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.

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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 It is this preparation which has been used in the experiments described here, in which α-oxoglutarate has been replaced by DPNH, and fatty acid synthesis followed in the spectrophotometer, by observing the utilization of DPNH.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

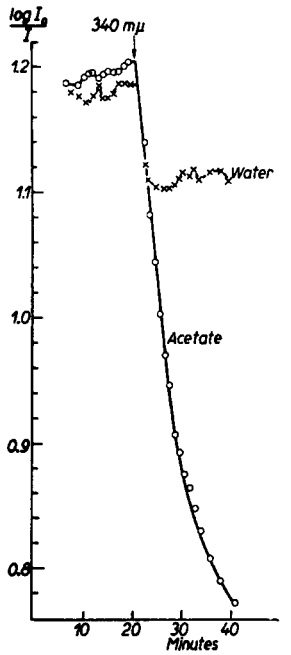

Fig. 1. Oxidation of DPNH by the fatty acid synthesizing enzyme system. The reaction was started by the addition at the arrow of 60 μM K-acetate to the assay system.

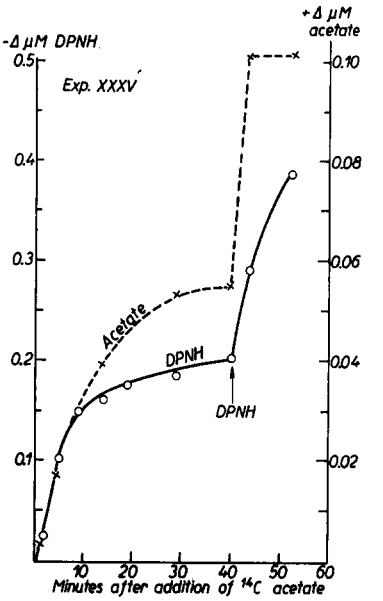

Fig. 2. Utilization of DPNH and incorporation of ^{14}C-acetate into fatty acids. At the arrow (40 min) a second addition of 0.4 μM of DPNH was made.

or of protein. The reaction can be re-started immediately by the addition of further DPNH, but not by the addition of more CoA, protein, or of synthetically prepared crotonyl-CoA. During the early part of this second phase the DPNH:acetate ratio approaches 2:1, suggesting the utilization of pre-formed intermediates.

The nature of these intermediates was further investigated in an experiment, similar to that shown in Fig. 2, in which these intermediates were isolated as their hydroxamates[3] at various intervals of time after the start of the reaction. These hydroxamates were submitted to chromatography in an octyl alcohol-formic acid-water solvent system[4], and the paper strips so obtained scanned for radioactivity in a self-recording scanner. This showed: (i) initially a 70 and 5 fold excess of acetyl-CoA and β-hydroxybutyryl-CoA respectively over all other intermediates, the latter accounting for the high DPNH:acetate ratio; (ii) an accumulation of crotonyl-CoA, butyryl-CoA and of β-hydroxyoctanoyl-CoA (identified by its R_F value) and the appearance of a little octanoyl-CoA during the first phase of the reaction; (iii) a marked increase in butyryl-CoA and octanoyl-CoA after the second addition of DPNH, at the expense of crotonyl-CoA and β-hydroxy-octanoyl-CoA; (iv) the appearance of very little hexanoyl-CoA, and then only at the end of the second phase of the reaction.

This suggests that two possible rate limiting steps operating in the system, either singly or together, might be (i) the step from β-hydroxy-acyl-CoA to unsaturated fatty acyl-CoA possibly due to inhibition by β-keto-acyl-CoA; (ii) the step from unsaturated fatty acyl-CoA to saturated fatty acyl-CoA, possibly reflecting a defect in the electon transfer mechanism at the point.

The mechanism of electron transfer from DPNH to unsaturated fatty acyl-CoA is still obscure. Previous work reported using highly purified preparations of the enzyme that catalyses this step shows that this enzyme is incapable of reacting with DPNH[5,6]. In the mammary gland preparation this electron transfer does not involve DPNH-cytochrome *c* reductase, for reduced cytochrome *c* and DPN will not substitute for DPNH, and the preparation contains negligible DPNH-cytochrome *c* reductase activity of the type described by MAHLER *et al.*[7]. However, our preparation shows very marked diaphorase activity using 2:6-dichlorophenol-indophenol as electron acceptor, the pH optimum of this reaction being at 6.2. At pH 8.0 the rate of DPNH oxidation in the presence of the dye is still five times greater than that observed in the fatty acid synthesizing system. Possibly this diaphorase activity represents the "short-circuiting" by the

dye of a system responsible for the transfer of electrons from DPNH to the enzyme that is responsible for catalyzing the step from unsaturated to saturated fatty acyl-CoA. This would represent an alternative pathway for electrons to that provided through DPNH-cytochrome *c* reductase and the cytochrome system.

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DPN- und TPN-spezifische Nukleosidasen in Erythrozyten

Im Verlauf von Untersuchungen über die Reifung von Kaninchen-Erythrozyten, in denen das Verhalten von DPN und TPN untersucht wurde, zeigte sich, dass trotz schneller DPN-Spaltung durch Erythrozyten-Hämolysate, TPN nur sehr langsam angegriffen wird. Dies gilt für den Abbau von zelleigenen, ebenso wie von zugesetzten Pyridinnukleotiden. In Retikulozyten war die DPN-Spaltung um weniges, die TPN-Spaltung um vieles höher als in Normozyten. Diese Beobachtungen legten den Gedanken nahe, dass in Erythrozyten spezifische Fermente für die DPN- und TPN-Spaltung existieren. Es handelt sich um Nukleosidasen, wie sich aus dem Vergleich zwischen den enzymatisch-optisch bestimmten DPN- (mit krist. Alkoholdehydrase[1]) und TPN- (mit Zwischenferment[2]) Werten einerseits und den Nikotinsäureamid-Ribose-Werten (fluorometrisch[3]) andererseits ergibt. Beide Nukleosidasewirkungen sind durch Nikotinsäureamid hemmbar.

Es gelingt durch Extraktion mit $NaHCO_3$-Lösung aus Azetonpulver von Retikulozytenstroma eine lösliche DPN-Nukleosidase abzutrennen. Die Lösung zeigt keine TPN-spaltende Aktivität, während die Suspension noch TPN spaltet (Tabelle I).

TABELLE I

ABTRENNUNG DER DPN- VON DER TPN-NUKLEOSIDASE

0.05 ml Enzymlösung (entspricht der Suspension bzw. Extrakt von 0.03 ml urspr. Zellen); Veronal-Azetat-Puffer pH 7.4; 190 μg DPN; 62μg TPN; Endvolumen 1.1 ml; Temperatur 37°C; Inkubationsdauer für DPN 30, für TPN 90 Minuten. Nichtgespaltenes DPN bzw. TPN wurde im Kochsaft fluorometrisch bestimmt[3].

Fraktion	*DPN*	*TPN*
$NaHCO_3$-Suspension des Azetonpulvers	145*	34
Überstehende Lösung der $NaHCO_3$-Suspension	40	0

* gespaltene Pyridinnukleotide in μg.

Somit ist die Existenz spezifischer Nukleosidasen für DPN und TPN sehr wahrscheinlich gemacht worden. Es bleibt zu untersuchen, ob ihr Vorkommen auf die roten Blutzellen beschränkt ist.

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