Acceleration of hepatic sterol synthesis after a single dose of the porphyrogenic chemical allylisopropylacetamide

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Abstract Hepatic sterol synthesis is accelerated in animals after a single dose of allylisopropylacetamide, a chemical known to be porphyrogenic. Evidence is provided to show that enhanced sterol biosynthesis is due to an increased formation of mevalonic acid, indicating that the primary site of action of the chemical is on one or more steps in the pathway from acetate to mevalonate.

Supplementary key words digitonin-precipitable sterols · porphyria

A number of chemicals and drugs have been found to produce a form of hepatic porphyria in animals (1). The experimental condition, characterized by increased synthesis of porphyrins and porphyrin precursors by the liver, mimics the inherited human hepatic porphyrias and has greatly facilitated the study of abnormal porphyrin metabolism and of the mechanisms regulating porphyrin–heme biosynthesis.

In 1963 Granick and Urata (2) demonstrated that administration of the porphyria-inducing compound DDC to guinea pigs invariably results in a greatly increased activity of hepatic δ-ALA synthetase, the initial and the rate-limiting enzyme in the biosynthetic pathway to the porphyrins. Subsequent investigations in chick embryo liver cell culture (3, 4), as well as in intact animals (5), provided evidence that DDC, AIA, and other porphyrogenic substances enhance the de novo formation of δ-ALA synthetase and do not merely activate a preexisting latent form of the enzyme.

Previous work from this laboratory (6) has shown that two porphyria-inducing compounds, DDC and AIA, given to experimental animals cause marked elevation of cholesterol, phospholipids, and total lipids in the serum. Among the various mechanisms postulated to explain these findings, the authors advanced the hypothesis that the observed hyperlipidemia might be the result of accelerated biosynthesis of different lipid components by the liver. In view of the established inducing effects of AIA and DDC on the level of hepatic δ-ALA synthetase, it seemed reasonable in fact to suggest that these chemicals may also be capable of inducing de novo formation of the enzyme(s) responsible for biosynthesis of cholesterol and other lipids in the liver. As a first step in the evaluation of this hypothesis, we decided to study, by isotopic methods, the rate of hepatic sterol synthesis in rats treated with AIA.

MATERIALS AND METHODS

Sodium [1-^{14}C]acetate (2 mCi/mmole) and tritiated water (24 mCi/g) were purchased from New England Nuclear Corp. [2-^{14}C]Mevalonate (dibenzylethylenediamine salt, 2.8 mCi/mmole) and tritium-labeled sodium acetate (217 mCi/mmole) were from Volk Radiochemical Co. AIA was a gift of Hoffmann-La Roche, Inc., Nutley, N.J. All solvents used were of reagent grade and appropriate solvents were distilled prior to use. Radioassay of digitonin-precipitable sterols isolated from rat livers was carried out using a Packard Tri-Carb series 314E and a Nuclear Chicago Mark I liquid scintillation spectrometer.

Treatment of animals

Female Sprague-Dawley rats weighing 100–200 g were used in all experiments. Rats were kept in individual cages with free access to Purina rat chow and water. The animal room was windowless and artificially lighted from 7 a.m. to 7 p.m.

After a 36-hr fast, experimental rats were administered subcutaneously 500 mg of AIA/kg in physiological saline. Control animals received equivalent amounts of physiological saline. 12 hr later each animal was given labeled pre-

Abbreviations: ALA, aminolevulinic acid; HMG, β-hydroxy-β-methylglutaric acid; DPS, digitonin-precipitable sterols; AIA, allylisopropylacetamide; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine.

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cursors, intravenously or intraperitoneally, as specified in the tables. 30 min after the administration of isotope, the rats were killed by decapitation, and their livers were removed, gently blotted, and weighed. The rats were always killed between 8 and 9 a.m. to minimize diurnal variation of cholesterol synthesis (7, 8).

Liver slices (500 mg) from AIA-treated and control animals, obtained with a Stadie-Riggs tissue slicer and approximately 0.5 mm in thickness, were incubated in Krebs-Ringer phosphate solution containing ^3H_2O and [1-^{14}C]acetate. The digitonin-precipitable sterols from liver slices were isolated, and incorporation of radioactivity was measured as described below.

