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HYDROGEN TRANSFER IN FATTY ACID SYNTHESIS BY RAT LIVER AND MAMMARY-GLAND CELL-FREE PREPARATIONS STUDIED WITH TRITIUM-LABELED PYRIDINE NUCLEOTIDES AND GLUCOSE

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SUMMARY

Incorporation of tritium from position 4 of the pyridine ring of TPNH and DPNH into fatty acids was studied in systems composed of particle-free supernatant fractions plus microsomes prepared from rat livers and in particle-free supernatant fractions from lactating rat mammary glands.

1. Tritium from both nucleotides was incorporated into fatty acids, but that from TPNH was preferentially incorporated.

2. Tritium transfer from both α- and β-configurations on position 4 of TPNH was demonstrated, but that from the α- exceeded that from the β-configuration.

3. DPN^+ inhibited tritium transfer from [β-^{3}H]TPNH but not from [α-^{3}H]TPNH. The transfer from [β-^{3}H]DPNH was inhibited by TPN^+, and the transfer was negligible from [α-^{3}H]DPNH.

4. Two methods are presented for calculating integral changes in tritium activities on TPN^+ when the nucleotide is continuously oxidized and reduced.

5. Under certain conditions, in the mammary-gland system the value for

$$\frac{\text{tritium transfer from TPNH to fatty acids}}{^{14}\text{C conversion from [1-}^{14}\text{C]acetate to fatty acids}}$$

approached the theoretical value of two.

6. No tritium from pyridine nucleotides nor ^{14}C from acetate was incorporated into fatty acids in the absence of citrate.

7. Tritium from [1-^{3}H]glucose 6-phosphate was efficiently transferred through TPN^+ to fatty acids by lactating rat mammary-gland preparations.

INTRODUCTION

The biosynthesis of even-chain fatty acids by homogenate fractions devoid of mitochondria involves the intermediate condensation of malonyl-CoA with acetyl-CoA and the simultaneous removal of the carboxyl carbon of the malonyl-CoA[1-6]. The

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene.

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resulting fatty acid derivative[5,6] is saturated by two reductive steps, one leading to the formation of a β-hydroxyacyl-thiol ester, and the other to the saturated acyl derivative. TPNH utilization in the first reductive step has been demonstrated[5]. Studies of WAKIL *et al.*[6,9], LYNEN[5] and ABRAHAM *et al.*[7,8] indicated that the enzyme catalyzing the second step can utilize either TPNH or DPNH as coenzyme. The former is a more efficient hydrogen donor than the latter[5–10].

The purpose of the present investigation was to determine whether hydrogen from the four position of the pyridine rings of TPNH and DPNH can be transferred to fatty acids during their synthesis from acetate by homogenate fractions prepared from two types of rat tissues: lactating mammary gland and normal liver. VENNESLAND *et al.*[11–13] have shown that isotopic hydrogen introduced into the four position of the pyridine ring of DPN^+ or TPN^+ can be made to appear in either the α- or β-configuration. We have studied the efficiency of hydrogen transfer to fatty acids from these two isotopic isomers. A preliminary report of some of the findings has appeared[10].

EXPERIMENTAL

Animals and their treatment

Mammary glands were excised from lactating rats (300–400) g that had suckled at least five pups for 18–20 days. Livers were taken from normal male rats (200–250 g). All rats were of the Long-Evans strain, and had been raised on an adequate stock diet (Diablo Labration).

Tissue homogenates were prepared in 0.25 M sucrose and fractionated as described previously[8].

Substrates

Labeled substrates: Potassium [1-^{14}C]acetate was prepared by the Grignard reaction[14]. Citrate labeled with ^{14}C in the tertiary carboxyl position was synthesized in this laboratory by the reaction of [^{14}C]hydrogen cyanide with diethyl-β-ketoglutarate, and isolated as the calcium salt[15]. It was converted to the tripotassium salt with the aid of Dowex-50 K^+ and used in that form. [1-^{14}C]Glucose 6-phosphate or [6-^{14}C]glucose 6-phosphate or [1-^{3}H]glucose 6-phosphate was prepared by phosphorylation of the corresponding labeled glucose with yeast hexokinase and ATP[16,17]. These singly-labeled phosphorylated hexoses were isolated as their barium salts by the method of UMBREIT *et al.*[18], and converted to the potassium salts by the use of Dowex-50 K^+.

Oxidized DPN (DPN^+) and TPN (TPN^+) were labeled with tritium in the four position of the pyridine ring by a modification of the method outlined by SAN PIETRO[19] for labeling pyridine nucleotides with deuterium from D_2O. The ^{3}H-labeled water used in the preparation of the labeled nucleotides contained 8 C in 2 ml, and was purchased from the New England Nuclear Corporation. The oxidized ^{3}H-labeled nucleotides, which were purified by several lyophilizations and water–acetone precipitations, were finally obtained as a dry powder. [4-^{3}H]DPN^+ and [4-^{3}H]TPN^+, denote label of ^{3}H at position 4 of the pyridine ring.

The [4-^{3}H]DPN^+ and [4-^{3}H]TPN^+ were reduced with purified enzyme preparations, and the amounts of reduced nucleotides formed were determined from the difference in absorbancy at 340 mμ of the two labeled nucleotides before and after

enzymic treatment[20]. Care was taken to avoid excess amounts of substrate in the preparation of the reduced labeled nucleotides, and the reactions were taken as far as possible toward completion. VENNESLAND *et al.*[11] have shown that the glucose 6-phosphate dehydrogenase system is β-specific in the reduction of TPN^+, transferring the hydrogen from the substrate glucose 6-phosphate to the β-configuration on the four position of the pyridine ring of TPN^+. Since the nucleotide [4-^{3}H]TPN^+ already contained isotope in the four position of the pyridine ring, the introduction of unlabeled hydrogen into the β-stereo-configuration by the glucose 6-phosphate dehydrogenase system yielded a reduced nucleotide with ^{3}H in the α-position. This reduced nucleotide is designated here [α-^{3}H]TPNH.

[β-^{3}H]TPNH was prepared from [4-^{3}H]TPN^+ with the aid of isocitric dehydrogenase and isocitrate[12].

[4-^{3}H]DPN^+ was reduced either with yeast alcohol dehydrogenase and ethanol[13], thereby yielding [β-^{3}H]DPNH, or with glutamic dehydrogenase and L-glutamate, yielding [α-^{3}H]DPNH[12].

Unlabeled substrates: CoA, TPN^+, DPN^+ and ATP were purchased from Pabst Laboratories; disodium glucose 6-phosphate and L-glutamic acid from California Corporation for Biochemical Research; reduced glutathione from Sigma Chemical Company; and glycylglycine from Nutritional Biochemical Corporation.

Purified enzymes: Yeast hexokinase and isocitric dehydrogenase were purchased from Sigma Chemical Company; glucose 6-phosphate dehydrogenase and glutamic dehydrogenase from C. F. Boehringer and Sons (Germany) and alcohol dehydrogenase from Nutritional Biochemical Corporation.

