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Time Study of Incorporation *in vivo* of Inorganic Phosphate (P^{32}) into Phospholipids of Mitochondria.* (30014)

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Phospholipids are found in the membranes (1), mitochondria, nuclei, and microsomes (2,3) of the cell. Paper chromatographic techniques are capable of providing composition of various phospholipids from tissues (4,5), and subcellular fraction(6,7) of the cell. The incorporation *in vivo* of inorganic phosphate (P_i^{32}) into the individual phospholipids of rat tissues has been reported(8, 9). However, an *in vivo* time study on uptake of P_i^{32} into individual phospholipids of liver mitochondria has not been reported. This study reports the data on a time study of the incorporation of P_i^{32} into mitochondrial phospholipids of rat liver and hamster liver and kidney.

Methods. Male albino rats of the Sprague-Dawley strain, previously maintained on Purina Laboratory chow, weighing 120-150 g, were used in the time uptake study. Male hamsters, weighing 120-160 g were maintained on Purina Laboratory chow pellets. The animals were injected with 200 μc of P_i^{32} and sacrificed at 0.5, 1, 2, 4, 6, 8, and 10 hours later by decapitation. The tissues were quickly removed, rinsed with cold water and placed on cracked ice. The organs were immediately weighed and homogenized in cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with teflon pestle. The kidney was diluted to 25 ml with the cold sucrose solution and the liver to a concentration of 1 g per 8 ml. Aliquots of 5 ml were pipetted into 10 ml of a cold solution of 10% trichloroacetic acid plus 0.4 M $MgCl_2 \cdot 6H_2O$(10) and kept 3°C overnight and inorganic phosphorus(11) and radioactivity(12) analysis performed on the supernatant. The remaining homogenates were centrifuged(13) in the cold for 15 minutes at 800 $\times$ *g* to remove the nuclei. The nuclear pellet was rehomogenized in sucrose and centrifuged and the supernatants combined. Mitochondria were separated from the combined supernatants by centrifugation at 10,000 $\times$ *g* for 15 minutes. The mitochondrial pellet was twice resuspended in sucrose and centrifuged to wash out contaminating microsomes. This procedure removed over 99% of the microsomes as determined by assaying for microsomal glucose-6-phosphatase(14).

The lipids were extracted from the mitochondria by extracting once with 8 ml of methanol and twice with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at room temperature. This was followed by 4 extractions with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at 55°C. Each extraction was for 15 minutes in a shaking water bath followed by centrifugation for 3 minutes at 1,600 $\times$ *g* to facilitate removal of the solvent. The organic solvents of the pooled extracts were evaporated on a Rinco Rotating Vacuum Evaporator (Rinco Instrument Co.) at 35° and the water removed by lyophilization in a Freeze-Dryer (Virtis Co., Inc.). The lipids were taken up in chloroform and the phospholipids separated by chromatography on silicic acid impregnated glass filter paper(15). The solvent system was diisobutyl ketone:acetic acid: water:benzene (160:50:8:7, v/v). The chromatograms were dried, stained with Rhodamine 6G and the phospholipids identified with ultraviolet light(16). The individual phosphatides were extracted with 1 N metha-

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TABLE I. Time Study of Incorporation *in vivo* of P_i^{32} into Mitochondria Phospholipids of Rat Liver.

