The production of ^{32}P-labeled mitochondrial nucleotides

While studying the role of ATP* in the regulation of mitochondrial pathways[1,2], it was considered desirable to obtain information concerning the various nucleotides in isolated mitochondria, as well as to study the incorporation of ^{32}P into these fractions. Since the particles were to be used in experiments concerning oxidative phosphorylation, it was necessary to devise a method of labeling the intramitochondrial nucleotides which did not necessitate any methodological derivation from the techniques of preparation routinely employed in these studies. This note describes such a technique and presents some related data on the rate of exchange between ^{32}P and homogenate organic phosphorus, and the identification of certain of the mitochondrial components.

Individual rats were sacrificed by decapitation, the livers removed rapidly and minced in cold 0.25 *M* sucrose. The mince was washed with sucrose and homogenized in 25 ml of 0.25 *M* containing 0.2 ml of carrier free $H_3{}^{32}PO_4$ (10 mc/ml) using a Potter-Elvehjem type homogenizer with a teflon pestle. The homogenates were then diluted to approximately 125 ml with cold 0.25 *M* sucrose and centrifuged at 1600 *g* (International Refrigerated Centrifuge, Model PRI, Head No. 840) for 10 minutes. The supernate was then decanted and centrifuged for 15 minutes at 4100 *g* (5000 r.p.m.) at 0°C. The resulting pellet was washed twice with cold sucrose. Prior to the last washing, three pellets were combined to provide sufficient material for chromatographic analysis and centrifuged for 15 minutes. The final pellet contained mitochondria capable of carrying on efficient succinate linked oxidative phosphorylation. Phosphorylation was determined according to a modification[3,4] of the MARTIN AND DOTY method[5].

The rate of exchange between ^{32}P and organic P in homogenates at 1–4°C is shown in Fig. 1. Also shown is the incorporation of ^{32}P into the 7 minute hydrolyzable phosphate. Zero time coincides with the initiation of homogenization. Following a thirty second homogenization, the suspension was swirled in an ice bath, and aliquots periodically removed for the determination of phosphorylation. It is seen from Fig. 1 that a rapid exchange, which approaches an asymptote at 6 minutes, occurs between inorganic ^{32}P and organic ^{32}P. The exchange into the 7 minute labile fraction parallels this and remains fairly constant over the 20 minute period shown.

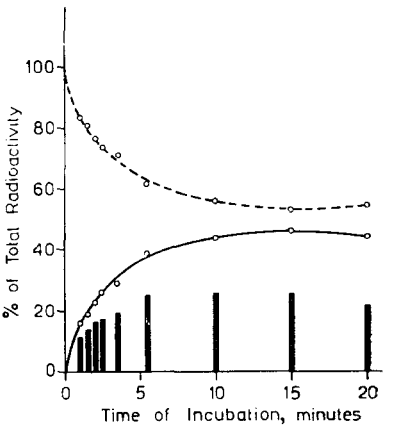

Fig 1. The incorporation of carrier free ^{32}P into total organic and 7 minute hydrolyzable organic phosphorus fractions of rat liver homogenates at 0°C. --O--O-- = inorganic ^{32}P, —O—O— = total organic ^{32}P, —▮—▮—= 7 minute hydrolyzable organic ^{32}P. Zero time corresponds to initiation of homogenization.

The chromatographic separation of acid-soluble nucleotides was based on the method of HURLBERT *et al.*[6]. The final mitochondrial suspension was fixed with an equal volume of 10% TCA. During chromatography 5 ml samples were collected each 15 minutes and E_{260} and radioactivity determined. The extinction was read in a Beckman DU spectrophotometer and radioactivity determined in the slightly modified apparatus described by LINDBERG AND ERNSTER[7].

Fig. 2 presents the superimposed radioactivity and E_{260} readings for each 5 ml fraction. The bases for naming the various fractions are, (1) the order and magnitude of appearance of the various nucleotides as compared with those for whole liver reported by HURLBERT *et al.*[6], (2) the characteristic ultra violet absorption curves[8], and (3) behavior in paper chromatography using the solvent systems of ZETTERSTRÖM AND LJUNGGREN[9] (solvent I, *iso*butyric acid-ammonia) and MARKHAM AND SMITH[10] (solvent II, propanol-ammonium sulfate).