Incubation procedures

The composition of the two basic incubation media used is given below:

Compound	*Lactating mammary-gland system (μmoles)*	*Liver system (μmoles)*
Glycylglycine–KOH buffer	120 (pH 7.2)	120 (pH 7.5)
$KHCO_3$	5	5
$MgCl_2$	35	35
$MnCl_2$	1	1
GSH	30	30
ATP	5	24
CoA	0.05	0.05
Potassium acetate	3	3

Other additions are recorded in the tables and figures. All substrates and cofactors were contained in a volume of 1.0 ml. In the mammary-gland experiments the reactions were started by addition of 0.75 ml of the particle-free supernatant fraction obtained from lactating rat mammary-gland homogenates which had been centrifuged at 80000 × *g* for 45 min after removal of nuclei, cell debris, fat and mitochondria[8]. The final volume of the incubation mixture was therefore 1.75 ml. In the liver experiments, the reactions were started by addition of 0.75 ml of the particle-free supernatant fraction plus 0.05 ml of a microsomal suspension, both prepared from the

same liver homogenized in 0.25 M sucrose[7]. The total volume in this case was 1.80 ml. The reaction mixtures were incubated at 30° with air as the gas phase.

Analytical procedures

Fatty acids[8] and CO_2 (see ref. 7) were isolated as described elsewhere. Protein was determined by the biuret method of GORNALL *et al.*[21].

Radioactivity in [^{14}C]fatty acids and ^{3}H compounds was determined with the automatic Packard Tri-Carb liquid scintillation spectrometer. The scintillation mixture used for assay of [^{14}C]fatty acids was composed of 0.5–1.0 ml hexane, in which the fatty acids were dissolved, and 10 ml toluene containing 24 mg of PPO. The counting efficiency was between 50 and 60 %. The scintillation mixture used in the assay of the ^{3}H-labeled fatty acid consisted of 0.5–1.0 ml of hexane containing the fatty acid and 10 ml toluene in which were dissolved 48 mg of PPO and 1 mg of POPOP. The counting efficiency in this case was 18 %. The scintillation mixture used for the assay of ^{3}H-labeled, water-soluble compounds consisted of 0.02 ml water in which the labeled compounds were dissolved, 2.0 ml of absolute methanol, and 8.0 ml of toluene containing 48 mg PPO and 1 mg POPOP. The counting efficiency was 10 %.

RESULTS

In order to study incorporation of tritium from a pyridine nucleotide into a compound in a multi-enzyme system we must know the rate at which the pyridine nucleotide is oxidized. The oxidation of TPNH has been studied[16] by the use of isotope techniques that involved the formation of $^{14}CO_2$ from [1-^{14}C]glucose 6-phosphate, [6-^{14}C]citrate and [4-^{14}C]malate. These techniques enabled us to estimate TPNH formation from each of those compounds, even when they were combined with each other, a procedure not possible with spectrophotometric methods. As shown previously[22], TPNH oxidation is the rate-limiting step in the oxidation of glucose 6-phosphate, malate and citrate, in both the lactating mammary gland and the liver homogenate systems, under conditions wherein the amount of TPN^+ or TPNH added was small compared with the TPNH-generating capacity of the dehydrogenases present.

Fatty acid synthesis in the lactating rat mammary gland[8,23] and rat-liver systems[7,23] proceeds only when isocitrate is simultaneously oxidized. Thus, an integral change of ^{3}H on [4-^{3}H]TPN^+ must be considered when evaluating incorporation of ^{3}H from labeled pyridine nucleotides into fatty acids. Hence, the results are presented first as a direct percentage of the ^{3}H on labeled nucleotides incorporated into fatty acids by the two homogenate systems, and are then calculated, taking into consideration the integral change of specific activity of the labeled hydrogen on those nucleotides.

Experiments with particle-free supernatant fractions prepared from mammary gland of lactating rats in the presence of citrate:

Incorporation of tritium from ^{3}H-labeled reduced pyridine nucleotides into fatty acids

Experiments with [α-^{3}H]TPNH and [β-^{3}H]TPNH: The transfer of ^{3}H from [α-^{3}H]TPNH and [β-^{3}H]TPNH to fatty acids as a function of time is shown in Fig. 1. Incorporation of ^{3}H into fatty acids from [α-^{3}H]TPNH was more than twice that from [β-^{3}H]TPNH at all time intervals. In the presence of DPN^+, the transfer of

^3H from [β-^3H]TPNH into fatty acids was slightly decreased, whereas the transfer from the α-position was increased.

Experiments with [α-^3H]DPNH and [β-^3H]DPNH: The time course of tritium transfer from [α-^3H]DPNH and [β-^3H]DPNH to fatty acids is shown in Fig. 2. Only about 1 % of the tritium of the former was incorporated into fatty acids in 1 h. When compared with the incorporation of tritium from [α-^3H]TPNH or [β-^3H]TPNH, the values obtained with [α-^3H]DPNH are very small. The addition of TPN^+ to the incubation medium caused only a small depression in the incorporation.

About 3 % of the tritium from [β-^3H]DPNH was incorporated into fatty acids (Fig. 2), about the same amount as that transferred from [β-^3H]TPNH. In this case, the addition of TPN^+ resulted in a marked depression (from about 3 to 1 %) of the ^3H transferred to fatty acids. It should be noted that the addition of DPN^+ decreased incorporation of ^3H from [β-^3H]TPNH into fatty acids by about 25 % (from 4 to 3 % (Fig. 1)). Apparently, a β-specific reductive step in fatty acid synthesis can be performed with either TPNH or DPNH, but the former is somewhat more readily utilized

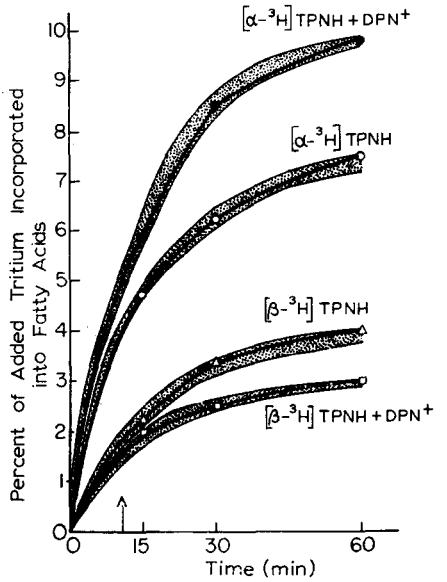

Fig. 1. Per cent of ^3H from [α-^3H]TPNH and [β-^3H]TPNH incorporated into fatty acids by supernatant fractions prepared from lactating rat mammary-gland homogenates. To the standard incubation medium were added 25 μmoles of potassium citrate and 1 μmole of either [α-^3H]TPNH or [β-^3H]TPNH ($4 \cdot 10^5$ counts/min). Unlabeled DPN^+ (1 μmole) was added as indicated. The closely agreeing percentages (shown by ●—●, ○—○, △—△, □—□) of ^3H on the nucleotide transferred to fatty acids obtained in 3 experiments with supernatant fractions were averaged and plotted. The shaded areas represent the standard error limits of the loss of ^3H from TPNH calculated from oxidation studies as discussed on p. 251 and 252 (see also ref. 22). Maximum incorporation of ^3H into fatty acids for each case studied was taken as 100 %. The arrow on the time scale represents one half-life time of TPNH (for explanation see text) which was calculated from either (a) the oxidation studies with TPNH-generating substrates or (b) the slope of the above curves.