Time after adm P_i^{32} (hr)	No. of rats	Specific activities					
		Phospholipid fractions					
		Total phospholipids	Phosphatidyl inositol	Sphingo-myelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
.5	4	8.1 ± 1.1 (1.0 ± .01)	3.0 ± 1.1 (.4 ± .1)	2.2 ± .6 (.3 ± .1)	3.8 ± .6 (.5 ± .1)	2.3 ± .8 (.3 ± .1)	2.1 ± 1.1 (.2 ± .1)
1	4	13.1 ± 4.3 (1.4 ± .2)	6.6 ± 2.2 (.7 ± .1)	6.5 ± 1.7 (.7 ± .2)	8.4 ± 5.1 (1.5 ± .2)	5.1 ± 1.5 (.5 ± .1)	6.5 ± 1.1 (.7 ± .1)
2	4	22.5 ± 3.0 (2.9 ± .3)	8.7 ± 2.2 (1.0 ± .2)	8.6 ± 1.6 (1.3 ± .4)	18.6 ± 2.6 (2.9 ± .6)	10.2 ± 1.0 (1.4 ± .2)	14.3 ± 3.2 (1.7 ± .3)
4	4	31.1 ± 3.4 (4.5 ± .7)	14.4 ± 2.9 (2.2 ± .3)	21.5 ± 4.4 (3.5 ± .2)	34.0 ± 5.0 (5.5 ± .3)	19.5 ± 1.2 (2.3 ± .6)	30.6 ± 3.4 (4.5 ± .3)
6	4	45.7 ± 8.0 (9.8 ± .6)	30.6 ± 6.2 (6.5 ± .6)	26.9 ± 2.7 (5.5 ± 1.1)	59.6 ± 7.7 (11.9 ± .6)	39.7 ± 6.1 (7.7 ± 1.3)	61.5 ± 5.7 (12.6 ± 1.5)
8	4	50.0 ± 6.1 (14.0 ± 2.3)	29.6 ± 3.3 (8.0 ± .9)	28.4 ± 5.3 (7.5 ± 1.2)	64.4 ± 4.9 (17.4 ± 1.2)	44.6 ± 11.0 (9.1 ± 1.1)	60.3 ± 5.8 (15.7 ± .8)
10	4	45.4 ± 4.1 (15.2 ± 1.8)	30.7 ± 2.6 (10.5 ± .6)	29.5 ± 3.7 (10.4 ± 1.9)	59.0 ± 6.1 (19.3 ± 1.2)	48.9 ± 2.9 (15.6 ± 2.0)	59.5 ± 5.9 (19.9 ± 2.1)

Specific activity (counts/min/mg of P). Numbers preceded by ± are standard deviations. Values in parentheses are relative specific activities × 100 (specific activity of phospholipid P/specific activity of inorganic P_i).

nolic HCl. The hydrolysate was evaporated to dryness, digested for 30 minutes with 0.2 ml of H_2SO_4-HNO_3 (1:1), allowed to cool, and 3 drops of HNO_3 were added and digestion continued for an additional 15 minutes. The samples were then neutralized to pH 6-7 with NH_4OH with phenol red as internal indicator. Inorganic phosphorus was determined by the method of Fiske and Subbarow (11). An aliquot of the colored solution of the phosphate analysis was pipetted into a steel planchet containing a drop of concentrated NaOH solution and dried under a heat lamp. The radioactivity was determined by placing the planchet under an ultra-thin window, gas flow Geiger tube (Tracerlab TGC-14) and correcting for decay. From the phosphorus and radioactivity analysis the specific activity (counts per minute/mg of phosphorus) was calculated. The relative specific activities(17) of individual phospholipids were calculated. The relative specific activity is the ratio of specific activity of phospholipids to specific activity of inorganic phosphorus and expresses the relationship between the isotopic concentration in a compound and in its precursor(18). Under the conditions of our experiment, an increase in the formation of phospholipids may be reasonably assumed when higher values for specific and relative specific activities are found. Various factors such as the difference in the role of absorption and in the equilibrium between intra- and extracellular inorganic phosphorus with different specific activities, may conceivably produce changes in the specific activity of the inorganic fraction. However, a ratio of the specific activities, or relative specific activity, should provide a means of eliminating the effects of such factors.

