Biochemical Pharmacology, Vol. 26, pp. 481–484. Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF DRUGS WHICH ALTER MICROSOMAL DRUG-METABOLIZING ENZYME ACTIVITY ON RAT HEPATIC CHOLESTEROL BIOSYNTHESIS*

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(*Received* 7 *June* 1976; *accepted* 27 *August* 1976)

Abstract—Effects on cholesterol biosynthesis of compounds known to alter hepatic microsomal drug metabolism were assessed in rats *in vivo* by measuring the simultaneous incorporation of tracer quantities of [1-^{14}C]acetate and $^{3}H_2O$ into cholesterol. The incorporation of both substrates was increased with phenobarbital and decreased with $CoCl_2$ treatment. No change in incorporation of either substrate was noted when rats were treated with metyrapone, piperonyl butoxide or pregnenolone-16α-carbonitrile (PCN). The effect of these compounds on the assessment *in vivo* of cholesterol biosynthesis may be accurately characterized by use of either precursor as substrate. Compounds which induce or inhibit rat hepatic microsomal drug metabolism do not necessarily alter hepatic cholesterol biosynthesis.

Radiolabeled acetate has been commonly used as a precursor to measure cholesterol biosynthesis [1–3] *in vivo* and *in vitro*. *In vivo*, acetate utilized in the form of acetyl CoA for cholesterol biosynthesis is of extramitochondrial origin [4]. Barth *et al.* [5], using the isolated perfused rat liver, showed that [^{14}C]acetate was not distributed uniformly intra- and extramitochondrially. Dietschy and McGarry [6] recently presented evidence that [^{14}C]acetate incorporation into cholesterol is not a valid measure of cholesterol biosynthesis in some experimental situations. Their conclusions were based on observed changes in the specific activity of the intramitochondrial acetate pool caused by alterations in the metabolic state of the rat. Starvation, for example, produced a change in the intramitochondrial [^{14}C]acetate pool size [7].

Jones and Armstrong [8] reported a 4-fold increase in [^{14}C]acetate incorporation into hepatic cholesterol after hamsters had been treated with phenobarbital. Wada *et al.* [9] reported increased incorporation of [^{14}C]acetate, [^{14}C]mevalonate and [^{14}C]squalene into cholesterol in rats treated with phenobarbital and increased [^{14}C]mevalonate incorporation into cholesterol after 3-methylcholanthrene treatment. Both phenobarbital and 3-methylcholanthrene induce an increase in the activity of drug-metabolizing enzyme systems [10–13]. On the other hand, β-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A) is an inhibitor of cholesterol biosynthesis [14, 15] as well as microsomal drug metabolism [16, 17].

This study was undertaken to determine if changes in hepatic cholesterol biosynthesis were a general response in rats treated with drugs that induce or inhibit hepatic microsomal drug-metabolizing enzyme activity and whether the changes were accurately reflected by the incorporation of [1-^{14}C]acetate into cholesterol. Here we report a comparison of [1-^{14}C]-acetate and $^{3}H_2O$ incorporation *in vivo* into cholesterol in animals pretreated with phenobarbital, $CoCl_2$, metyrapone, piperonyl butoxide, and pregnenolone-16α-carbonitrile (PCN).

MATERIALS AND METHODS

Male Charles River rats (100–125 g) were housed individually and fed Purina rat chow and water *ad lib.* The animal quarters were maintained at 22.5 ± 1°. An alternating light and dark cycle was maintained from 6:00 a.m. to 6:00 p.m. and 6:00 p.m. to 6:00 a.m. respectively. Treated rats were injected intraperitoneally with phenobarbital, 75 mg/kg daily × 3; cobalt chloride ($CoCl_2$), 60 mg/kg daily × 2; metyrapone, 60 mg/kg 30 min before the radiolabeled substrates; piperonyl butoxide, 1 g/kg 30 min before the radiolabeled substrates; or PCN, 50 mg/kg daily × 4. Piperonyl butoxide and PCN were administered in propylene glycol. The other drugs were dissolved in normal saline. All drugs were given in a volume of 0.2 ml/100 g body weight and control animals received the same volume of the corresponding solvent. Each group consisted of seven animals.

