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COLORIMETRIC ESTIMATE OF CHOLESTEROGENESIS IN LIVERS PERFUSED IN THE PRESENCE OF *TRANS*-1,4-bis-(2-CHLOROBENZYLAMINOMETHYL)-CYCLOHEXANE DIHYDROCHLORIDE (AY-9944)

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Abstract—AY-9944 [*trans*-1,4-bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride] inhibits the conversion of sterol intermediates to cholesterol. The appearance of sterol intermediates, measured colorimetrically as "fast-acting" sterols, has been used as an estimate of hepatic cholesterogenesis *in vivo* after treatment with AY-9944. The appearance of fast-acting sterols as an estimate of cholesterogenesis was studied in the isolated rat liver, perfused *in vitro* in the presence of AY-9944, with or without oleic acid. Oleic acid elevated the appearance of fast-acting sterols in the liver and the perfusate. The secretion of fast-acting sterols by the perfused liver correlates with the activity of microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterogenesis, and the synthesis of fast-acting sterols. The latter also correlates with the activity of HMG-CoA reductase. These observations are in agreement with previous reports of stimulation of the secretion and synthesis of cholesterol by liver perfused with oleic acid. Based on these findings, it is suggested that the level of fast-acting sterols after treatment with AY-9944 may be used as a colorimetric estimate for the synthesis and distribution of cholesterol in the isolated perfused rat liver.

AY-9944 [*trans*-1,4-bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride], like triparanol, is an inhibitor of cholesterogenesis which leads to an accumulation of sterol intermediates in tissue. After its introduction [1, 2], it was found that the compound apparently inhibits the conversion of 7-dehydrocholesterol to cholesterol [3, 4]. The accumulation of sterol intermediates has been used as an estimate of cholesterogenesis [1–5]. Horton *et al.* [5] developed a method to estimate cholesterogenesis in the intact rat treated with AY-9944 by measuring the appearance of 7-dehydrocholesterol as a "fast-acting" sterol in rat plasma. "Fast-acting" sterols refer to sterols which react with Liebermann–Burchard reagent to produce maximal color within 2 min in contrast to cholesterol, a "slow-acting" sterol, which produces maximal color after 30 min [6]. Horton *et al.* [5] reported a significant correlation between the concentration of fast-acting sterols in the blood and hepatic cholesterogenesis, estimated by the incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols by liver slices of the same animals. Measuring the level of fast-acting sterols in the blood as an indicator for changes in hepatic synthesis of cholesterol has been used extensively in studying the effects of carcinogenic compounds on hepatic cholesterogenesis [7–9].

The possibility of using the appearance of fast-acting sterols after treatment with AY-9944 as an estimate of hepatic cholesterogenesis was tested in the isolated perfused rat liver. In previous work, it had been found that oleic acid stimulated the secretion and synthesis of cholesterol by the perfused liver [10, 11]. The increase in hepatic cholesterogenesis was estimated both by the increase in the incorporation of tritium from tritiated water into cholesterol [10], and separately [11] by the increase in the activity of microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the rate-limiting enzyme of cholesterogenesis.

It was observed in this study that the addition of AY-9944 resulted in the appearance of fast-acting sterols in the liver and the perfusate. In addition, oleic acid stimulated the accumulation of fast-acting sterols and the activity of microsomal HMG-CoA reductase.

EXPERIMENTAL

Male Sprague–Dawley rats from Murphy Breeding Labs, Inc., Plainfield, IN, were used in this study. The rats were housed, with lighting from 5:00 a.m. to 5:00 p.m., and were fed Wayne Lab-blox and water *ad lib.* The livers were removed between 8:50 and 9:50 a.m. and were perfused *in vitro* using the apparatus and conditions described previously [10–12]. The perfusion medium consisted of Krebs–Ringer bicarbonate buffer (pH 7.4), 100 mg glucose/100 ml of solution, and 3% of purified bovine serum albumin [10]. Following perfusion for a 20-min period of equilibration, a sample of the perfusate was taken for analysis; a solution of AY-9944 in 0.9% NaCl was added to the perfusate pool to obtain a concentration of 1×10^{-5} M AY-9944, and infusion into the perfusate was started. The infusate (pH 7.4) contained 0.9% NaCl, 1×10^{-5} M AY-9944, and 3% purified bovine serum albumin alone or complexed with 14.16 μmoles oleic acid/ml of infusate. The infusate was delivered to the perfusate at a constant rate of 11.7 ml/hr for the 3-hr period of perfusion.