Fraction I in Fig. 2 corresponds to CMP regarding its elution position from the Dowex column, but not when chromatographed on paper using solvent systems I and II. The ultra violet absorption spectrum of fraction I does not correspond to CMP and this fraction is as yet unidentified. Fraction II corresponds to the DPN position from the column as well as in solvent systems

* The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; UTP, uridinetriphosphate; UDP, uridinediphosphate; UDP-X_3, carbohydrate derivative of UDP; UMP, uridinemonophosphate; UM-5-P, uridine-5'-monophosphate; CMP, cytidinemonophosphate; ITP, inosinetriphosphate; GTP, guanosinetriphosphate; DPN, diphosphopyridine nucleotide; FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide; TCA, trichloroacetic acid; F-A, formic acid; A-F, ammonium formate; E_{260}, extinction at 260 mμ.

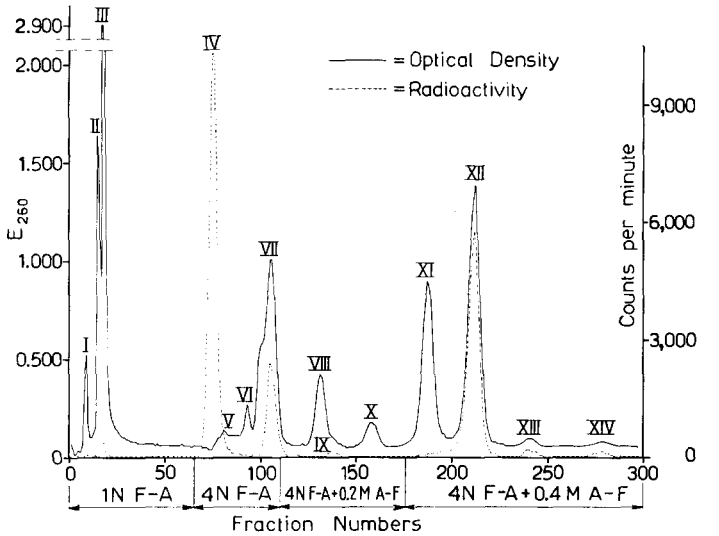

Fig. 2. E_{260} and radioactivity (——— = E_{260}, ----- = radioactivity) values of chromatographic fractions of TCA-soluble fraction of rat liver mitochondria prepared with $H_3{}^{32}PO_4$. The radioactivity scale represents the number of counts/minute/15 microliters.

I and II. Spraying with 1 *N* NaCN results in a flourescent spot. Fraction III parallels AM-5-P from the column and in systems I and II. The ultra violet curve is identical to AMP. It may be seen that a small amount of activity which, incidentally, travels with the ultra violet quenching spot on paper, is present in this fraction. Fraction IV has been identified as inorganic orthophosphate in solvents I and II. The magnitude of this fraction might well be due to a rupture of labile phosphate bonds during the TCA extraction procedure. Fraction V is as yet unidentified. Fraction VI corresponds to UM-5-P regarding column position, solvents I and II and the characteristic ultra violet absorption curve. The position of elution from the Dowex column, movement in solvent systems I and II, and the characteristic absorption curve all identify fraction VII as ADP. The activity of this fraction does not separate from the quenching spot during paper chromatography. Fraction VIII, which was originally thought to be FAD because of its intense yellow fluorescence, does not exhibit the typical absorption peaks of FAD and FMN at 370 and 450 mμ for FAD and FMN and does not correspond to either in solvent system I. This fraction is as yet unidentified. Fraction IX has not been identified, nor has fraction X. Fraction XI, which is of interest because of its magnitude in mitochondrial preparations, corresponds to UDP or UDP-X_3 as to its column elution position. The absorption curve, however, is not typical of UDP, having a λ min. point at 243 mμ and a λ max. point at 258 mμ at pH 2. In solvent system II this fraction separates into two components, one an ultra violet quenching spot and the other, immobile, exhibiting a white-yellow fluorescence. The fluorescent spot, when eluted, does not correspond to a flavin compound when its absorption spectrum is examined. When eluted, the quenching spot's absorption spectrum resembles those of the adenine and uridine bases at pH 2. However, upon hydrolysis in 1 *N* acid for 1 hour at 100°C and subsequent chromatography in systems I and II, this compound corresponds to none of the monophosphates of adenine, uridine, or cytidine. Nor does this compound have the characteristic movement of ITP in these solvent systems. Prior to the separation of the two components of fraction XI by paper chromatography, and upon hydrolysis, one ultra violet quenching spot is observed near the moving front while AMP, UMP and CMP remain near the base of the paper in solvent system I. In solvent system II, the same hydrolysate results in two spots, one just below AMP, and one just above AMP between the latter and UMP. When the hydrolysate of the ultra violet quenching component is chromatographed in system II, one spot is observed above CMP, the nucleotide which moves the farthest in this system. The components of this fraction are as yet unidentified. The position of elution from the column, the ultra violet spectrum, the movement in systems I and II and the high radioactive incorporation of fraction XII are all characteristic of ATP. Although the two fractions following ATP are eluted in positions characteristic of GTP and UTP in the HURLBERT system for whole liver, they have not been identified in mitochondria. Larger quantities of these fractions are being obtained for analysis. Although their absolute concentrations appear rather low, their specific activities must be quite

