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EFFECT OF PHENOBARBITAL ON THE INCORPORATION OF ^{3}H OR ^{14}C LABELED PRECURSORS INTO FATTY ACIDS

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Abstract—The incorporation of various ^{3}H or ^{14}C labeled precursors into hepatic fatty acids was studied in control and phenobarbital-treated rats. *In vitro*, phenobarbital had no effect on fatty acid synthesis from the tritiated precursors $^{3}H_2O$, 1-^{3}H glucose, 2-^{3}H lactate, 2,3-^{3}H succinate and 2-^{3}H acetyl CoA or from the ^{14}C labeled precursors 1-^{14}C acetate and 1,3-^{14}C malonyl CoA in liver supernatant or supernatant + microsomes preparations. *In vivo*, phenobarbital stimulated the incorporation of 1-^{14}C acetate, $^{3}H_2O$, 2-^{3}H lactate and 2,3-^{3}H succinate but had no stimulatory effect on the incorporation of 1-^{3}H glucose. The activities of lactic dehydrogenase, glucose 6-phosphate dehydrogenase and succinate cytochrome *c* reductase were not modified by administration of phenobarbital but that of NADPH-cytochrome *c* reductase was increased. These results indicate that the NADH and NADPH pools are not quite in equilibrium, and that the endoplasmic reticulum probably competes with the cytoplasm for NADPH utilization and thus may play a part in the regulation of fatty acid synthesis. The increased incorporation of ^{14}C precursors observed *in vivo* in phenobarbital-treated rats was not due to stimulation of the synthesis of the key enzymes of fatty acid synthesis but could be related to an activation of these enzymes which, *in vivo*, are probably fixed on the endoplasmic reticulum.

PREVIOUS studies have shown that phenobarbital increases the intrahepatic concentration of fatty acids but as yet no explanation of this phenomenon has been forthcoming. *In vitro*, phenobarbital seems to have no effect on fatty acid synthesis nor on esterification.[1–3] This study was undertaken to determine whether phenobarbital behaves differently *in vivo*. Although *in vitro*, fatty acid synthesis takes place mainly in particle-free liver supernatant,[4] *in vivo* the predominant site of hepatic fatty acid synthesis seems to be the endoplasmic reticulum[5–7] the amount of which has been shown to be increased by phenobarbital.[8]

Furthermore, since phenobarbital stimulates the NADPH cytochrome *c* oxidase activity of liver microsomes[9] it appeared to us of interest to investigate whether NADPH oxidation by the endoplasmic reticulum interfered with the hydrogenations occuring in the course of fatty acid synthesis.

METHODS

Experiments in vivo. First experiment: female rats (300–350 g) fed *ad lib.* with Nafag cubes from 4 to 7 a.m. daily received an i.p. injection (80 mg/kg) of phenobarbital or 0·9% NaCl 48 and 24 hr before the experiment. On the third day they received an i.v. injection of 1-^{14}C acetate (10 μCi, 61 mCi/mM) or 2,3-^{3}H succinate (200 μCi, 114·5 mCi/mM). The rats were killed 15 min later and the livers were

divided in two parts, one part was immediately saponified and the other was homogenized in 9 vol. of 0·25 M sucrose and centrifuged for 10 min at 1000 *g* to remove nuclei and cell fragments. The supernatant was centrifuged for 10 min at 15,000 *g*. The resulting mitochondrial pellet was collected. The 15,000 *g* supernatant was then centrifuged at 105,000 *g* for 1 hr to sediment the microsomes. Samples were taken from the different fractions for spectrophotometric determination of proteins (biuret method), mitochondrial succinate dehydrogenase activity,[10] supernatant glucose 6-phosphate dehydrogenase[11] and lactic dehydrogenase[12] and microsomal NADPH-cytochrome *c* reductase.[13] The entire operation was carried out between 0 and 4°. After saponification of the samples and removal of unsaponifiable material, fatty acids were extracted from determination of their radioactivity in a liquid scintillation spectrometer.

Second and third experiments: the conditions were identical to those of experiment 1, except that the precursors were 1-^{3}H glucose (100 μCi, 7·6 mCi/mM) and 1-^{14}C acetate (10 μCi, 60 mCi/mM) in the second experiment, and 2-^{3}H lactate (100 μCi, 5 mCi/24 mM) and 1-^{14}C acetate (60 mCi/mM) in the third.

Fourth experiment: the conditions were identical to those of experiment 1, except that rats were fasted for 24 hr, that phenobarbital was administered 72, 48 and 24 hr before the experiment and that the radioactive precursors were 1-^{14}C acetate (60 mCi/mM) and 2-^{3}H lactate (5 mCi/24 mM).