In order to discount the possibility that a major fraction of the radioactivity in sterols from the livers of fasting animals could be the result of intestinal sterol synthesis, the following experiment was performed. AIA was administered to fasting animals as before. 12 hr later animals were anesthetized with ether and their abdominal cavities were opened, and after ligation of the superior mesenteric artery their intestines from the pylorus to the rectum were removed. Blood loss was kept within minimal limits by careful hemostasis. 100 mCi of [1-^{14}C]acetate was then injected directly into the portal vein, and exactly 15 min later livers were removed and gently blotted. The experimental procedure was carried out between 8 a.m. and 12 noon, alternating experimental and control animals to minimize variations due to cyclic rhythm of cholesterol synthesis.

Isolation of DPS from liver and measurement of radioactivity

The liver from each rat was hydrolyzed by refluxing for 3 hr in 15% ethanolic KOH, and the unsaponifiable lipids were extracted as described by Clayton, Nelson, and Frantz (9). Sterols were precipitated as digitonides according to the method of Sperry (10). Free sterols obtained by treatment of digitonides with pyridine were dissolved in 10 ml of benzene. The Liebermann-Burchard color assay on an aliquot of this solution revealed the presence of cholesterol only. No sterol that gave Liebermann-Burchard color at 1.5 min after the addition of reagent was detected. This is not meant to imply that all the radioactivity found was present only in cholesterol, the possibility being that some of it could still be trapped in companion sterols. The total amount of radioactivity was computed from measurements on an aliquot of the above solution.

In experiments in which only a single isotope, ^{14}C or ^{3}H, was used, the scintillation fluid was 0.3% 2,5-diphenyloxazole in toluene. The counting efficiency for ^{3}H was 16% and for ^{14}C was 40–50%. In experiments in which double isotopes were employed, the scintillation fluid was 0.4% 2,5-diphenyloxazole in toluene containing 0.005% 1,4-bis-2-(5-phenyloxazolyl)-benzene, and the ^{14}C and ^{3}H activities were determined simultaneously at settings where spillover of tritium counts into the ^{14}C channel was zero and the spillover of ^{14}C counts into the tritium channel was 12%. The tritium activity reported is therefore corrected for this spillover. The efficiency of counting ^{14}C and ^{3}H with the Packard Tri-Carb spectrometer at these settings was 52% and 12%, respectively. The results of in vitro liver slice experiments were obtained by measuring radioactivity with a Nuclear Chicago Mark I spectrometer equipped with an external standard. The ^{14}C and ^{3}H activities in DPS obtained from liver slices were corrected for quenching by the channels ratio method (11). The spillover of ^{14}C counts into the tritium channel was 9%, and the counting efficiencies for ^{3}H and ^{14}C were 33% and 66%, respectively.

RESULTS

Both experimental and control animals were fasted for a total of 48 hr (except where indicated otherwise) prior to administration of isotope in order to eliminate the effect of variable food intake on the rate of hepatic sterol synthesis and also to enhance the possible inductive effect of AIA because, at least in the case of hepatic δ-ALA synthetase, fasting has been shown to enhance this effect (12). This period of starvation is also similar to the one recently studied by Shapiro and Rodwell (13). A 12-hr interval between the administration of AIA and the injection of isotope was selected arbitrarily on the basis of previous experience by others regarding rhythmic variations in the amount of HMG CoA reductase (14, 15) and on the cytochemical alterations in liver after AIA treatment (16).

The liver cholesterol of experimental rats, when expressed as milligrams per gram of liver, was always lower than in control rats, as shown in Tables 1, 4, and 5. This was due to the fact that livers of animals receiving AIA were considerably larger than those of the control group. The average increase in liver weight of experimental over control rats was 16%, expressed per 100 g of body weight.