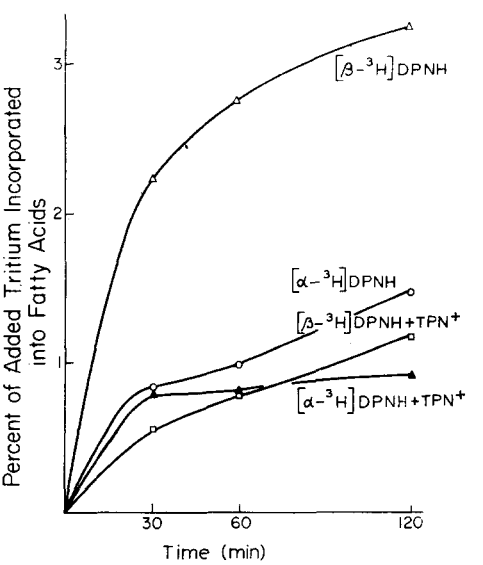

Fig. 2. Per cent incorporation of ^3H from [α-^3H]DPNH and [β-^3H]DPNH into fatty acids by supernatant fractions obtained from lactating rat mammary-gland homogenates. To the standard incubation medium were added 25 μmoles of potassium citrate and 1 μmole of either [α-^3H]DPNH or [β-^3H]DPNH ($4 \cdot 10^5$ counts/min). Unlabeled TPN^+ (1 μmole) was added as indicated. See Fig. 1 and text for other details.

Incorporation of ^3H from ^3H-labeled oxidized pyridine nucleotides into fatty acids

Experiments with [4-^3H]TPN$^+$: The percentages of the tritium of the [4-^{3}H]-TPN$^+$ incorporated into fatty acids, as a function of time, are presented in Fig. 3. When citrate was the sole TPNH-generating substrate* (curve B), ^{3}H incorporation into fatty acids was about 9% after 2 h of incubation. The addition of DPN$^+$ did not change significantly the rate of incorporation (curve A).

When the sole TPNH-generating substrate was glucose 6-phosphate**, virtually no tritium was transferred from [4-^{3}H]TPN$^+$ to fatty acids. This was not unexpected, since [1-^{14}C]acetate was not converted to fatty acids by our lactating mammary-gland system in the absence of added citrate[8,24]. In contrast to this lack of ^{3}H incorporation into fatty acids when glucose 6-phosphate served as sole TPNH-generating substrate, the addition of citrate to the medium, which already contained glucose 6-phosphate, did result in a considerably higher incorporation of ^{3}H from [4-^{3}H]TPN$^+$ into fatty

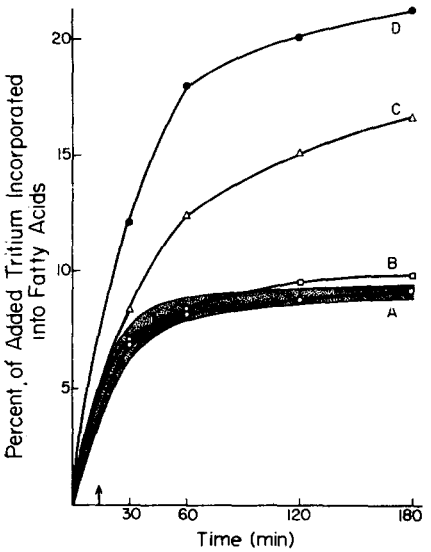

Fig. 3. Per cent incorporation of ^{3}H from [4-^{3}H]TPN$^+$ into fatty acids by lactating rat mammary-gland supernatant fractions. To the standard incubation medium were added 25 μmoles of potassium citrate and 1 μmole of [4-^{3}H]TPN$^+$ ($4 \cdot 10^5$ counts/min; curve B). In addition, 1 μmole of unlabeled DPN$^+$ (curve A), 10 μmoles of glucose 6-phosphate (curve C), 1 μmole of unlabeled DPN$^+$ and 10 μmoles of glucose 6-phosphate (curve D) were added. ●—●, △—△, □—□, ○—○, the percentages of ^{3}H transferred to fatty acids. For explanation of the shaded area and other details see Fig. 1. The arrow on the time scale represents one half-life time of TPNH.

acids than was observed with citrate alone (curve C). Under these conditions, *i.e.*, when glucose 6-phosphate and citrate were present***, the addition of DPN$^+$ resulted in a further increase of ^{3}H transfer from [4-^{3}H]TPN$^+$ to fatty acids (curve D). In the experiments with [α-^{3}H]TPNH (Fig. 1) the addition of DPN$^+$ increased the incorporation of tritium into fatty acids. In the presence of glucose 6-phosphate and citrate (as TPNH-generating substrates), the conversion of the ^{3}H of [4-^{3}H]TPN$^+$ to fatty acids was increased in the presence of DPN$^+$. Therefore, [α-^{3}H]TPNH was apparently the preferentially formed pyridine nucleotide in the experiments in which both glucose 6-phosphate and citrate were present.

Experiments with [4-^3H]DPN$^+$: Fig. 4 shows the results of experiments in which the conversion of ^{3}H from the four position of the pyridine ring of [4-^{3}H]DPN$^+$ into

* In this case ^{3}H will appear only in the β-position of TPNH.
** In this case ^{3}H will appear only in the α-position of TPNH.
*** In this case ^{3}H will appear in both positions, but preferentially in the α-position.

fatty acids was studied as a function of time. In all cases the transfer of ^3H from [4-^3H]DPN$^+$ was less than one tenth that observed with [4-^3H]TPN$^+$. The addition to the medium of glucose 6-phosphate, which already contained citrate, significantly decreased ^3H incorporation into fatty acids from [4-^3H]DPN$^+$. This is in contrast to the observed increase in ^3H incorporation into fatty acids from [4-^3H]TPN$^+$ under these same conditions (Fig. 3).

The low incorporation of ^3H from [4-^3H]DPN$^+$ is not surprising in view of the fact that no DPNH-generating substrate was added to the incubation media. The effect of DPNH-producing substrates upon the conversion of ^3H from [4-^3H]DPN$^+$ into fatty acids was not studied because knowledge concerning oxidation–reduction velocities of this pyridine nucleotide is not available at present.

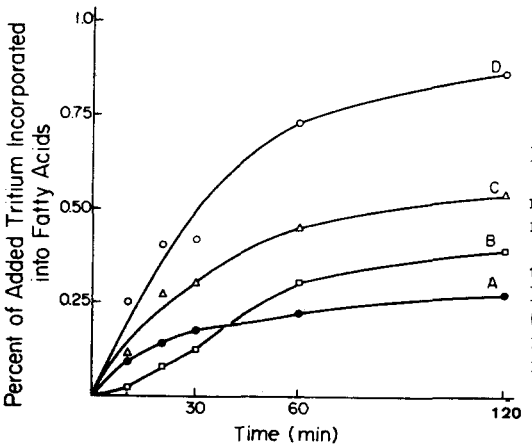

Fig. 4. Per cent incorporation of ^3H from [4-^3H]DPN$^+$ into fatty acids by lactating rat mammary gland supernatant fractions. 25 μmoles of potassium citrate and 1 μmole of [4-^3H]DPN$^+$ ($4 \cdot 10^5$ counts/min) were added to the standard incubation mixture (curve D). In addition, 10 μmoles of glucose 6-phosphate (curve A), or 1 μmole of unlabeled TPN$^+$ plus 10 μmoles of glucose 6-phosphate (curve B), or 1 μmole of unlabeled TPN$^+$ (curve C) were added.

Incorporation of ^3H from [1-^3H]glucose 6-phosphate into fatty acids

In order to study the transfer of ^3H from [1-^3H]glucose 6-phosphate to fatty acids via TPN$^+$ in experiments with multienzyme systems, it must be shown that only the ^3H, and separated from the original glucose carbon, is incorporated into fatty acids. This was demonstrated by incubating [1-^{14}C]glucose 6-phosphate with particle-free supernatant fractions obtained from the mammary glands of lactating rats with and without citrate. In such experiments no ^{14}C was recovered in the isolated fatty acids (curve A, Fig. 5). Thus ^3H from [1-^3H]glucose 6-phosphate was not transferred to fatty acid in the form of acetyl-CoA.

