The incorporation *in vitro* of [*Me*-^{14}C]choline into the phospholipids of rat-liver mitochondria

In the considerable work that has been carried out on the biosynthesis of phosphatidyl choline in liver mitochondria *in vitro*[1-5], labelled phosphoryl choline has been used almost exclusively as precursor. In earlier experiments, the cofactors required for the incorporation of free choline into phosphatidyl choline were described[6], yet it was suggested in a later paper[7] that choline had been incorporated into stearyl choline, which was found to behave similarly to phosphatidyl choline on alumina columns.

The present work was undertaken to determine the optimal conditions for the incorporation of [*Me*-^{14}C]choline into phosphatidyl choline. The labelled choline chloride was incubated with freshly prepared rat-liver mitochondria for 1 h at 37°. The reaction was stopped by the addition of ice-cold 50 % TCA to give a final concn. of 5 % TCA. To remove the labelled choline chloride which had not been incorporated, the precipitate was washed extensively with 5 % TCA containing 0.002 *M* unlabelled choline chloride. The control experiment (Table I) showed that virtually all of the unreacted [*Me*-^{14}C]choline chloride had been removed from the phospholipids. The lipids were extracted from the precipitate with chloroform-methanol. The extract was taken to dryness under reduced pressure to split proteolipids and the residue extracted overnight with chloroform. The procedures described above were based on the method of MARINETTI *et al.*[8].

The phospholipids were isolated from this extract by silicic acid column chromatography as outlined by FILLERUP AND MEAD[9], but using a simplified system of solvents for elution. The eluate containing the phospholipids was taken to dryness and the

TABLE I

INCORPORATION OF [*Me*-^{14}C]CHOLINE INTO MITOCHONDRIAL PHOSPHOLIPIDS

Each system contained: 1.5 ml of mitochondrial suspension in 0.25 *M* sucrose, 0.25 ml of 1.25 *M* sucrose, 0.5 ml of 0.1 *M* phosphate buffer, pH 7.4, 0.2 ml of 0.2 *M* $MgCl_2$, and 5 μmoles choline chloride containing 0.5 μC. The final vol. was 3.0 ml. The cofactors were added as shown below.

Expt.	*Cofactors*	*Specific activity**
I	Control (zero-time)	54
	None	1250
	ATP (10 μmoles)	1292
	ATP (10 μmoles) + CTP (1 μmole)	1104
	ATP (10 μmoles) + CTP (1 μmole) + 0.2 ml supernatant	1079
II	None	1341
	CMP (8 μmoles)	3480
	ATP (10 μmoles) + CoASH (1 μmole)	1670
	ATP (10 μmoles) + CoASH (1 μmole) + CMP (8 μmoles)	7810
III	None	1190
	CMP (4 μmoles)	2080
	CMP (4 μmoles) + ATP (8 μmoles)	1740
	CMP (4 μmoles) + ATP (8 μmoles) + CTP (1 μmole)	1755
	ATP (8 μmoles) + CTP (1 μmole)	1368

* Disintegrations/min/mg P.

Abbreviations: TCA, trichloracetic acid; ATP, adenosine triphosphate; CTP, cytidine triphosphate; CMP, cytidine monophosphate; CoASH, reduced coenzyme A.

specific activity determined by liquid scintillation counting. Using this method of counting a very high efficiency (about 80 %) was obtained. It had the advantage that ^{14}C-labelled stearic acid could be used as an internal standard. Thereby, the decreased rate of counting due to the inherent colour of the phospholipids could be corrected. At the same time, it was possible to convert the counts obtained into disintegrations.

Table I shows that neither ATP, nor ATP + CTP, brought about an increased rate of incorporation of choline, even in the presence of native supernatant containing microsomes and soluble proteins. CMP, however, stimulated the incorporation considerably and a combination of CMP + ATP + CoASH was still more effective in this respect (see Expt. II). The observation that CMP alone stimulated the incorporation of choline might be explained by the fact that it was phosphorylated to CTP by the ATP present originally in the mitochondria. Experiment III shows that this is not so, since the addition of ATP did not increase the incorporation beyond that due to CMP.

The radioactive phospholipid was tentatively identified as phosphatidyl choline by alkaline hydrolysis of the total phospholipids and separation of the resulting diesters on ion-exchange resins[10]. Glyceryl-phosphoryl-choline was the only diester which was found to be radioactive.

Experiments using cations and anions in the absence of other cofactors showed that Ca^{++} ($3.7 \cdot 10^{-3}$ *M*) alone gave a 20-fold increase, and Ca^{++} ($3.7 \cdot 10^{-3}$ *M*) + $HPO_4^{=}$ ($3.3 \cdot 10^{-3}$ *M*) a 35-fold increase in the rate of incorporation. Other divalent metals (Mn, Co, Zn, Ni, Hg, Ba) did not stimulate the incorporation except for Mg^{++}, which caused a very slight stimulation. One of the possible explanations of the effect brought about by Ca^{++} could be the presence of a phospholipase D. So far, there have not been any reports of the occurrence of this enzyme in animal tissues, except for some indications that it might occur in extracts of rat intestinal mucosa[11]. Though phospholipase D is an enzyme liberating choline from phosphatidyl choline, the reverse reaction would become more significant in the presence of excess choline, and might be detectable by using labelled choline.

More detailed information on these studies will be described elsewhere.

We should like to thank Professor A. C. FRAZER for his interest and helpful discussion.

This work was supported by grants from the Medical Research Council.

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Received November 28th, 1958