Incorporation of ^{14}C from [1-^{14}C]acetate and tritium from body water into DPS of AIA-treated as well as untreated rats was taken as an index of the rate of sterol synthesis in the liver of intact rats and in liver slices. A question may be raised as to whether the use of this index rather than measurements of labeling cholesterol per se could have accounted for the apparent increase in the rate of synthesis in animals treated with AIA. It appears most likely, however, that any error of this kind would have been in the direction of minimizing the difference between the control and treated animals. When the sterols recovered from digitonides from an in vivo experiment were chromatographed on silicic acid (17), it was found that the incorporation of radioactivity into cholesterol alone was 3.3 times higher in the experimental group (3000 dpm of

TABLE 1. Effect of allylisopropylacetamide on incorporation of ^{14}C from acetate into liver digitonin-precipitable sterols in intact rats

Group	Treatment	Liver Weight	Cholesterol	Activity of Liver DPS
		g	*mg/g liver*	*dpm/liver* × 10^{-2}
1	Saline (24)[a]	3.2 ± 0.6[b]	2.3 ± 0.4	385 ± 480
2	AIA (24)	3.7 ± 0.7	2.0 ± 0.3	861 ± 1446
P (1, 2)		<0.02	<0.01	<0.1
3	Saline (10)	5.0 ± 0.4	2.4 ± 0.3	240 ± 393
4	AIA (10)	5.8 ± 0.5	2.0 ± 0.2	1026 ± 615
P (3, 4)		<0.01	<0.01	<0.001
5	Saline (5)	4.8 ± 0.5	2.8 ± 0.2	99 ± 109
6	AIA (5)	5.5 ± 0.1	2.6 ± 0.2	243 ± 116
P (5, 6)		<0.05	<0.05	<0.05
7	Saline (5)	4.5 ± 0.5	2.6 ± 0.2	63 ± 37
8	AIA (5)	5.5 ± 0.4	2.4 ± 0.1	205 ± 64
P (7, 8)		<0.02	<0.05	<0.001
9	Saline (6)	4.8 ± 0.4	3.0 ± 0.2	204 ± 124
10	AIA (6)	5.3 ± 0.4	2.7 ± 0.1	392 ± 150
P (9, 10)		<0.05	<0.001	<0.025

Animals in groups 1–4 were given 10 μCi of [1-^{14}C]acetate. Animals in groups 5–10 were given 4.51 μCi of [1-^{14}C]acetate. All animals received the isotope intraperitoneally except those in groups 3 and 4, which received it intravenously. Animals in groups 9 and 10 were allowed free access to food and water until AIA treatment. All animals were killed 30 min after administration of isotope except those in groups 7 and 8, which were killed 6 hr after isotope administration.

[a] Number of animals in each group.

[b] Values are means ± SD.

^{14}C) than in the control group (900 dpm of ^{14}C). The activity in sterols other than cholesterol made up only about 15% of the total in the controls, while in the treated rats, 45% was in other sterols. Although no measurements were made of radioactivity in sterols not precipitated with digitonin and in squalene, we may safely assume that this radioactivity, as with precipitable companion sterols, was also higher in the treated rats.

The data on the incorporation of ^{14}C from acetate and ^{3}H from body water are presented in Tables 1–5. Animals in groups 1 and 2 in Table 1 that received the isotope intraperitoneally show a large variation in the activity of liver DPS, which renders the difference between the control and the experimental groups statistically nonsignificant but does not detract from the fact that it represents a trend in the same direction as in all of the subsequent experiments. This was shown to be especially true in the other experiments in which intraperitoneal injection of isotope was employed and the differences in the incorporation of [^{14}C]acetate into liver DPS of control and experimental animals 30 min (groups 5 and 6) and 6 hr (groups 7 and 8) after the isotope was administered were found to be statistically significant (Table 1). Although there is no satisfactory explanation for large variations from animal to animal in some experiments, we may point out that variations of the same order of magnitude in studies with dogs or rats in which cholesterol synthesis was measured have also been observed by others (18–20). Perhaps it may

TABLE 2. Effect of allylisopropylacetamide on incorporation of ^{3}H and ^{14}C into rat liver digitonin-precipitable sterols in vitro

Treatment	Activity of Liver DPS		$^{3}H/^{14}C$ × 10^{-2}
	^{3}H	^{14}C	
	dpm × 10^{-3}		
Saline (10)[a]	42 ± 17[b,c]	0.12 ± 0.06[b]	3.49 ± 0.20
AIA (11)	165 ± 135	0.55 ± 0.45	3.01 ± 0.18

Each vessel contained 2.0 ml of Krebs-Ringer phosphate solution, 22 mCi of [^{3}H]water, 0.16 μCi of [^{14}C]acetate (2.16 mg), and 0.5 g of liver slices, approximately 0.5 mm in thickness, cut with a Stadie-Riggs slicer. Temperature, 37°C; pH, 7.4; time of incubation, 3 hr; atmosphere, 100% O_2.