Results. Table I shows a time study of incorporation of P_i^{32} into the mitochondrial phospholipids of the liver from the rat up to 10 hours after injection of the labeled phosphate. Phosphatidyl choline has the greatest specific activity up to 4 hours following administration of the isotope. Dawson(9) and Marinetti *et al*(8) have reported in whole liver studies that the incorporation of P_i^{32} into phosphatidyl ethanolamine is greater than the other phospholipids up to 21 hours following administration of the isotope. Marinetti *et al*(8) was able to demonstrate in the P_i^{32} incorporation studies of whole liver that the specific activities of phosphatidyl ethanolamine reached a peak before phosphatidyl choline and subsequently fell below phosphatidyl choline. The latter continues to be elevated. This would indicate in the whole liver that phosphatidyl ethanolamine may be a

precursor of phosphatidyl choline or represents some interconversion of the phospholipids. Artom(19) has demonstrated in rat liver homogenate the methylation of phosphatidyl monomethylethanolamine and dimethylethanolamine to form phosphatidyl choline. Borkenhagen, Kennedy and Fielding (20) have reported enzymatic conversion of phosphatidyl serine to phosphatidyl ethanolamine in rat liver. If the specific activity curves of the various phospholipids are plotted against time it is apparent from the data there is no evidence of any interconversion of phospholipids in liver mitochondria, *i.e.*,

TABLE II. Time Study of Incorporation *in vivo* of P_i^{32} into Mitochondria Phospholipids of Hamster Liver.

Time after adm P_i^{32} (hr)	No. of Hamsters	Specific activities					
			Phospholipid fractions				
		Total phospholipids	Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
.5	8	2.6 ± .9 (.4 ± .1)	3.9 ± .3 (.5 ± .1)	3.3 ± .8 (.5 ± .9)	1.2 ± .1 (.2 ± .1)	3.0 ± 1.5 (.5 ± .2)	.8 ± .1 (.1 ± .1)
1	8	7.1 ± .6 (.6 ± .1)	8.1 ± 1.2 (.8 ± .1)	7.0 ± 5.5 (2.4 ± .3)	3.5 ± .4 (.6 ± .2)	4.6 ± 1.8 (.6 ± .1)	2.3 ± .8 (.4 ± .1)
2	8	10.4 ± 1.4 (1.4 ± .1)	8.8 ± .5 (1.6 ± .4)	6.4 ± 5.7 (2.4 ± 1.6)	8.3 ± .9 (.8 ± .4)	9.7 ± 3.1 (1.1 ± .4)	4.1 ± .3 (.8 ± .2)
4	4	16.5 ± .6 (2.8 ± .5)	18.8 ± 1.2 (2.3 ± .3)	13.2 ± 2.1 (2.8 ± .9)	14.8 ± 3.3 (2.4 ± .2)	17.1 ± .5 (2.1 ± .2)	13.4 ± .8 (2.0 ± .2)
6	4	23.8 ± 2.8 (4.3 ± .3)	26.0 ± 4.3 (4.9 ± .4)	31.1 ± 2.8 (6.5 ± .8)	22.8 ± 1.3 (4.6 ± .3)	21.1 ± 2.5 (3.0 ± .1)	17.2 ± 1.5 (3.5 ± .2)
8	4	27.9 ± 1.6 (5.8 ± .5)	25.5 ± 1.1 (5.7 ± .5)	12.7 ± 1.2 (3.0 ± .3)	27.2 ± 1.3 (5.9 ± .3)	19.9 ± 7.8 (3.8 ± .6)	22.9 ± 1.2 (4.5 ± .2)
10	4	31.0 ± 6.0 (7.1 ± .6)	25.5 ± 2.9 (6.0 ± .4)	10.7 ± .8 (3.2 ± .9)	35.5 ± 5.8 (7.5 ± .4)	23.3 ± 3.9 (5.4 ± .4)	24.8 ± 4.0 (5.7 ± .3)

Specific activity (counts/min/mg of P). Numbers preceded by ± are standard deviations. Values in parentheses are relative specific activities × 100 (specific activity of phospholipid P/specific activity of inorganic P_i).

TABLE III. Time Study of Incorporation *in vivo* of P_i^{32} into Mitochondria Phospholipids of Hamster Kidney.