high and thus may be of importance. It is possible that these fractions represent nucleotide tetra- or higher polyphosphates of unknown significance such as those reported recently by SACKS[11,12] and MARRIAN[13] in liver and commercial ATP preparations.

The present work provides a method of labeling certain of the nucleotides of liver mitochondria without any deviation from the usual methods of preparation and isolation. It is expected that such a method may become valuable in studies of the role of nucleotide levels in mitochondrial metabolism. Further work, attempting to identify the unknown fractions, is in progress.

The authors are indebted to Professor OLOV LINDBERG, LARS ERNSTER and HANS LÖW for valuable discussions during this investigation.

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Received July 8th, 1955

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Fatty acid synthesis by soluble enzyme preparations from rabbit mammary gland

Recently, POPJÁK AND TIETZ showed that soluble enzyme preparations from lactating rat mammary gland were capable of synthesizing fatty acids from ^{14}C labelled acetate, in the presence of ATP and α-oxoglutarate[1]. The reaction was shown to be coenzyme A dependent[2]. We have now shown that rabbit mammary gland extracts, prepared and assayed as described previously for rat mammary gland extracts[1], show the same fatty acid synthesizing activity. This activity per mg protein can be increased some 4–8 fold by precipitating the rabbit mammary gland extract by saturation with ammonium sulphate, followed by refractionation with ammonium sulphate between the limits of 35–60 % saturation. It is this preparation which has been used in the experiments described here, in which α-oxoglutarate has been replaced by DPNH, and fatty acid synthesis followed in the spectrophotometer, by observing the utilization of DPNH.

The assay system contains: 30 μM ATP, 2 μM CoA, 0.4–0.8 μM DPNH, 30 μM cysteine, 100 μM Tris buffer, pH 9.4, 20 μM $MgCl_2$, 5–60 μM acetate and about 10 mg protein. The final volume of the reaction mixture is 3.0 ml, the final pH is 8.0. The mixture is incubated for 5 minutes at 38°C, to eliminate a small blank reaction utilizing DPNH, and then transferred to a cuvette read at 340 mμ, and the reaction started by the addition of acetate (Fig. 1). An immediate decrease in optical density occurs, which is not observed in the absence of acetate, CoA or ATP, and which only proceeds at one third the rate in the absence of cysteine. Very little fatty acid synthesis occurs in the absence of DPNH, as measured by the incorporation of labelled acetate into fatty acid.

The course of fatty acid synthesis, as measured by the incorporation of label, follows closely the utilization of DPNH (Fig. 2). In this experiment ^{14}C labelled acetate was used, and the fatty acids isolated and estimated as previously described[1]. During the early part of the reaction the DPNH: acetate ratio is about 6:1, but falls as the reaction proceeds. When half the DPNH has been utilized the reaction comes to a standstill, independent of the starting concentration of DPNH