The oxidation of 1 mole of [1-^3H]glucose 6-phosphate to pentose phosphate and CO_2 involves the reduction of 2 moles of TPN$^+$, yielding 1 mole of [^3H]TPN and 1 mole of TPNH. This is borne out by our finding that no gluconic acid 6-phosphate accumulated during the oxidation of glucose 6-phosphate under our assay conditions. Thus, in order to obtain the actual amounts of hydrogen from [1-^3H]glucose 6-phosphate incorporated into fatty acid via TPNH, the values found for tritium incorporation must be multiplied by 2.

Almost no ^3H was incorporated into fatty acids from [1-^3H]glucose 6-phosphate in the absence of citrate (curve B, Fig. 5). The incorporation of hydrogen from [1-^3H]glucose 6-phosphate into fatty acids in the presence of both citrate and TPN$^+$ was somewhat higher than that of ^{14}C from [1-^{14}C]acetate under the same incubation

conditions (compare curve E with curve D). When DPN^+ was added to the system containing glucose 6-phosphate, acetate, citrate and TPN^+, the conversion of hydrogen from [1-^{3}H]glucose 6-phosphate (curve G) and ^{14}C from [1-^{14}C]acetate to fatty acids (curve F) was significantly increased. Again, hydrogen incorporation was considerably higher than ^{14}C incorporation.

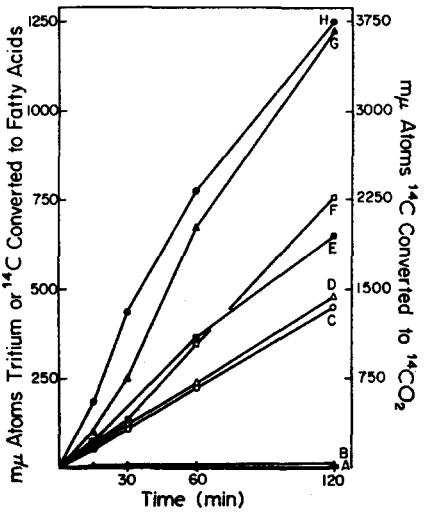

Fig. 5. A comparison between hydrogen transfer from [1-^{3}H]glucose 6-phosphate and ^{14}C conversion from [1-^{14}C]acetate and [1-^{14}C]glucose 6-phosphate to fatty acids by the lactating mammary-gland system. The following were added to the standard incubation medium:

Curve	*Compound added*						*Radioactive products isolated*
	Citrate (25 μmoles)	*Glucose 6-phosphate (10 μmoles)*	*Unlabeled*			*[1-^{14}C]-Acetate ($5 \cdot 10^5$ counts/min)*	
			DPN^+ (1 μmole)	*TPN^+ (1 μmole)*	*TPNH (1 μmole)*		
A	+(Unlabeled)	[1-^{14}C]	None	+	None	None	[^{14}C]fatty acids
B	None	[1-^{3}H]	+	None	+	None	[^{3}H]fatty acids
C	[6-^{14}C]	+(Unlabeled)	None	None	+	None	$^{14}CO_2$
D	+(Unlabeled)	+(Unlabeled)	None	None	+	+	[^{14}C]fatty acids
E	+(Unlabeled)	[1-^{3}H]	None	+	None	None	[^{3}H]fatty acids
F	+(Unlabeled)	+(Unlabeled)	+	None	+	+	[^{14}C]fatty acids
G	+(Unlabeled)	[1-^{3}H]	+	None	+	None	[^{3}H]fatty acids
H	+(Unlabeled)	[1-^{14}C]	+	None	+	None	$^{14}CO_2$

Each value is the average of results obtained in 2 experiments. Protein concentration was 5.7 and 5.9 mg per incubation mixture, and the final incubation volume was 1.75 ml.

The generation of TPNH from [1-^{14}C]glucose 6-phosphate (curve H) and from [6-^{14}C]citrate (curve C) is also shown in Fig. 5 as a function of time. The slope depicting incorporation of ^3H from [1-^{3}H]glucose 6-phosphate (curve G) into fatty acids followed closely that for oxidation of [1-^{14}C]glucose 6-phosphate (curve H).

Comparison of the incorporation of ^3H from [^3H]pyridine nucleotides and [1-^3H]glucose 6-phosphate into fatty acids by lactating rat mammary gland and rat-liver preparations:

Experiments with ^3H-labeled reduced pyridine nucleotides

The results obtained in the experiments in which the ^3H-labeled reduced pyridine nucleotides were incubated with the mammary-gland supernatant fractions are recorded in Table I. Incorporation of isotope from both α- and β-forms of [^3H]TPNH and [^3H]DPNH into fatty acids by mammary-gland system was observed only when citrate was added to the medium.

TABLE I

INCORPORATION OF ^3H FROM [4-^3H]DPNH AND [4-^3H]TPNH, INTO FATTY ACIDS BY NORMAL RAT-LIVER HOMOGENATE PREPARATIONS (SUPERNATANT + MICROSOMES) AND BY PARTICLE-FREE, LACTATING RAT MAMMARY-GLAND HOMOGENATE FRACTIONS

0.75 ml particle-free supernatant fraction prepared from a 3:1 homogenate of liver in 0.25 M sucrose plus 0.05 ml of the microsomal suspension obtained from the same liver was incubated for 1 h at 30° in air. 0.75 ml of particle-free supernatant fraction prepared as above from the mammary gland of lactating rats was incubated as described above. In both cases, unless otherwise specified, the medium was composed of 120 μmoles of glycylglycine buffer (pH 7.5 for liver experiments and pH 7.2 for mammary-gland experiments), 5 μmoles $KHCO_3$, 35 μmoles $MgCl_2$, 1.0 μmole $MnCl_2$, 30 μmoles reduced glutathione, 0.05 μmole CoA, 24 μmoles ATP for liver experiments or 5 μmoles for mammary-gland experiments, 3 μmoles potassium acetate, and 1 μmole of the labeled or unlabeled pyridine nucleotides, all in a final volume of 1.80 ml for liver experiments and 1.75 ml for mammary-gland experiments. The labeled pyridine nucleotides contained about 1 μC ^3H/μmole, and potassium citrate was added as indicated below. Each value recorded below is the average, and its standard error, of the results of 5 experiments with 5 rats.

*Citrate**	*Pyridine nucleotides added*				*^3H recovered in isolated fatty acids (%)*	
	Labeled		*Unlabeled*		*Mammary-gland system*	*Liver system*
	[4-^3H]DPNH	*[4-^3H]TPNH*	*DPN+*	*TPN+*		
+		α-			7.5 ± 1.55	0.30 ± 0.05
+		α-	+		10.5 ± 1.17	0.31 ± 0.01
+		β-			3.9 ± 0.90	0.35 ± 0 03
+		β-	+		3.0 ± 0.81	0.33 ± 0.02
		α-			0.07 ± 0.02	
		β-			0.05 ± 0.02	
+	α-				2.2 ± 0.50	0.11 ± 0.01
+	α-			+	1.6 ± 0.30	0.09 ± 0.02
+	β-				3.6 ± 0.22	0.13 ± 0.02
+	β-			+	1.7 ± 0.25	0.09 ± 0.01
	α-				0.02 ± 0.004	
	β-				0.04 ± 0.01	

* 25 μmoles for mammary-gland experiments and 37.5 μmoles for liver experiments.

