LABELING OF MITOCHONDRIAL PHOSPHATIDYL INOSITOL PHOSPHATE BY P_i^{32} AND BY γP^{32}ATP

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Garbus et al. (1963) reported labeling of mitochondrial phospholipid following short incubations with P_i^{32}. The rapidly labeled lipid has been identified by means of alkaline hydrolysis as phosphatidyl inositol phosphate (Garbus et al., 1963; Gaillard and Hawthorne, 1963; Morgan, 1964). Inhibitors of oxidative phosphorylation blocked incorporation of P_i^{32} into lipid, although the reported effects of oligomycin were variable (Garbus et al., 1963; Michell et al., 1964). While such studies suggest that a high energy intermediate is required for the phosphorylation of phosphatidyl inositol, no evidence for its nature has been presented. This communication shows that the fraction of radioactivity in γP^{32}ATP incorporated into lipid is greater than that incorporated from P_i^{32} and further that inhibitors of oxidative phosphorylation, particularly atractyloside, can inhibit the labeling of lipid from P_i^{32} but have no effect on labeling from γP^{32}ATP.

MATERIALS AND METHODS

$Na_3P^{32}O_4$ (P_i^{32}) (IsoServe, Cambridge, Mass.) was diluted with unlabeled carrier on arrival. The atractyloside samples were gifts from Dr. A. Lehninger and from Dr. A. Bruni. Oligomycin, antimycin, and hexokinase (type IV) were purchased from Sigma Chemical Co. γP^{32}ATP was prepared and purified by the method of Glynn and Chappel (1964).

Guinea pig liver mitochondria, prepared in 0.25 M sucrose by the method of Schneider (1948), were washed 1-2x with sucrose and used immediately. The incubation procedure was that described by Garbus et al. (1963). Mitochondria from 0.5 g of guinea pig liver and additions were suspended in a total volume of 1.2 ml. Following incubation, 4.5 ml of chloroform-methanol (1:2) were added and the mixture was dispersed in the incubation tube by agitation in an ultrasonic bath. An additional 1.5 ml of chloroform were added followed by 1.5 ml of 2 M KCl in 0.5 M phosphate buffer (pH 7.4). The mixture was filtered through glass wool and the lower layer was then evaporated to dryness. Six ml of chloroform-

TABLE 1. Incorporation of P_i^{32} and $\gamma P^{32}ATP$ into Mitochondrial Lipid in 5 min

	$+P_i^{32}$	$+ATP^{32}$
	cpm	cpm
Complete system	7,723	9,792
* 0 time	104	260
- Mg^{++}	1,295	1,891
- Glutamate	8,060	10,425
+ 10 μmoles EDTA	372	1,857

The complete system contained 10 μmoles of $MgCl_2$, 20 μmoles of Tris-HCl (pH 7.4), 15 μmoles of K glutamate, 2x washed mitochondria from 0.5 g of liver, and either 0.13 μmoles of $Na_3P^{32}O_4$ (4.6 x 10^6 cpm) or 0.13 μmoles of $\gamma P^{32}ATP$ (1.1 x 10^6 cpm) in a total volume of 1.2 ml. The mitochondria were preincubated at 30° for 5 min before adding the radioactive substrates and then incubated for 5 min. The lipid was extracted and counted as described in the text.

* Solvent added immediately _after_ the addition of radioactive substrates.

methanol-conc. HCl (200:100:1) were added and the solution was washed with 1.5 ml of 0.05 M H_3PO_4. The upper layer was removed and the lower layer was washed with 3.5 ml of equilibrated upper phase (Folch et al., (1957) which was 0.1 N with respect to HCl. The lower phase was dried under nitrogen, dissolved in a Hyamine-ethanol-toluene counting system and counted in a liquid scintillation spectrometer. This extraction method removed contaminating P_i^{32}.

Incorporation of P_i^{32} and γP^{32}ATP into mitochondrial lipid. As shown in Table 1, almost 4x more phosphate is incorporated from γP^{32}ATP than from P_i^{32} following a 5 min incubation. At shorter time periods (1-2 min), ATP is 8-10x more effective than P_i. Since the amounts of endogenous ATP and P_i in the mitochondria were not established, the absolute efficiency of these two precursors is not known. However, addition of unlabeled phosphate had little effect on γP^{32}ATP incorporation, while the presence of substantial amounts of unlabeled ATP greatly depressed the incorporation of P_i^{32}. Deletion of glutamate had practically no effect. Mg^{++} stimulated lipid labeling from both γP^{32}ATP and P_i^{32}.

The effect of ATP trapping agents on the labeling of lipid. The addition of hexokinase and either glucose or deoxyglucose to the mitochondrial system decreased incorporation from both P_i^{32} and γP^{32}ATP (Table 2). This result is consistent with the hypothesis that ATP is the precursor of the lipid phosphate.

