivity of the mitochondrial phospholipids formed in the presence of [^{32}P]GP. Neither lecithin nor kephalins were found to be labelled. This does not seem surprising since, according to KENNEDY[22] phosphatidic acid is the chief intermediate product in the synthesis of phospholipids and since only phosphatidic acid, cardiolipin and inositol phosphatides contain the phosphate moiety derived from α-glycerophosphate. However, phosphatidic acid was likewise the main labelled phospholipid component, when [^{14}C]oleic acid was used as tracer[2]. This seems to indicate that phosphatidic acid formed during mitochondrial contraction is not only an intermediate product in the synthesis of other phospholipids, but may play an important, although still obscure, role in the mechanism of mitochondrial contraction.

One can only speculate on the possible role of phospholipid synthesis in mitochondrial contraction. Three posibilities seem worth considering:

1. Swelling of mitochondria is presumably accompanied by some structural change in mitochondrial membranes. As lipoprotein complexes are the main constituents of mitochondrial membranes, it is possible that splitting of some phospholipids may be partly responsible for these changes. Thus, the resynthesis of these phospholipids may serve to reconstitute the damaged membranes. It is known[23] that the mitochondrial membrane is negatively charged, and it has been suggested[24] that negatively charged groups, possibly those of phospholipids, may control the transport across the membrane. Phosphatidic acid is one of the most negatively charged phospholipids and therefore its role in controlling the transport of molecules through mitochondrial membrane may be of particular importance.

2. Synthesis of phospholipids may be related to changes in the structure of some mitochondrial proteins. The view that the shrinkage of mitochondria may be due to some contractile proteins has been put forward by LEHNINGER[25]. Furthermore, JUDAH[26] has observed phosphorylation of some mitochondrial proteins during the contraction induced by ATP. However, nothing is known about the relationship of lipids to contractile proteins.

3. Synthesis of phospholipids may be related to active transport of water and of other substances across mitochondrial membranes. The extrusion of water from the mitochondria may bear some resemblance to the active transport of ions through membranes or it may be accompanied by active transport of some other molecules. If this were the case, the stimulation of phospholipid synthesis in response to mitochondrial contraction would be similar to the "phospholipid effect" observed by HOKIN *et al.*[27-30].

Many data have been accumulated in recent years which point to phosphatidic acid as a metabolically active substance involved in the transport of various organic and inorganic compounds through cell membranes[27-33]. On the basis of numerous studies, HOKIN AND HOKIN[34] assume that the exchange of the phosphate moiety in phosphatidic acid may be part of a carrier mechanism in the transport of various molecules across membranes. They presented a scheme[30] whereby phosphatidic acid, which is presumably part of the lipoprotein complexes in the membranes, functions as a carrier for ions through the membrane. The scheme is based on the combined actions of diglyceride kinase and phosphatidic acid phosphatase which result not only in the synthesis and breakdown of the phosphatidic acid but also in ATPase activity. Furthermore, the ATPase activity has been shown to be closely related to the action of the sodium–potassium pumps in both nerve[35] and erythrocyte[36] membranes. Thus, the utilization of ATP for the synthesis of phosphatidic acid could represent the input of energy for transport.

In the present investigation a similar biochemical response was observed on contraction of the mitochondria as has been observed by others for the stimulation of the transport of various molecules across membranes. One may suppose, therefore, that phosphatidic acid formed during the contraction is involved in the mechanism of the active extrusion of water from the mitochondria.

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REFERENCES

1 A. L. LEHNINGER AND L. F. REMMERT, *J. Biol. Chem.*, 234 (1959) 2459.
2 L. WOJTCZAK AND A. L. LEHNINGER, *Biochim. Biophys. Acta*, 51 (1961) 442.
3 A. L. LEHNINGER, B. L. RAY AND M. SCHNEIDER, *J. Biophys. Biochem. Cytol.*, 5 (1959) 97.
4 A. L. LEHNINGER, *J. Biol. Chem.*, 234 (1959) 2465.
5 A. L. LEHNINGER, *J. Biol. Chem.*, 234 (1959) 2185.
6 G. V. MARINETTI AND E. STOTZ, *Biochim. Biophys. Acta*, 21 (1956) 168.
7 O. W. THIELE AND W. WOBER, *Z. Physiol. Chem.*, 326 (1961) 89.
8 G. V. MARINETTI, J. ERBLAND AND J. KOCHEN, *Federation Proc.*, 16 (1957) 837.
9 R. M. C. DAWSON, *Biochim. Biophys Acta*, 14 (1954) 374.
10 S. COHEN AND D. SCOTT, *Science*, 111 (1950) 2890.
11 G. WYATT, T. LONGHEED AND S. WYATT, *J. Gen. Physiol.*, 39 (1956) 853.
12 D. C. MORTINER, *Can. J. Chem.*, 30 (1952) 653.
13 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
14 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
15 E. P. KENNEDY, *J. Biol. Chem.*, 201 (1953) 399.

[16] A. L. LEHNINGER, *J. Biochem. (Tokyo)*, 49 (1961) 553.
[17] A. L. LEHNINGER, *Biochim. Biophys. Acta*, 48 (1961) 324.
[18] G. HÜBSCHER AND B. CLARK, *Biochim. Biophys. Acta*, 41 (1960) 45.
[19] L. W. WHEELDON, *J. Lipid Res.*, 1 (1960) 439.
[20] A. KORNBERG AND W. E. PRICER, Jr., *J. Am. Chem. Soc.*, 74 (1952) 1617.
[21] A. L. LEHNINGER, *Biochim. Biophys. Acta*, 37 (1960) 387.
[22] E. P. KENNEDY, *Federation Proc.*, 16 (1957) 847.
[23] H. PAULY, L. PACKER AND H. P. SCHWAN, *J. Biophys. Biochem. Cytol.*, 7 (1960) 589.
[24] J. M. MACHINIST, M. L. DAS, F. L. CRANE AND E. E. JACOBS, *Biochem. Biophys. Res. Commun.*, 6 (1962) 475.
[25] A. L. LEHNINGER, *Federation Proc.*, 19 (1960) 952.
[26] J. D. JUDAH, *Nature*, 187 (1960) 506.
[27] M. R. HOKIN, L. E. HOKIN, M. SAFFRAN, A. V. SCHALLY AND B. U. ZIMMERMANN, *J. Biol. Chem.*, 233 (1958) 811.
[28] M. R. HOKIN, B. G. BENFEY AND L. E. HOKIN, *J. Biol. Chem.*, 233 (1958) 814.
[29] L. D. EGGMAN AND L. E. HOKIN, *J. Biol. Chem.*, 235 (1960) 2569.
[30] L. E. HOKIN AND M. R. HOKIN, *J. Gen. Physiol.*, 44 (1960) 61.
[31] L. E. HOKIN AND A. L. SHERWIN, *J. Physiol. (London)*, 135 (1957) 18.
[32] L. E. HOKIN AND M. R. HOKIN, *J. Biol. Chem.*, 233 (1958) 805.
[33] A. M. WOODIN, *Biochem. J.*, 82 (1962) 9.
[34] L. E. HOKIN AND M. R. HOKIN, *Nature*, 189 (1961) 836.
[35] J. C. SKOU, *Biochim. Biophys. Acta*, 23 (1957) 394.
[36] R. L. POST, C. R. MERRIT, C. R. KINSOLVING AND C. D. ALBRIGHT, *J. Biol. Chem.*, 235 (1960) 1796.