[a] Number of animals in each group.

[b] $P < 0.001$.

[c] Values are means ± SD.

be argued that, because the animals were fasted throughout the experimental period, hepatic sterol synthesis could be presumed to be minimal, and in this particular event the source of radioactivity in liver sterols may largely be the contribution of intestinal sterol synthesis. This may also explain the fact that statistical variation was less when the isotope was given intravenously as opposed to when it was given via the intraperitoneal route.

In order to confirm that the radioactivity measured in liver sterols was primarily a reflection of hepatic sterol synthesis, three different experiments were performed. First, in an in vitro experiment, ability of liver slices from AIA-treated and control animals to incorporate ^{3}H from body water and ^{14}C from acetate was measured. As can be seen from results in Table 2, liver slices from treated animals incorporated four times more ^{3}H and ^{14}C into DPS than did the slices from the untreated animals. In another experiment, both experimental and control animals were allowed free access to food and water until AIA treatment. 12 hr after AIA, each animal received 4.51 μCi of [1-^{14}C]acetate intraperitoneally, and 30 min and 6 hr after the isotope was given, animals were killed by decapitation. Liver sterols were isolated and incorporation of radioactivity into DPS was measured as described. The results presented in Table 1 (groups 9 and 10) clearly indicate an almost twofold increase in the activity of liver DPS from treated animals compared with their controls. These experiments, of course, do not rule out the possibility that intestinal sterol synthesis may also have been stimulated after treatment with AIA, but, in any case, they show an increase in the ability of livers from AIA-treated animals to incorporate radioactivity into sterols from their precursors. However, even more cogent evidence that sterol synthesis by the liver is significantly enhanced after a single dose of AIA was obtained by measurement of radioactivity in liver DPS from animals whose intestines from the pylorus to the rectum were removed surgically 12 hr after AIA treatment. The experimental conditions were as described earlier, and the data presented in Table 3 indicate a more

TABLE 3. Effect of allylisopropylacetamide on incorporation of ^{14}C from sodium [1-^{14}C]acetate into rat liver sterols after bowel removal

Treatment	Total Cholesterol	Activity of Liver DPS
	mg/liver	*dpm/liver* × 10^{-3}
AIA (6)[a]	12.9 ± 1.0[b]	115 ± 55
Saline (6)	12.2 ± 0.9	18 ± 21
P	<0.025	<0.005

All animals were injected with 100 mCi of [1-^{14}C]acetate directly into the portal vein and killed 15 min later.
[a] Number of animals in each group.
[b] Values are means ± SD.

TABLE 4. Effect of allylisopropylacetamide on incorporation of ^{3}H from body water into liver digitonin-precipitable sterols in intact rats

Treatment	Liver Weight	Cholesterol	Activity in DPS
	g	*mg/g liver*	*dpm/liver* × 10^{-2}
Saline	5.4 ± 0.4[a]	2.6 ± 0.4	34 ± 21
AIA	6.6 ± 0.3	2.3 ± 0.1	108 ± 47
P	<0.01	<0.02	<0.001

All animals were given 50 mCi of [^{3}H]water intravenously and were killed 30 min later. Each group contained 10 animals.
[a] Values are means ± SD.

than sixfold increase in the incorporation of radioactivity into liver sterols isolated from experimental animals over that obtained from control animals. The data from these experiments taken together offer convincing evidence that what was principally observed under these conditions was an acceleration of hepatic sterol synthesis induced by AIA.