Effect of inhibitors of oxidative phosphorylation. Since the results suggested that P_i enters the lipid *via* ATP, studies were performed with known inhibitors or uncouplers of oxidative phosphorylation (Table 3). It is seen that these agents inhibit the incorporation of radioactivity from P_i^{32} with very little effect on γP^{32}ATP. In contrast to a previous report (Garbus et al., 1963) oligomycin also inhibited the labeling of lipid by P_i^{32}. Atractyloside is particularly effective in blocking incorporation of P_i^{32} into lipid, yet has no effect or even stimulates

TABLE 2. Effect of the Addition of Hexokinase and Glucose or Deoxyglucose on the Rapid Labeling of Lipid by P_i^{32} or ATP^{32}

	$+P_i^{32}$	$+ATP^{32}$
	cpm	cpm
Complete	12,720	20,316
0 time	172	155
+ 25 µmoles glucose	13,206	20,679
+ 25 µmoles glucose + 1.5 mg hexokinase	3,408	2,523
+ 25 µmoles deoxyglucose	12,258	19,942
+ 25 µmoles deoxyglucose + 1.5 mg hexokinase	4,305	2,475

The incubation conditions were the same as described under Table 1. 0.13 µmoles of P_i^{32} (1.12×10^7 cpm) or 0.13 µmoles of $\gamma P^{32}ATP$ (8.3×10^6 cpm) was used per tube.

the incorporation of $\gamma P^{32}ATP$. We also found that under conditions in which lipid labeling from P_i^{32} was 80% inhibited by oligomycin or completely inhibited by atractyloside, the conversion of P_i^{32} to ATP as measured by the method of Nielsen and Lehninger (1955) was only 20% inhibited.

Characterization of the labeled lipid. The labeled lipids were identified by alkaline hydrolysis (Hübscher et al., 1960) followed by separation of the water-soluble glycerylphosphoryl derivatives by paper chromatography or paper electrophoresis. Carrier brain inositide was added to the lipid before hydrolysis. Two solvent systems, butanol-acetic acid-water (5:4:1) (Gray, 1964) and isopropanol-ammonia-water (6:3:1) (Hendrickson and Ballou, 1964), were used to develop the paper chromatograms. High voltage electrophoresis of hydrolysates was performed

TABLE 3. Effect of the Addition of Inhibitors

Addition	Final Concentration	$+P_i^{32}$	$+ATP^{32}$
		cpm	cpm
None	-	8,114	23,907
None (0 time)	-	222	817
DNP	10^{-4}M	630	14,486
Arsenate	10^{-3}M	3,059	17,154
CN^-	5.0×10^{-4}M	144	16,574
N_3^-	5.0×10^{-4}M	3,621	24,480
Antimycin	1 μg/1.2 ml	334	20,441
Oligomycin	1 μg/1.2 ml	2,914	
"	2 μg/1.2 ml	1,915	25,434
Atractyloside	5.0×10^{-5}M	62	
"	5.0×10^{-6}M	46	24,934
None	-	10,126	21,325
None (0 time)	-	133	606
Atractyloside	2.5×10^{-5}M	37	
"	5.0×10^{-6}M	82	
"	2.5×10^{-6}M	211	47,320
"	5.0×10^{-7}M	8,592	
"	5.0×10^{-8}M	9,406	

The incubation conditions were the same as described in Table 1. Different experiments contained different amounts of radioactivity in 0.13 μmoles of P_i or ATP and results were normalized to 10^7 cpm added for each labeled substrate. The mitochondria were preincubated for 5 min with the inhibitors (in water or ethanol) before adding the radioactive substrates.

according to the conditions of Seiffert and Agranoff (1965). In each instance, 85-95% of the radioactivity was found in the glycerylphosphoryl inositol phosphate fraction. This was true when either P_i^{32} or γP^{32}ATP was used as the radioactive precursor. Some radioactivity was found in phosphatidic acid. This was established by the recovery of α-glycerophosphate after the same hydrolytic, chromatographic, and electrophoretic procedures. The phosphatidic acid fraction increased with time after an initial lag period. In 20 min incubations, phosphatidic acid represents most of the total radioactivity in mitochondrial lipid.

DISCUSSION

These experiments indicate that in mitochondria, ATP is a more direct precursor of phosphatidyl inositol phosphate than is P_i. This is in agreement with results found for other investigators in brain (Colodzin and Kennedy, 1964) and in erythrocyte membranes (Hokin and Hokin, 1964). It should be pointed out that in all of these experiments, labeling by ATP does not distinguish between _de novo_ synthesis of phosphatidyl inositol phosphate and an exchange reaction involving ATP and phosphatidyl inositol phosphate.

The physiological significance of the rapid labeling is of interest. It is not likely that this lipid plays a direct role in oxidative phosphorylation, but it might be involved in transport across the mitochondrial membrane. In this respect, it is significant that inhibition of P_i incorporation into lipid is produced by concentrations of atractyloside which are sufficiently low not to block P_i incorporation into ATP. This inhibitor has been postulated to block the inward transport of ADP (Heldt et al., 1965). The present studies suggest that atractyloside inhibits the outward transport of ATP through the mitochondrial membrane. It would also appear that the lipid itself is on the outside of the mitochondrial membrane. The exact nature of atractyloside inhibition awaits further elucidation of the mechanism

of ATP transport across the mitochondrial membrane.

The inhibition of labeling by oligomycin cannot be due entirely to its block of a terminal step of oxidative phosphorylation. A disportionately large inhibition of lipid labeling from P_i^{32} by oligomycin compared with the inhibition of ATP synthesis by the drug was observed. It is thus probable that the action of oligomycin is more complex than has previously been believed.

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