Time after adm P_i^{32} (hr)	No. of Hamsters	Specific activities					
			Phospholipid fractions				
		Total phospholipids	Phosphatidyl inositol	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
.5	4	6.9 ± .7 (.6 ± .1)	9.1 ± 1.8 (.8 ± .1)	2.7 ± .8 (.2 ± .1)	4.7 ± .7 (.3 ± .01)	2.9 ± .4 (.2 ± .01)	.5 ± .1 (.1 ± .01)
1	4	12.3 ± .9 (.9 ± .1)	15.4 ± 1.3 (1.7 ± .4)	3.9 ± .7 (.3 ± .01)	16.0 ± 1.2 (1.4 ± .1)	5.5 ± 1.1 (.4 ± .1)	1.2 ± .01 (.1 ± .01)
2	4	18.6 ± 1.5 (1.8 ± .01)	26.1 ± 2.6 (2.8 ± .4)	6.1 ± 2.1 (1.0 ± .1)	24.8 ± 2.2 (3.5 ± .2)	12.4 ± 1.0 (1.2 ± .1)	3.4 ± .5 (.3 ± .1)
4	4	28.2 ± 1.4 (5.0 ± .5)	35.8 ± 3.4 (6.9 ± 1.5)	14.4 ± 1.5 (2.5 ± .4)	53.2 ± 5.4 (7.9 ± .6)	31.1 ± 5.3 (3.5 ± .4)	7.0 ± 1.1 (1.2 ± .1)
6	4	26.3 ± 1.5 (6.2 ± .8)	35.9 ± 6.4 (7.1 ± 1.4)	26.3 ± 1.1 (6.6 ± .8)	55.4 ± 2.2 (13.0 ± 1.4)	45.0 ± 6.5 (12.0 ± 1.0)	11.3 ± .7 (2.7 ± .1)
8	4	29.1 ± 2.1 (7.6 ± .9)	44.9 ± 6.7 (13.0 ± 1.7)	23.3 ± .8 (6.8 ± .6)	55.7 ± 8.2 (17.0 ± 1.6)	46.3 ± 3.6 (12.0 ± 1.0)	12.9 ± 1.0 (3.4 ± .3)
10	4	28.8 ± 1.9 (9.6 ± .9)	38.5 ± 5.9 (13.0 ± 4.1)	25.8 ± 1.7 (7.7 ± .1)	57.0 ± 4.3 (21.0 ± 1.6)	45.8 ± 1.5 (14.0 ± 2.1)	12.8 ± 1.0 (4.8 ± .5)

Specific activity (counts/min/mg of P). Numbers preceded by ± are standard deviations. Values in parentheses are relative specific activities × 100 (specific activity of phospholipid P/specific activity of inorganic P_i).

phosphatidyl ethanolamine to phosphatidyl choline or phosphatidyl ethanolamine to phosphatidyl serine. Table II shows data of a time study of incorporation of P_i^{32} into mitochondrial phospholipids of the liver of the hamster. It is apparent that there is very little difference in incorporation of P_i^{32} into the various phospholipids of the liver of the hamster. Table III shows data of a time study of incorporation of P_i^{32} into mitochondrial phospholipids of the kidney of the hamster. Phosphatidyl choline has the greatest incorporation of the isotope. The smallest amount of P_i^{32} incorporated occurred in phosphatidyl ethanolamine. It is apparent from the data that the specific activity time curves give no evidence of any interconversion of the phospholipids in mitochondria.

Summary. *In vivo* incorporation of P_i^{32} into the phospholipids of rat liver mitochondria, hamster liver and kidney mitochondria was investigated. The time course from 0.5 to 10 hours is given. The specific activity time curves give no evidence of any interconversion of the phospholipids in mitochondria.

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Fatty Acid Synthesis *in vitro* in a System from Rat Liver.* (30015)

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The experiments to be reported were carried out to investigate factors which may control the rate of fatty acid synthesis in a supernatant-microsomal system from rat liver. This synthesis comprises the following reactions: (a) activation of CO_2 with utilization of ATP in the presence of an enzyme which contains biotin, (b) carboxylation of acetyl-CoA with the activated CO_2 to form malonyl-CoA, (c) condensation of the malonyl-CoA with acetyl-CoA with the liberation, as CO_2 of the carbon previously fixed into acetyl-CoA, forming a β-ketoacyl derivative, and (d) subsequently the acyl derivatives are

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