In the liver experiments no clear difference was observed in the percentages of ^3H from [α-^3H]TPNH and [β-^3H]TPNH incorporated into fatty acids. Nor was a difference discernible in the transfer of ^3H from the two steroisomers of [4-^3H]-DPNH. Both forms of the [4-^3H]TPNH, however, were about three times as efficient in donating ^3H as were the corresponding forms of [^3H]DPNH.

Experiments with ^3H-labeled oxidized pyridine nucleotides

The percentages of the incubated ^3H incorporated into fatty acids, under varying cofactor conditions, are recorded in Table II. In the absence of citrate, incorporation

of isotope from either oxidized nucleotide was negligible in the experiments with both tissue preparations. In the presence of citrate alone, ^3H transfer from [4-^3H]-TPN$^+$ was considerable in the experiments with both tissue preparations; under identical incubation conditions, incorporation of acetate carbon into fatty acids was also shown to require the presence of citrate or isocitrate[7,8,24]. The addition of glucose 6-phosphate to a medium which already contained citrate further enhanced ^3H transfer from [4-^3H]TPN$^+$ to fatty acids, particularly in the mammary-gland system.

TABLE II

INCORPORATION OF ^3H FROM [4-^3H]TPN$^+$ AND [4-^3H]DPN$^+$ INTO FATTY ACIDS BY NORMAL RAT-LIVER HOMOGENATE PREPARATIONS (SUPERNATANT + MICROSOMES) AND BY PARTICLE-FREE LACTATING RAT MAMMARY-GLAND HOMOGENATE FRACTIONS

For preparation of homogenate fractions and incubation conditions see Table I. Potassium citrate or potassium glucose 6-phosphate was added as indicated below. Each value recorded below is the average, and its standard error, of the results obtained in 5 experiments.

*Citrate**	*Glucose 6-phosphate (10 μmoles)*	*Pyridine nucleotides added*				*^3H recovered in isolated fatty acid (%)*	
		Labeled		*Unlabeled*		*Mammary-gland system*	*Liver system*
		[4-^3H]DPN$^+$	*[4-^3H]TPN$^+$*	*DPN$^+$*	*TPN$^+$*		
+			+			6.0 ± 1.4	1.33 ± 0.15
+			+	+		5.0 ± 1.5	1.32 ± 0.15
+	+		+			12.0 ± 2.4	1.60 ± 0.14
+	+		+	+		17.6 ± 2.3	1.70 ± 0.10
			+			0.05 ± 0.01	0.38 ± 0.02
	+		+			0.06 ± 0.01	0.32 ± 0.03
+		+				2.43 ± 0.5	0.09 ± 0.01
+		+			+	0.50 ± 0.1	
+	+	+				0.75 ± 0.15	0.06 ± 0.005
+	+	+			+	0.36 ± 0.05	0.05 ± 0.01
		+				0.03 ± 0.005	0.04 ± 0.006
	+	+				0.02 ± 0.004	

* 25 μmoles for mammary-gland experiments and 37.5 μmoles for liver experiments.

In the experiments with [4-^3H]DPN$^+$ significant transfer of ^3H to fatty acids occurred, again only in the presence of citrate. In the experiments with both liver and mammary-gland systems the amounts of ^3H transferred from [4-^3H]DPN$^+$ were much less than those from [4-^3H]TPN$^+$.

Experiments with [1-^3H]glucose 6-phosphate

Almost no ^3H was transferred from [1-^3H]glucose 6-phosphate to fatty acids in the absence of citrate (Table III). Under all conditions studied, the yields of [^3H]fatty acid in the experiments with the supernatant fractions of mammary glands exceeded by far those with the combined supernatant plus microsomes fractions of liver.

When TPNH was added to the incubation mixture, incorporation of ^3H from [1-^3H]glucose 6-phosphate into fatty acids was lower than when TPN$^+$ was added. This difference in the action of the two nucleotides apparently resulted from a diluting effect of the unlabeled hydrogen on the four position of the pyridine ring of TPNH. Here again the addition of DPN$^+$ to the medium containing TPNH increased the amount of ^3H transferred to fatty acids.

TABLE III

THE TRANSFER OF ^3H FROM [1-^3H]GLUCOSE 6-PHOSPHATE TO FATTY ACIDS BY NORMAL RAT-LIVER HOMOGENATE PREPARATIONS (SUPERNATANT + MICROSOMES) AND BY PARTICLE-FREE LACTATING RAT MAMMARY-GLAND HOMOGENATE FRACTIONS

For liver, 18.3 mg supernatant protein plus 3.6 mg microsomal protein (Expt. 1) and 16.8 mg supernatant protein plus 3.8 mg microsomal protein (Expt. 2) and for mammary gland 5.7 mg supernatant protein (Expt. 1) and 5.9 mg supernatant protein (Expt. 2) were incubated for 2 h. Preparation of homogenate fractions and other incubation conditions are recorded in Table I. Additional substrates and cofactors were added as indicated below. All media contained 10 μmoles of [1-^3H]glucose 6-phosphate ($2.9 \cdot 10^5$ counts/min for liver and $2.3 \cdot 10^5$ counts/min for mammary gland).

Additions to medium				*Liver*		*Mammary gland*	
*Citrate**	*TPN+ (0.5 µmole)*	*DPN+ (0.5 µmole)*	*TPNH (0.5 µmole)*	*Expt. 1 (counts/min)*	*Expt. 2 (counts/min)*	*Expt. 1 (counts/min)*	*Expt. 2 (counts/min)*
+	+	None	None	154	100	14 100	12 400
+	None	None	+	53	60	7 900	7 900
+	+	+	None	122	150	25 400	25 300
None	+	None	None	0	0	100	50

* 25 μmoles for mammary-gland experiments and 37.5 μmoles for liver experiments.

*Calculation of the amounts of hydrogen from reduced pyridine nucleotides incorporated into fatty acids**

The loss of ^3H from the labeled nucleotides depends upon the oxidation rate of the reduced forms. Dilution of the tritium activity is dependent upon the reduction rate of the oxidized form. Both the TPNH oxidation rate and the TPN^+ reduction rate with different substrates have been studied in this laboratory in liver and lactating mammary-gland systems under conditions in which TPN^+ was added in limited amounts[22]. In the presence of excess amounts of a TPNH-generating substrate, such as glucose 6-phosphate or citrate, the oxidation and reduction rates of the triphosphopyridine nucleotide are equal. Therefore, when an amount of triphosphopyridine nucleotide equal to the amount added is oxidized, the specific activity of the hydrogen on the four position of the pyridine ring will be half that of the initial specific activity. The time at which this occurs is defined here as the half-life time of the added [^3H]-triphosphopyridine nucleotide (see Figs. 1 and 3).

When the method based on $^{14}CO_2$ production from ^{14}C substrates was used to calculate TPNH generation in the lactating rat mammary-gland system[16,25], an average reduction of 936 mμmoles TPN^+/mg supernatant protein/2 h was obtained with glucose 6-phosphate and citrate as TPNH-generating substrates[22]. For example, in an experiment in which the amount of supernatant protein incubated was 8.8 mg, it can be calculated that 68.6 mμmoles of TPNH were generated/min. Since 1000 mμmoles of [4-^3H]TPNH were added to the medium at the start of the incubation period, the average half-life time of ^3H on the added [4-^3H]TPNH was 1000/68.6 or 14.6 min. The values differed somewhat among the animals studied.