Fifth experiment: the conditions were identical to those of experiment 1, except that the radioactive precursors were ^3H_2O (1 mCi/0·3 ml) and 1-^{14}C acetate (10 μCi, 60 mCi/mM) and that rats were killed 30 min post injection.

All of these experiments were repeated at least twice.

Experiments in vitro. Animals were treated as for *in vivo* experiments. Livers were homogenized and fractions separated as previously described. Exactly 1 or 2 mg of protein from particle-free supernatant or supernatant + microsomes was incubated in 1 ml of phosphate buffer 0·05 M (pH 6·5) containing in the first experiment 5 mM mercaptoethanol 1 mM NADPH, 0·1 mM (5 μCi) 2-^{3}H acetyl CoA, 0·1 mCi (0·25 μCi) 1,3-^{14}C malonyl CoA and in the second experiment 8 mM ATP, 1 mM $MnCl_2$, 70 mM $MgCl_2$, 20 mM Na citrate, 0·1 mM CoA, 30 mM cystéine, 20 mM $KHCO_3$, 0·5 mM NAD, 0·5 mM NADP and the labeled precursors 2,3-^{3}H succinate, 1-^{3}H glucose, 2-^{3}H lactate, 1-^{14}C acetate (10 mM) or ^3H_2O. The reaction mixtures were incubated at 37°C for 15 min in the first experiment and for 45 min in the second.

Reactions were stopped by addition of KOH and alcohol and the tubes were boiled for 3 hr before extraction of fatty acids.

RESULTS

Experiments in vivo. The results of these experiments are shown in Table 1. A significant increase in fatty acid radioactivity was observed in the experiment with 1-^{14}C acetate as well as in that with 2,3-^{3}H succinate ($P < 0·001$). The values obtained for both precursors were parallel in the three fractions studied. Similar results were obtained with 2-^{3}H lactate and ^3H_2O. However, when 1-^{3}H glucose and 1-^{14}C acetate were injected under the same conditions, only the incorporation from 1-^{14}C acetate was significantly increased. A similar experiment carried out with 24 hr-fasted

TABLE 1. EFFECT OF PHENOBARBITAL ON FATTY ACID SYNTHESIS *in vivo*

	Phenobarbital			Control rats		
Fed animals	1-^{14}C Acetate	2,3-^{3}H Succinate	^{3}H/^{14}C	1-^{14}C Acetate	2,3-^{3}H Succinate	^{3}H/^{14}C
Total radioactivity of liver fatty acids	568,000	168,000	0·26	283,000	81,400	0·29
% of Total radioactivity in: Supernatant	14·2	11·9	0·21	11·9	8·5	0·29
% of Total radioactivity in: Microsomes	71·6	74·6	0·25	74·6	78·9	0·30
% of Total radioactivity in: Mitochondria	14·2	13·5	0·22	13·5	12·6	0·26
	1-^{14}C Acetate	1-^{3}H Glucose	^{3}H/^{14}C	1-^{14}C Acetate	1-^{3}H Glucose	^{3}H/^{14}C
Total radioactivity of liver fatty acids	455,000	33,100	0·07	236,000	29,500	0·12
	1-^{14}C Acetate	2-^{3}H Lactate	^{3}H/^{14}C	1-^{14}C Acetate	2-^{3}H Lactate	^{3}H/^{14}C
Total radioactivity of liver fatty acids	670,000	76,500	0·124	276,400	32,000	0·117
	1-^{14}C Acetate	$^{3}H_2O$	^{3}H/^{14}C	1-^{14}C Acetate	$^{3}H_2O$	^{3}H/^{14}C
Total radioactivity of liver fatty acids	577,800	730	0·128	348,800	250	0·073
Fasted animals	1-^{14}C Acetate	2-^{3}H Lactate	^{3}H/^{14}C	1-^{14}C Acetate	2-^{3}H Lactate	^{3}H/^{14}C
Total radioactivity of liver fatty acids	132,200	13,320	0·110	100,000	11,500	0·100

The results represent the radioactivity of fatty acids expressed in dpm for a dose of 10 μCi of each precursor.
All experiments were carried out with six animals in each group.