Pretreatment was followed by intraperitoneal administration of 10 μmole's [1-^{14}C]sodium acetate/100 g body weight (New England Nuclear, sp. act. 1 mCi/m-mole) and 0.25 m-mole $^{3}H_2O$/100 g body weight (New England Nuclear, sp. act. 18 mCi/m-mole) in 0.2 ml saline/100 g body weight. One hr later, the rats were lightly anesthetized with ether and

* Supported by Grant GM 15956 from the United States Public Health Service.

† Recipient of a Medical Student Research Fellowship in Pharmacology-Clinical Pharmacology from the Pharmaceutical Manufacturers Association Foundation.

exsanguinated by cardiac puncture for measurement of serum cholesterol. The liver was excised and quickly cooled on ice after excess blood was absorbed with a paper towel. Two 1-g pieces of each liver were saponified with 15 ml of 10% KOH in 70% ethanol by heating at 70° for 1 hr, then extracted with three 10-ml portions of petroleum ether. The extracts were combined, washed with 10 ml water and evaporated to dryness. The resulting residue was dissolved in ethanol–acetone (1:1, v/v), and 10 ml 0.5% digitonin (Fisher) was added to isolate the resulting insoluble cholesterol digitonide. The precipitate was washed sequentially with 10 ml water, 10 ml acetone–ether (2:1, v/v), and 10 ml ether. The sterol digitonide (minor quantities of non-cholesterol sterols may also be present) was dried overnight in a vacuum desiccator containing phosphorous pentoxide. Accurately weighed (3–5 mg) quantities of sterol digitonide were burned in a Packard model 306 tissue oxidizer to separate and prepare the ^3H and ^{14}C for counting. Radioactivity was measured with a Packard Tri-Carb model 3320 liquid scintillation spectrometer and corrected for quenching by external standardization.

Another portion of the sterol digitonide was cleaved in pyridine and extracted with ether [18]. Separation of the component sterols was achieved by a modification of the method of Gibbons and Mitroupoulos [19]. The ether extract was applied to silicic acid N (Macherey-Nagel) thin-layer plates. At one edge of the plate a cholesterol standard was spotted. The plates were developed in chloroform–benzene (80:20). The cholesterol standard was located by heating the plate after coating the edge with 90% trichloroacetic acid. The area of the plate corresponding to cholesterol (R_f 0.33) was eluted with chloroform. Desmosterol (1 mg) was added to provide adequate material for isolation and the chloroform evaporated. The sterols were acetylated with pyridine–acetic anhydride and separated on a 1 × 10 cm $AgNO_3$–silicic acid column [20]. The eluate was collected in fractions, evaporated and 10 ml AquaSol (New England Nuclear) added for liquid scintillation counting.

Duplicate 0.5-g pieces of liver were homogenized in 9.5 ml isopropyl alcohol, and the suspensions centrifuged and analyzed for cholesterol. Serum and hepatic cholesterol concentrations were determined by Autoanalyzer using Technicon procedure N24A. Data were analyzed by one-way analysis of variance and Student's *t*-test using a Hewlett-Packard model 9810 or an Olivetti 101 Programma calculator.

RESULTS

None of the modifiers produced a significant change in the serum cholesterol at the doses studied (Table 1). Phenobarbital and $CoCl_2$ significantly increased the hepatic cholesterol concentration (Table 1). [^{14}C]acetate incorporation into sterol digitonide was significantly increased in phenobarbital-treated animals as compared to their respective control group, whereas $CoCl_2$-treated animals showed a significant decrease as compared to their controls (Table 2). ^3H_2O incorporation into sterol digitonide, in a similar fashion, was significantly increased in animals treated with phenobarbital and decreased in animals treated with $CoCl_2$. No differences between treated and untreated groups were observed with the other modifiers studied (Table 2). Although a significant variation in control values was noted between experiments, the small standard error among rats in the same group attests to the similarity of conditions within each experiment.

With the exception of the $CoCl_2$-treated rats, the radio-activity contained in the sterol digitonide was associated almost entirely with cholesterol (Table 3), as demonstrated by purification of the sterols by thin-layer and column chromatography following cleavage of the digitonide. In the $CoCl_2$-treated animals, 15.9 per cent of the digitonin-precipitable fraction was associated with 28, 29 and 30 carbon sterols.