The half-life time of TPNH was determined by still another method. Since

* ^3H incorporation into fatty acids from water can be ignored under the conditions of our studies: 1.8 ml of incubation mixture contain 100 mmoles of water; therefore, if the $4 \cdot 10^5$ counts per min of ^3H added as labeled nucleotide were all converted to water before being converted to fatty acids, the specific activity of the water would be $4 \cdot 10^5$ counts/min/100 mmoles, or 4 counts per μmole of water.

acetate incorporation into fatty acids was linear with time in the experiments with supernatant fractions of lactating mammary glands (Fig. 8), we have assumed, in the alternative calculation, that hydrogen incorporation from the four position of the pyridine ring of TPNH into fatty acids is also linear with time. Therefore the slope of the curves obtained when tritium incorporation into fatty acids from [α-^{3}H]-TPNH, [β-^{3}H]TPNH and [4-^{3}H]TPN$^+$ is plotted against time should reflect the changes in the specific activities of the hydrogen on the labeled triphosphopyridine nucleotide, provided that only one TPNH-generating substrate is used.

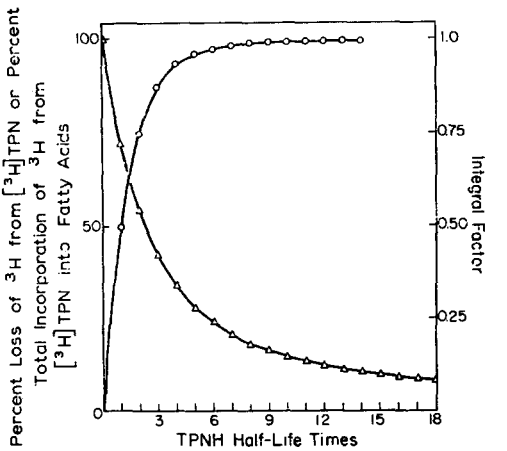

Fig. 6. Relation between loss of ^{3}H from a pyridine nucleotide and time, and between the integral change of specific activity on the pyridine nucleotide and time. O—O, represents ^{3}H loss and Δ—Δ represents $\int_0^T S_t = S_0\, e^{\frac{-\ln 2 t}{T}}$. For explanation see text.

The theoretical relation between the loss of ^{3}H from the four position of the pyridine ring of the labeled nucleotide and its half-life time is plotted in Fig. 6*.

* The well-established equation[26] for radioactive decay was applied:

$$S_t = S_0\, e^{-\lambda t} \tag{1}$$

where S_0 = initial specific activity, S_t = specific activity at time t, and λ = disintegration constant = $\ln 2/t_{1/2}$. Then by integrating between the limits of o and time T, we obtained the average specific activity, or $\bar{S}$, in the following manner:

$$\bar{S} = \frac{1}{T}\int_0^T S_t\, dt \tag{2}$$

or

$$= \frac{1}{T}\int_0^T S_0\, e^{-\lambda t}\, dt \tag{3}$$

then

$$= \frac{S_0}{T}\left(\frac{1}{\lambda} - \frac{e^{-\lambda T}}{\lambda}\right) \tag{4}$$

or

$$= \frac{S_0}{\lambda T}\left(1 - e^{-\lambda T}\right) \tag{5}$$

But substituting $x t_{1/2}$ for T (where x = the number of half-lives), we obtain:

$$\bar{S} = \frac{S_0}{x \ln 2}\left(1 - \frac{1}{2^x}\right) \tag{6}$$

The slope of this curve (Δ) must be identical with that depicting the theoretically expected maximum incorporation of ^3H into a compound. Thus, when these theoretical curves are constructed for each experiment and plotted as shown in Figs. 1 and 3, they should approximate closely the curves depicting the experimentally determined percentages of tritium from [α-^{3}H]TPNH, [β-^{3}H]TPNH and [4-^{3}H]TPN$^+$ incorporated into fatty acids. Fig. 1 shows that these theoretical considerations are met since the curves for the experimentally observed percentages of ^3H from [α-^{3}H]TPNH and [β-^{3}H]TPNH incorporated into fatty acids lie well within the error limits (shaded areas in Figs. 1 and 3) of the TPNH half-life curves as determined from the substrate oxidation studies[22]. However, in Fig. 3, the curves of the percentages of added ^3H in [4-^{3}H]TPN$^+$ incorporated into fatty acids, in the presence of both citrate and glucose 6-phosphate as TPNH-generating substrates, do not fall off at the same rate as does the curve calculated for tritium loss from the pyridine nucleotide. This is due, in part, to the shuttling of tritium between the α- and β-configuration on the four position of the pyridine ring of TPNH. This shuttling, which results from the fact that both glucose 6-phosphate oxidizing enzymes are β-specific, and the isocitric dehydrogenase is α-specific, slows down the decline in the specific activity of the ^3H on the pyridine nucleotide.

It should be recalled that, in the studies with [α-^{3}H]TPNH and [β-^{3}H]TPNH recorded above, the extent of incorporation of ^3H from [α-^{3}H]TPNH into fatty acids by supernatant fractions of lactating rat mammary glands was more than twice that observed from [β-^{3}H]TPNH. In this preparation we have shown: (a) that the conversion of [1-^{14}C]glucose 6-phosphate to $^{14}CO_2$ is much more rapid than the conversion of [6-^{14}C]citrate to $^{14}CO_2$ (see ref. 22); (b) that the enzymes concerned with the oxidation and hence the production of TPNH from glucose 6-phosphate, are more active than those that generate TPNH from citrate[22]. Hence, another reason for the higher incorporation of ^3H from [4-^{3}H]TPN$^+$ into fatty acids in the presence of glucose 6-phosphate and citrate must lie in the β-specific reduction of [4-^{3}H]TPN$^+$ shifting the ^3H into the α-position as a result of the preferential oxidation of glucose 6-phos-

TABLE IV

CALCULATED CHANGES IN ACTIVITIES OF ^3H AT THE FOUR POSITION OF THE PYRIDINE RING OF TPN WITH TIME

Half-life times of TPNH	*Estimated integral change factor*	*Calculated* ^3H activity at carbon 4 on pyridine ring of TPN$^+$ ($1 \cdot 10^5$ counts/min)*
0	1	4.00
0–1	0.725	2.90
0–2	0.545	2.18
0–3	0.423	1.69
0–4	0.340	1.36
0–5	0.280	1.12
0–6	0.240	0.96
0–7	0.205	0.82
0–8	0.181	0.73
0–9	0.163	0.65
0–10	0.145	0.58

* Determined from Eqn. 6. See footnote on p. 252.

phate. On the other hand, when citrate is the sole TPNH-generating substrate from [4-^{3}H]TPN$^+$, ^{3}H will appear only in the β-position. Therefore a correspondence in the slopes of curves A and B shown in Fig. 3 with the slope of the curve (Fig. 6) calculated for the ^{3}H lost from the pyridine nucleotides was not unexpected.

The change in average specific activity of ^{3}H on the pyridine nucleotide due to loss of isotopic hydrogen from the four position of the pyridine ring during the incubation period was calculated for each experiment as the integral of the equation: $S_t = S_0\, e^{-\lambda t}$ (see ref. 26). Examples of this type of calculation are recorded in Table IV. The last column shows the average counts/min of tritium on the pyridine nucleotide in relation to the half-life time of TPNH.