It is possible that changes observed in the incorporation of radioactivity when acetate is the precursor may be due to changes in the acetate pool size rather than to a change in the catalytic activity of the cholesterol biosynthetic pathway in the liver of AIA-treated rats. Whether AIA acts to modify the pool size of acetate and its derivatives may be evaluated by measurement of incorporation of radioactivity into sterols from a precursor such as $^{3}H_2O$, which is not subject to change in this fashion. In this event, the conditions that require formation of new C—H bonds of cholesterol will result in the incorporation of tritium atoms from tritiated water into cholesterol and this will depend only on the number of molecules of substrate converted into product. Accordingly, data in Table 4 show that AIA produces a threefold increase in the incorporation of ^{3}H into liver sterols. Moreover, the data from the liver slice experiment in Table 2 show that incorporation of both tritium and acetate into sterols is increased by AIA. The $^{3}H/^{14}C$ ratio, which remains approximately unchanged for both experimental and control animals, reflects a similar degree of incorporation from both precursors. These results lead to the conclusion that the increase in cholesterol synthesis brought about by AIA treatment is most likely due to an increase in the catalytic activity of the hepatic pathway for cholesterol biosynthesis.

Since the formation of mevalonic acid from HMG CoA is very sensitive to changes in physiological conditions, we considered it possible that AIA may exert its effect by altering the rate of formation of mevalonic acid from acetate. In order to test this hypothesis we administered simultaneously [^{3}H]acetate and [2-^{14}C]mevalonate to rats given AIA and measured the incorporation of radioactivity into the liver sterols. The results shown in Table 5 indicate that, although the incorporation of tritium from acetate in liver DPS is increased in the experimental group, the difference in the ^{14}C activity of liver as a result of mevalonate conversion to DPS in both control and experimental animals is not significant. These results permit the conclusion that the observed increase in sterol biosynthesis is due to an increased formation of mevalonic acid from acetate, and they further suggest that the primary site of action of AIA as it affects cholesterol synthesis is on one or more steps in the pathway from acetate to mevalonate.

DISCUSSION

Prolonged fasting results in a decrease in body and liver weights of rats. However, in spite of a loss in body weight a single dose of AIA actually produced an increase in liver weight and overcame the effects of fasting. However, Taddeini, Nordstrom, and Watson (6) have previously observed that considerable growth of the liver in the AIA-treated rabbits, whether due to hypertrophy or cell multiplication, reflected new formation of liver cell protoplasm. Hypertrophy of the hepatocytes after AIA injection to rats has also been reported (16). Decrease in cholesterol concentration thus appears to be a consequence of increased liver size. These effects of AIA treatment closely parallel those produced by whole body X-irradiation of fasted rats described by Gould, Bell, and Lilly (21). Our results showing an increased rate of cholesterol synthesis are in good agreement with the suggestion originally made by Frantz, Schneider, and Hinkelman (20) of an inverse relationship between the rate of synthesis and liver choles-

TABLE 5. Effect of allylisopropylacetamide on incorporation of ^{3}H from acetate and ^{14}C from mevalonic acid into liver digitonin-precipitable sterols in intact rats

Treatment	Liver Weight	Cholesterol	Activity of Liver DPS	
			^{3}H	^{14}C
	g	*mg/g liver*	*dpm/liver* × 10^{-2}	
Saline	5.6 ± 0.6[a]	2.7 ± 0.2	675 ± 493	985 ± 331
AIA	6.4 ± 0.7	2.4 ± 0.3	2205 ± 1496	1163 ± 572
P	<0.01	<0.01	<0.001	NS[b]

2 μCi of [2-^{14}C]mevalonate and 100 μCi of [^{3}H]acetate were administered simultaneously to each rat through a tail vein 30 min before the animal was killed. Each group contained 14 animals.
[a] Values are means ± SD.
[b] Not significant.

terol concentration. The data in the present report further show that AIA-induced acceleration of cholesterol synthesis may be accounted for by an increased formation of mevalonic acid from acetate. Stimuli that produce a rise and fall in the rate of hepatic cholesterogenesis have been identified with concomitant alterations in the amount of the regulatory enzyme HMG CoA reductase (15, 22). It is therefore tempting to speculate that increased formation of mevalonate in this instance may also reflect a similar change in HMG CoA reductase.

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