DISCUSSION

Nearly every tissue in the body can synthesize cholesterol, the intestine and liver being quantitatively most important [21]. Unless appropriate conditions are used, administration *in vivo* of a radioactive precursor followed by measurement of radioactive cholesterol in a specific organ may not represent simply biosynthesis by that organ, but also the result of transport to or away from the organ in question. The method used here assesses hepatic cholesterol biosynthesis with no contribution from cholesterol synthesized in the intestine and transported to the liver. Chaikoff *et al* [22] showed that cholesterol absorbed from the gut was transported quantitatively into the lymph ducts and directly into the blood stream via the thoracic duct. Lindsey and Wilson [23] subsequently demonstrated, using [^{14}C]acetate as a precursor, that cholesterol synthesized in the rat intestine was not excreted into the lymph to any significant

Table 1. Effect of pretreatment on tissue cholesterol concentrations*

	Serum cholesterol (mg/dl)		Hepatic cholesterol (mg/g liver)	
	Experimental ($N = 7$)	Control ($N = 7$)	Experimental ($N = 7$)	Control ($N = 7$)
Phenobarbital	62.3 ± 2.9	57.5 ± 3.8	2.76 ± 0.02†	2.68 ± 0.03
Cobalt chloride	57.8 ± 7.4	52.1 ± 2.6	2.79 ± 0.07†	2.49 ± 0.04
Metyrapone	54.6 ± 1.6	54.7 ± 2.3	2.69 ± 0.04	2.75 ± 0.06
Piperonyl butoxide	58.5 ± 3.2	59.0 ± 1.7	2.61 ± 0.03	2.65 ± 0.03
Pregnenolone-16α-carbonitrile (PCN)	69.0 ± 2.6	70.6 ± 3.1	2.75 ± 0.04	2.75 ± 0.02

* See Materials and Methods for dose and duration of pretreatment with the various compounds.
† $P < 0.05$.

Table 2. Pretreatment effects on substrate incorporation into sterol digitonide*

	[^{14}C]acetate incorporation into sterol digitonide (pmoles/g liver × 10^2)		^3H_2O incorporation into sterol dignitonide (pmoles/g liver)	
	Experimental ($N = 7$)	Control ($N = 7$)	Experimental ($N = 7$)	Control ($N = 7$)
Phenobarbital	8.85 ± 0.82†	6.56 ± 0.54	15.67 ± 0.76†	10.23 ± 0.57
Cobalt chloride	2.26 ± 0.76†	7.58 ± 0.83	3.40 ± 0.80†	10.18 ± 0.58
Metyrapone	13.08 ± 0.87	10.89 ± 0.99	12.11 ± 0.70	13.01 ± 1.26
Piperonyl butoxide	11.23 ± 1.24	8.79 ± 0.74	13.33 ± 0.25	12.35 ± 0.64
Pregnenolone-16α-carbonitrile (PCN)	12.03 ± 0.83	10.97 ± 0.74	17.61 ± 1.01	17·57 ± 1.20

* See Materials and Methods for dose and duration of pretreatment with the various compounds.
† $P < 0.05$.

degree during the first hour after radiolabel injection. They also showed that with lymphatic diversion no labeled cholesterol appeared in the bile. Thus, radiolabeled cholesterol appearing in the rat liver 1 hr after administration of radiolabeled precursor must have been synthesized in the liver.