The amounts of hydrogen incorporated into fatty acids from [α-^{3}H]TPNH and [β-^{3}H]TPNH (shown in Fig. 7) and from [4-^{3}H]TPN$^+$ (shown in Fig. 8) were calculated

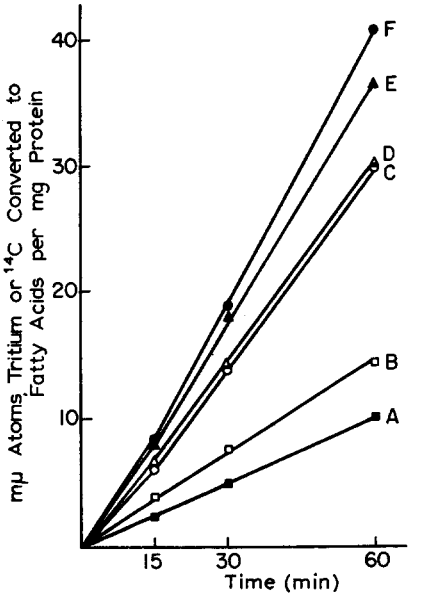

Fig. 7. Comparison between the incorporation of ^{14}C from [1-^{14}C]acetate and the calculated hydrogen transfer from [α-^{3}H]TPNH and [β-^{3}H]TPNH into fatty acids by supernatant fraction of lactating mammary glands. In addition to the standard incubation mixture and 25 μmoles of potassium citrate, the following were added: 1 μmole of [β-^{3}H]TPNH ($4\cdot10^5$ counts/min) and 1 μmole of unlabeled DPN$^+$ (curve A); 1 μmole of [β-^{3}H]TPNH ($4\cdot10^5$ counts/min) (curve B); 1 μmole of [α-^{3}H]TPNH ($4\cdot10^5$ counts/min) (curve C); potassium [1-^{14}C]acetate ($5\cdot10^5$ counts/min), 1 μmole of unlabeled TPNH and 1 μmole of unlabeled DPN$^+$ (curve D); potassium [1-^{14}C]acetate ($5\cdot10^5$ counts/min) and 1 μmole of unlabeled TPNH (curve E); and 1 μmole of [α-^{3}H]TPNH ($4\cdot10^5$ counts/min) and 1 μmole of unlabeled DPN$^+$ (curve F).

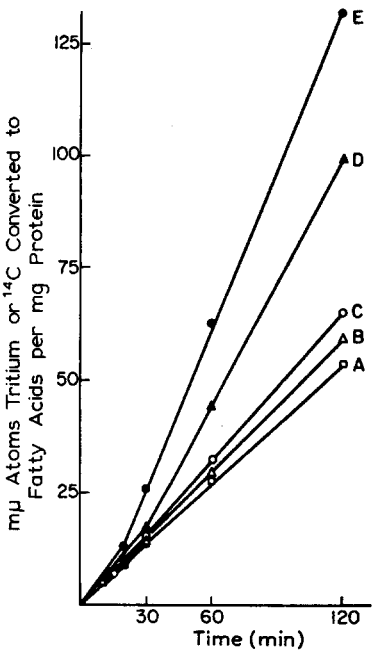

Fig. 8. Comparison between the incorporation of ^{14}C from [1-^{14}C]acetate and the calculated hydrogen transfer from [4-^{3}H]TPN$^+$ into fatty acids by the lactating mammary-gland system. In addition to the standard incubation mixture and 25 μmoles of potassium citrate, the following were added: 1 μmole of [4-^{3}H]TPN$^+$ ($4\cdot10^5$ counts/min) and 1 μmole of unlabeled DPN$^+$ (curve A); 1 μmole of [4-^{3}H]-TPN$^+$ ($4\cdot10^5$ counts/min) (curve B); potassium [1-^{14}C]acetate ($5\cdot10^5$ counts/min), 1 μmole of unlabeled TPN$^+$, 1 μmole of unlabeled DPN$^+$ and 10 μmoles of glucose 6-phosphate (curve C); 1 μmole of [4-^{3}H]TPN$^+$ ($4\cdot10^5$ counts/min) and 10 μmoles of glucose 6-phosphate (curve D); and 1 μmole of [4-^{3}H]TPN$^+$ ($4\cdot10^5$ counts/min), 1 μmole of unlabeled DPN$^+$ and 10 μmoles of glucose 6-phosphate (curve E).

from the results presented in Figs. 1 and 3 and the integral changes in specific activities of the [^{3}H]triphosphopyridine nucleotides. The half-life time of TPNH was determined for each experiment, and the specific activities of hydrogen on the pyridine nucleotides, ranging from the start of the experiment to the moment when it was stopped, were calculated from the data in Table IV. The curves obtained with these calculated values (Figs. 7 and 8) are linear with time. The incorporation of ^{14}C from [1-^{14}C]acetate into fatty acids by the particle-free supernatant fraction of lactating mammary gland was also linear with time.

In the experiments with the liver system, no calculation of the hydrogen incorporation from either of the labeled, reduced pyridine nucleotides ([4-^{3}H]DPN and [4-^{3}H]TPN) into fatty acids was attempted because: (a) acetate incorporation into fatty acids was not linear throughout the entire incubation period, and (b) ^{3}H loss from the four position of the pyridine ring of the nucleotides was very high as compared with the fatty acids synthesized*. For the latter reason, no difference between the incorporation rates of ^{3}H from the α- and β-positions of the reduced pyridine nucleotides was discernible in our experiments.

DISCUSSION

The key role of citrate in fatty acid synthesis from [^{14}C]acetate has been repeatedly emphasized[7,8,24,25,27–30]. In our experiments, which were carried out with a liver system composed of a supernatant fraction plus microsomes[7,24] and a supernatant fraction of lactating mammary gland[8,24], practically no [^{14}C]fatty acids were recovered from [1-^{14}C]acetate in the absence of citrate. The present findings with ^{3}H demonstrate that, in the absence of citrate, no hydrogen was transferred to fatty acids from either of the reduced pyridine nucleotides (DPNH and TPNH) nor from [1-^{3}H]glucose 6-phosphate. Thus, this tricarboxylic acid is required not only for incorporation of acetate carbon into fatty acids but also for hydrogen transfer to fatty acids.

Although both TPNH and DPNH can serve as hydrogen donors for the reductive steps in the synthesis of saturated fatty acids, many investigators[5–8] have pointed to the superiority of TPNH in almost all systems so far studied**. This is fully borne out in the results presented here on the direct transfer of hydrogen from TPNH to fatty acids. TPNH served at least three times better as a hydrogen donor than did DPNH in both our liver and lactating mammary-gland preparations.

In the experiments with the mammary gland supernatant fraction, incorporation of tritium from [α-^{3}H]TPNH into fatty acids was significantly higher than that from [β-^{3}H]TPNH. In the experiments with [4-^{3}H]DPNH, however, recoveries of [^{3}H]fatty acids from [β-^{3}H]DPNH were somewhat higher than those from [α-^{3}H]DPNH. In this mammary-gland system, the transfer of ^{3}H to fatty acids was highest from [α-^{3}H]-TPNH; in contrast, ^{3}H transfer from [α-^{3}H]DPNH was extremely low. Apparently TPN; in contrast, ^{3}H transfer from [α-^{3}H]DPN was extremely low Apparently hydrogen is preferentially transferred from the α-position of TPNH to fatty acids in one of the two reductive steps in fatty acid synthesis.

* Fatty acid synthesis from [1-^{14}C]acetate by the rat-liver system was only 1/5–1/6 of that obtained with the mammary-gland system.

** BRADY[31] has reported that, in purified homogenate fractions obtained from young rat brains, TPNH functions as the only hydrogen donor for fatty acid synthesis.