At the termination of the experiments the livers were perfused with a single-pass of 20 ml of ice-cold 0.9% NaCl; adherent non-hepatic tissue was removed and the liver was blotted, weighed and minced in an ice-cold

beaker. The minced tissue was homogenized in 0.3 M sucrose and 10 mM 2-mercaptoethanol [13] to a final volume of 50 ml, and aliquots were taken for saponification. The perfusate and liver homogenate were saponified with 1 ml of aqueous KOH (1 g/ml) and 4 ml of ethanol for 60 min at 75°. The non-saponifiable fraction was extracted into hexane by the addition of 5 ml of water and 5 ml of hexane followed by vigorous shaking for 1 min. Aliquots of hexane were removed, dried under N_2, and analyzed colorimetrically for "fast-acting" (2 min) and "slow-acting" (33 min) sterols, as described by Moore and Baumann [6]. The stable Liebermann–Burchard reagent reported by Kim and Goldberg [14] was used. The amounts of fast-acting and slow-acting sterols were calculated, and corrections were made according to the procedure of Moore and Baumann [6]. The constants used in the calculations described by Moore and Baumann were determined experimentally with 7-dehydrocholesterol (Sigma, St. Louis, MO) and free cholesterol (Nu-Chek Prep., Elysian, MN). The remaining portion of liver homogenate was used for the isolation of microsomes as described previously [11]. Microsomal activity of HMG-CoA reductase was assayed by the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonic acid as before with the exception that LK5D thin-layer plates (Whatman, Clifton, NJ) were used for the separation of mevalonate lactone [11].

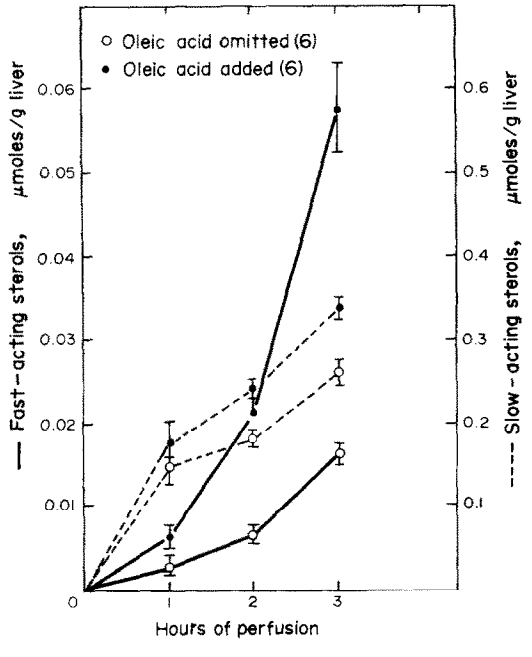

Fig. 1. Cumulative secretion of fast-acting and slow-acting sterols by perfused rat livers treated with AY-9944. The data shown are mean values ± S.E.M. for the cumulative secretion of sterols by the perfused liver. Samples of perfusate were taken at time 0 and after 1, 2 and 3 hr of perfusion. The output of sterols at time 0 was subtracted from the cumulative secretion. Except for the secretion of slow-acting sterols after the first hour of perfusion, all other comparisons between groups with or without oleic acid are significant at $P < 0.05$ by Student's t-test. Numbers in parentheses indicate the number of observations for each point.