TABLE 2. EFFECT OF PHENOBARBITAL ON FATTY ACID SYNTHESIS *in vitro*

	Phenobarbital			Control rats		
Experiment 1	^{3}H Acetyl CoA	1,3-^{14}C Malonyl CoA	^{3}H/^{14}C	^{3}H Acetyl CoA	1,3-^{14}C Malonyl CoA	^{3}H/^{14}C
Supernatant (105,000 *g*)	896,000	1,428,000	0·64	912,000	1,552,000	0·63
Supernatant (15,000 *g*) = (supernatant + microsomes)	1,110,000	1,776,000	0·56	1,168,000	1,908,000	0·61
Experiment 2	1-^{3}H Glucose	1-^{14}C Acetate	^{3}H/^{14}C	1-^{3}H Glucose	1-^{14}C Acetate	^{3}H/^{14}C
Supernatant (15,000 *g*) = (supernatant + microsomes)	1,200	1,450	0·82	1,180	1,600	0·73
	2-^{3}H Lactate 2,380	1-^{14}C Acetate 1,250	^{3}H/^{14}C 1·90	2-^{3}H Lactate 2,500	1-^{14}C Acetate 1,335	^{3}H/^{14}C 1·88
	2,3-^{3}H Succinate 1,780	1-^{14}C Acetate 1,265	^{3}H/^{14}C 1·41	2,3-^{3}H Succinate 1,570	1-^{14}C Acetate 1,200	^{3}H/^{14}C 1·30
	^3H_2O 60	1-^{14}C Acetate 1,120	^{3}H/^{14}C 0·005	^3H_2O 61	1-^{14}C Acetate 1,200	^{3}H/^{14}C 0·005

All results were expressed in dpm for a dose of 10 μCi of each precursor/mg protein. Differences between controls and phenobarbital-treated rats were not significant.

TABLE 3. THE EFFECT OF PHENOBARBITAL ON THE ACTIVITY OF LIVER ENZYMES

	Lactic dehydrogenase	Glucose 6-phosphate dehydrogenase	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
Control rats (6)	23,200 ± 1600	4·11 ± 0·15	158 ± 20	0·164 ± 0·14
Phenobarbital-treated rats (6)	23,700 ± 1700	4·27 ± 0·17	141 ± 16	0·353 ± 0·26

Definition of enzymatic units: Lactic dehydrogenase, amount of enzyme which converts 1 μmol of substrate/min at 25° (Racker units); glucose 6-phosphate dehydrogenase (Bücher units); succinate-cytochrome *c* reductase (nmoles substrate reduced min^{-1} mg $protein^{-1}$); NADPH-cytochrome *c* reductase, μmoles cytochrome *c* reduced min^{-1} mg $protein^{-1}$.

The number of animals used in each experiment is in parentheses.

rats showed no re-establishment of lipogenesis by phenobarbital from 1-^{14}C acetate or from 2-^{3}H lactate.

Experiments in vitro. The results of these experiments are shown in Table 2. In the first experiment, the incorporation of 2-^{3}H acetyl CoA and 1,3-^{14}C malonyl CoA was compared. No differences were observed between control and phenobarbital-treated rats in the presence of the 105,000 *g* particle-free supernatant or the 15,000 *g* supernatant + microsomes. We also compared the incorporation of different tritiated precursors capable of supplying hydrogen for fatty acid synthesis. Again, no differences were observed between control and phenobarbital-treated rats for 1-^{3}H glucose, 2-^{3}H lactate, 2,3-^{3}H succinate or $^{3}H_2O$. The latter was used because of the possibility of hydroxylation reactions in endoplasmic reticulum.

Enzyme assays. The activities of lactic dehydrogenase, glucose 6-phosphate dehydrogenase and succinate cytochrome *c* reductase were not significantly modified by administration of phenobarbital. In contrast, the activity of NADPH-cytochrome *c* reductase of control rats was about half that of phenobarbital-treated rats (Table 3).

DISCUSSION

The results reported here provide evidence that, *in vivo*, phenobarbital has a similar stimulatory effect on fatty acid synthesis from acetate as on cholesterol biosynthesis.[14] Opinions regarding the *in vitro* action of phenobarbital on cholesterol synthesis are divergent, ranging from the suggestion that phenobarbital could increase cholesterol synthesis from acetate[15,16] to the opinion that it efficiently stimulates this synthesis only from mevalonate.

The results of our experiments show that phenobarbital has no effect on fatty acid synthesis *in vitro*. The choice of acetyl CoA and malonyl CoA as precursors was designed to eliminate the activation steps of fatty acid synthesis and the need for ATP in order to determine whether phenobarbital modifies the activity of the enzymes of fatty acid synthesis. Our results, which are in agreement with those of Tepperman,[1] exclude the possibility of a stimulatory effect of phenobarbital on these enzymes. Although the action of phenobarbital on cholesterol biosynthesis is relatively easy to understand since several steps of its synthesis occur in the endoplasmic reticulum, its effect on fatty acid synthesis is rather difficult to explain. We have therefore tried to determine which subcellular fraction could account for the increase in fatty acid synthesis observed *in vivo*. We found no modification of the distribution of the radioactivity in these fractions.