Phenobarbital [10–13] and PCN [24, 25] in the dose and duration of treatment used in our study were arbitrarily chosen as examples of potent "inducers" whereas metyrapone [26], piperonyl butoxide [27] and $CoCl_2$ [28] were chosen as inhibitors of hepatic microsomal drug-metabolizing enzyme activity. Previous investigators [9, 29–32] have reported that drugs which induce hepatic microsomal drug oxidation increase the incorporation of [^{14}C]acetate into cholesterol. Since many steps in the cholesterol biosynthetic pathway are present in the microsomes and require O_2 and NADPH, the possibility that a common enzyme, cytochrome P-450, mediates both drug biotransformation and portions of cholesterol biosynthesis has been suggested [9, 29, 31]. Recently, Gibbons and Mitroupoulos [33] presented convincing evidence for the participation of cytochrome P-450 in lanosterol 14α demethylation. However, it is unlikely that stimulation of lanosterol demethylation significantly affects [^{14}C]acetate incorporation into cholesterol as β-hydroxy-β-methyl glutaryl-CoA (HMG-CoA) reductase has been clearly demonstrated to be rate limiting for the entire pathway. Therefore, induction of HMG-CoA reductase, not cytochrome P-450, by phenobarbital is a more likely mechanism for the increased incorporation of acetate into cholesterol. HMG-CoA reductase activity is in fact increased by phenobarbital treatment in both hamsters and man [34, 35]. Failure of PCN to increase [^{14}C]acetate incorporation implies that it has little if any effect on HMG-CoA reductase activity. In contrast, the inhibition of any enzyme in the pathway to the degree that it becomes rate-limiting could result in decreased incorporation of [^{14}C]acetate into cholesterol. Thus, $CoCl_2$-mediated reduction in lanosterol 14α demethylase activity or any of the other enzymes in the pathway should give the result observed in this study. Failure of piperonyl butoxide and metyrapone to decrease [^{14}C]acetate incorporation suggests (a) failure to inhibit HMG-CoA reductase, and (b) failure to inhibit other enzymes in the pathway to an extent that the inhibited enzyme becomes rate-limiting for the pathway.

[^{14}C]acetate is commonly used in studies designed to assess the rate of cholesterol biosynthesis [1–3]. Dietschy and McGarry [6], however, have shown this is not a valid measure of the absolute synthetic rate of this substance, since [^{14}C]acetate does not equilibrate into the intracellular acetate pools. For example, in fasting animals with enhanced ketone synthesis and an increased intramitochondrial acetate pool, the above substrate gave a distorted assessment of these non-equilibrated intracellular pools [7].

[^{14}C]acetate, incorporated at the initial biosynthetic step, is subject to the effect of changes in intracellular acetate pool sizes and control of the rate-limiting enzyme for cholesterol biosynthesis, HMG-CoA reductase. The rapid distribution of ^3H_2O into the total body water and exchangeable hydrogen pool indicates that equilibrium across membranes and fluxes between cytoplasmic compartments do not affect its utilization in cholesterol biosynthesis [36]. ^3H_2O has

Table 3. Distribution expressed in per cent of radiolabel in sterols after pretreatment*

	Experimental			Control		
	Cholesterol	Desmosterol	Other sterols	Cholesterol	Desmosterol	Other sterols
Phenobarbital	98.8	1.2	0	100.0	0	0
Cobalt chloride	84.1	0	15.9†	99.8	0	0.2
Metyrapone	99.5	0	0.5	99.7	0	0.3
Piperonyl butoxide	99.8	0	0.2	97.9	2.0	0.1
Pregnenolone-16α-carbonitrile (PCN)	99.7	0	0.3	99.4	0	0.6

* See Materials and Methods for dose and duration of pretreatment with the various compounds.
† This fraction consists of 28, 29 and 30 carbon sterols.

been used previously to assess cholesterol biosynthesis [5, 37, 38].

The concomitant use of two cholesterol precursors, which are incorporated at different parts of the biosynthetic pathway, allows a comparison between them as a measure of cholesterol biosynthesis. Our study corroborates previous observations that phenobarbital pretreatment enhances [^{14}C]acetate incorporation into sterol digitonide [8, 9, 29] but also provides evidence that $^{3}H_2O$ incorporation parallels this. The inhibition of sterol biosynthesis by $CoCl_2$ is also demonstrated by the parallel decrease in [^{14}C]acetate and $^{3}H_2O$ incorporation into sterol digitonide. Although differences in mitocondrial acetate fluxes may invalidate the use of [^{14}C]acetate as substrate for cholesterol incorporation studies under some experimental conditions [5–7], acetate did satisfactorily assess cholesterol biosynthesis in the studies reported here. It may be necessary, however, to validate the use of this substrate for each experimental condition.

Drugs that induce or inhibit rat liver microsomal drug metabolism do not necessarily alter hepatic cholesterol biosynthesis.

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