The finding that the transfer of tritium from [β-^{3}H]TPNH into fatty acids by our lactating mammary-gland preparation was somewhat inhibited by addition of DPN^+, whereas the transfer from [β-^{3}H]DPNH was strongly inhibited by TPN^+, suggests that the other reductive step in fatty acid synthesis, in which both TPNH and DPNH can serve as hydrogen donors, preferentially utilizes the hydrogen in the β-position of these nucleotides. In this reductive step, TPNH also appears to be the superior hydrogen donor.

It has been suggested by LYNEN[5], from experiments with a highly purified yeast system, that the first reductive step in fatty acid synthesis, namely, that concerned with conversion of acetoacetyl-enzyme (enol form) to β-hydroxybutyryl-enzyme, is TPNH-specific. On the other hand, the second reductive step, namely, the conversion of the α,β-unsaturated acyl-enzyme to the corresponding saturated acyl-enzyme, can utilize either DPNH or TPNH. This latter proposal comes from LYNEN's observations that the final hydrogen donor participating in this reaction is FMN.

Provided the yeast system described by LYNEN[3-5] can be used as a model for the lactating rat mammary-gland system, the first reductive step, which requires TPNH, involves predominantly the α-hydrogen on the four position of the pyridine ring. The second reductive step, in which either DPNH or TPNH can serve as hydrogen donor, is β-specific. If FMN is a participant in the second reductive reaction, then the transfer of hydrogen from the β-position of the pyridine nucleotides to FMN, and subsequently to the fatty acid derivative, must also be β-stereospecific with respect to the four position of the pyridine ring of the nucleotides.

In the experiments in which [4-^{3}H]TPN^+ was incubated with citrate, the resulting [4-^{3}H]TPNH should have been labeled solely in the β-position (isocitric dehydrogenase is α-specific), whereas only [α-^{3}H]TPNH should have been produced from glucose 6-phosphate (the glucose 6-phosphate oxidizing enzymes are β-specific). Where both TPNH-generating substrates were added, the preferential form of the reduced nucleotide produced should have been [α-^{3}H]TPN in the mammary-gland system. It should be recalled that the addition of glucose 6-phosphate to the particle-free supernatant fractions obtained from mammary glands of lactating rats strongly inhibits citrate oxidation[8,23]. Since [α-^{3}H]TPNH is a better donor of ^{3}H for fatty acid synthesis than is [β-^{3}H]TPNH, the addition of glucose 6-phosphate to a system already containing citrate should have resulted in an increased incorporation of ^{3}H from [4-^{3}H]TPN^+ into fatty acids. This was actually found to be the case.

When glucose 6-phosphate was incubated with [4-^{3}H]TPN^+ in the absence of citrate, thereby placing the tritium in the α-position of TPNH, virtually no isotope was transferred to the synthesized fatty acids by the mammary-gland preparations. This is not surprising in view of the fact that practically no fatty acids were synthesized by our mammary-gland system in the absence of citrate[8,24]. We may therefore conclude: (a) that glucose 6-phosphate places the ^{3}H (from [4-^{3}H]TPN^+) in the proper stereoconfiguration on the four position of the pyridine ring for optimal transfer to fatty acids, (b) that some factor involved in the oxidation or utilization of citrate is required for fatty acid synthesis from acetate.

In the liver system we could not demonstrate a significant difference in ^{3}H transfer to fatty acids from [α-^{3}H]TPNH and [β-^{3}H]TPNH, probably because of at least two effects: (a) the high TPNH turnover in liver, and (b) the much lower fatty acid synthesis in the liver as compared with the lactating mammary-gland system.

Working with a purified enzyme system obtained from rat liver, BRADY *et al.*[32] found that ^{14}C incorporation from [1-^{14}C]malonyl-CoA was about five times higher than was ^{3}H transfer from [1-^{3}H]glucose into fatty acids. These workers suggested that there is a considerable isotope discrimination against ^{3}H, either in the transfer from glucose through TPN^+ or in the reductive steps of fatty acid synthesis. The results of the experiments with the mammary-gland fraction presented here indicate that the findings of BRADY *et al.*[32] are better explained on the basis of the stereo-configuration of the ^{3}H on the pyridine nucleotide. Since [1-^{3}H]glucose would yield [β-^{3}H]TPNH, we should expect, as these investigators found, that during short intervals of incubation, very little ^{3}H would appear in the isolated fatty acids*. Since, in longer incubation times, the ^{3}H would eventually appear in both α- and β-positions of TPNH, incorporation of ^{3}H into fatty acids would increase because ^{3}H from the α-position is more readily converted to fatty acids than is that from the β-position. Thus a ratio of ^{3}H to carbon somewhat lower than 1 would be expected with short incubation times, and the ratio would approach a theoretical value of about 2.0** as the time of incubation was prolonged. Presumably some isotope discrimination does exist but, as judged by the data presented here, it is apparently of a much lower order than that suggested by BRADY *et al.*[32].

The values for the ratio:

$$\frac{\text{m}\mu\text{atoms hydrogen transferred through TPNH to fatty acids}}{\text{m}\mu\text{atoms } ^{14}C \text{ from [1-}^{14}C\text{]acetate incorporated into fatty acids}}$$

calculated from the mammary-gland data shown in Figs. 7 and 8 are given below:

TPNH	*DPN+*	*Citrate*	*Glucose 6-phosphate*	*Ratio*
[α-^{3}H]	None	+	None	0.82
[α-^{3}H]	+	+	None	1.35
[β-^{3}H]	None	+	None	0.40
[β-^{3}H]	+	+	None	0.28
+ (Unlabeled)	None	+	[1-^{3}H]	1.54
+ (Unlabeled)	+	+	[1-^{3}H]	1.92

It is apparent that this ratio is greatly affected by the experimental conditions. It is greater than 1 when the incorporations of hydrogen from both α- and β-positions are summed. Since the ratios obtained in the experiments with [1-^{3}H]glucose 6-phosphate closely approached the theoretical value of about 2.0, isotope discrimination against tritium conversion to fatty acids could not have been very great under these conditions.

Another factor to be considered in the study of ^{3}H transfer from a substrate to fatty acids is the oxidation rate of the substrate. This becomes particularly important when more than one TPNH-generating substrate is present. For example, when [1-^{3}H]glucose 6-phosphate is added, its oxidation results in formation of equimolar amounts of gluconic acid 6-phosphate which, when oxidized to pentose phosphate and CO_2, results in a 1:1 dilution of the tritium on the β-position of TPNH***.

* [α-^{3}H]TPNH is a 2–3 times better donor of ^{3}H than is [β-^{3}H]TPNH (see Fig. 3).

** This theoretical ratio is based on the finding that tritium from [4-^{3}H]TPNH appears only on alternating carbons, beginning with the β-position[32].

*** Gluconic acid-6-phosphate dehydrogenase is β-specific[11].

This dilution will occur unless gluconic acid 6-phosphate dehydrogenase is removed from the system. We have previously shown that the oxidation of glucose 6-phosphate in the liver system is strongly inhibited by citrate[16] and that citrate oxidation in turn is inhibited in the presence of glucose 6-phosphate in our lactating rat mammary gland preparations. This accounts for the fact that incorporation of ^{3}H from [1-^{3}H]-glucose 6-phosphate into fatty acids by the lactating mammary-gland supernatant fraction is so much higher than that by the rat-liver system. A calculation of the actual hydrogen incorporation into fatty acids through TPNH should only be attempted from such experiments when the oxidation rates of the TPNH-generating substrates added are known.

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