Omura and Kuriyama[2] and Eriksson and Dallner,[3] all of whom used glycerol 2-^{3}H as the precursor observed no increase in esterification in the phenobarbital-treated rats. Nevertheless, we considered it of interest to verify their results under our experimental conditions and to see if phenobarbital stimulated the esterification of fatty acids, a phenomenon which takes place in the endoplasmic reticulum. We therefore administered 1-^{14}C pyruvate and isolated total lipids from mitochondrial, microsomal and supernatant fractions. Indeed, 1-^{14}C pyruvate can only be incorporated into the glycerol part of triglycerides or phospholipids and not into the fatty acids. The values found for control rats which were killed 15 min after the administration of 10 μCi of 1-^{14}C pyruvate were 2900 dis min^{-1} liver^{-1} and 2660 dis min^{-1} liver^{-1} for the phenobarbital-treated rats. The differences were not significant.

Thus, the increase of incorporation of various precursors into fatty acids after phenobarbital administration could not be attributed to a stimulation of esterification, nor could it be explained by a stimulation of the activity, nor of the synthesis of the key fatty acid synthesizing enzymes since no effect was detectable *in vitro* even in the experiments with 2-^{3}H acetyl or 1,3-^{14}C malonyl CoA as precursors.

On the other hand, it seemed to us that the phenobarbital could play a part in fatty acid synthesis by controlling the NADPH pool indispensable for this synthesis. In this context, we compared the incorporation into fatty acids of three ^{3}H precursors: 1-^{3}H glucose, which allows the formation of NADPH, 2-^{3}H lactate which reduces NAD, and 2,3-^{3}H succinate which transmits its ^{3}H into fatty acids by a mechanism which is still unknown. In fact, the last two precursors have been found to be excellent sources of hydrogen for mouse liver fatty acids.[18–23] Our results show, however, that in rat liver, in contrast to mouse liver, 1-^{3}H glucose is as good a source of hydrogen for fatty acids as 2-^{3}H lactate which could mean that the pentose pathway is more active in rat liver than in mouse liver. Although phenobarbital did not increase mitochondrial succinate dehydrogenase activity it did stimulate the incorporation into fatty acids from both 2,3-^{3}H succinate and 1-^{14}C acetate in the same proportion. It is difficult to say at present whether the increase in fatty acid synthesis has a bearing upon the increase of succinate incorporation, and as long as we are ignorant of the pathway followed by the tritium of 2,3-^{3}H succinate in its incorporation into fatty acids, this relationship cannot be established. However, the fact that 1-^{3}H glucose is the only precursor whose incorporation into fatty acids is not stimulated by phenobarbital could be explained by a decrease in the NADPH pool available for fatty acid synthesis in phenobarbital-treated rats or by an inhibition of the Embden–Meyerhof pathway. In order to eliminate this last possibility we studied the action of phenobarbital on the incorporation of glucose U-^{14}C into fatty acids. We found 77,400 dis/min in the fatty acids of the livers of control rats and 125,500 dis/min in those of phenobarbital-treated rats ($P < 0{\cdot}001$) 15 min after the administration of the radioactive precursor. It would seem, therefore that the Embden-Meyerhof pathway is not inhibited by phenobarbital. Since glucose 6-phosphate dehydrogenase is not inhibited by phenobarbital but is, in fact, slightly stimulated by it,[24] it is possible that the stimulation of microsomal NADPH-cytochrome *c* reductase following phenobarbital administration, consumes a part of the NADPH originating from the glucose oxidative pathway, thus lowering the NADPH pool available for fatty acid synthesis. The increased incorporation of 2-^{3}H lactate or 2,3-^{3}H succinate into fatty acids could mean that hydrogen from both these precursors does not participate in the same pool as ^{3}H which comes from 1-^{3}H glucose, or that an unknown enzyme responsible for the ^{3}H utilization of these precursors is stimulated. This last suggestion seems unlikely since a similar stimulation is obtained when $^{3}H_2O$ is the tritiated precursor. None of these suggestions, however, explains why, *in vivo*, phenobarbital stimulates the incorporation of most of the precursors into fatty acids. It is probable that phenobarbital can stimulate the activity of the key enzymes only if these enzymes are fixed on the endoplasmic reticulum as seems to be the case in the living animal,[5–7,27] and that when these enzymes have been liberated from these structures in the supernatant during homogenization, they become insensitive to the action of phenobarbital. Further studies are in progress to verify these hypotheses.

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