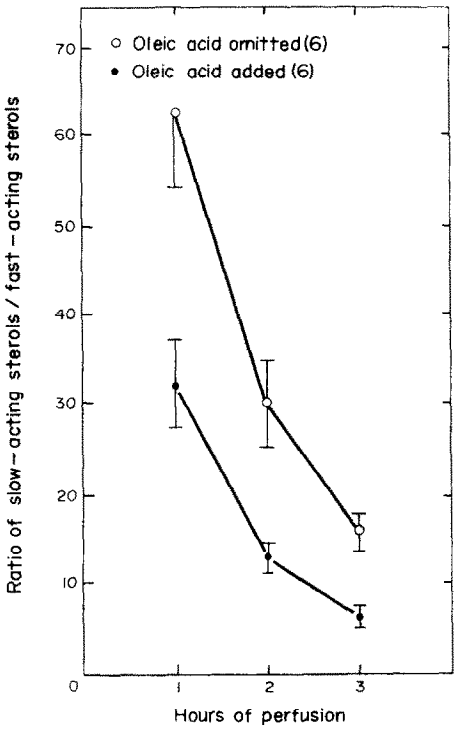

Fig. 2. Relationship between the secretion of slow-acting and fast-acting sterols by perfused livers treated with AY-9944. The data shown are the molar ratios of slow-acting sterols (μmoles/g of liver) secreted/fast-acting sterols (μmoles/g of liver) secreted at different time periods of perfusion calculated from Fig. 1. Data are mean values ± S.E.M., and differences between experiments with or without oleic acid at all three periods of determination are significant at $P < 0.05$, as determined by Student's t-test. Numbers in parentheses indicate the number of observations for each point.

RESULTS

The effect of oleic acid on the cumulative secretion of sterols by the perfused liver is shown in Fig. 1. In agreement with previous reports [10, 11], the addition of oleic acid elevated the secretion of sterols by the liver. The perfused liver secreted both the fast-acting and the slow-acting sterols. The secretion of both of these fractions was stimulated by oleic acid. The relationship between the secretion of slow-acting and fast-acting sterols is shown in Fig. 2. As the secretion of the fast-acting sterols is increased, the secretion of slow-acting sterols is reduced. The ratio of slow-acting/fast-acting sterols decreases by about 50 per cent after each hour of perfusion. The final ratio after 3 hr of perfusion, 15.9 ± 1.5 for experiments without oleate and 6.3 ± 0.9 for experiments with oleate, is similar to the ratio of slow-acting/fast-acting sterols for the liver in the respective groups (B/A, Table 1).

The effect of oleic acid on the amount of fast-acting and slow-acting sterols in the liver is shown in Table 1. In agreement with previous reports [10], the addition of oleic acid does not alter either the total amount of sterols in the liver (A + B, Table 1) or the amount of cholesterol, slow-acting sterols (B, Table 1). The concentration of fast-acting sterols in the liver, on the other hand, is elevated in experiments with oleic acid (A, Table 1).

Table 1. Concentration of fast-acting and slow-acting sterols in perfused liver treated with AY-9944*

Group	Fast-acting sterols (A) (μmoles/g liver)	Slow-acting sterols (B) (μmoles/g liver)	Total sterols (A + B)	Ratio (B/A)
(I) Oleic acid omitted (6)	0.32 ± 0.29	3.81 ± 0.19	4.08 ± 0.23	12.24 ± 1.08
(II) Oleic acid added (6)	0.61 ± 0.50	3.85 ± 0.26	4.46 ± 0.28	6.53 ± 0.53
Statistics I vs II	< 0.005	NS	NS	< 0.005

* Data shown are mean values ± S.E.M. The values are obtained from livers after 3 hr of perfusion in the presence of 1×10^{-5} M AY-9944. Numbers in parentheses represent the number of observations. The significance of differences was determined by Student's *t*-test. NS represents a P value greater than 0.05.

The relationship between the secretion of fast-acting sterols (y) and the activity of microsomal HMG-CoA reductase (x) can be described by the linear regression line $y = -0.003 + 0.16x$ ($r^2 = 0.77, p < 0.05$). This is in agreement with the correlation between the secretion of cholesterol and the activity of HMG-CoA reductase reported previously [11]. In addition, linear correlation between the secretion and the synthesis of fast-acting sterols (Fig. 3) and the latter with the activity of HMG-CoA reductase (Fig. 4) can be observed.

DISCUSSION

The addition of AY-9944 (1×10^{-5} M) resulted in the secretion and accumulation of fast-acting sterols in the isolated perfused rat liver. The concentration of AY-9944 used in this study is 10 times the amount required to inhibit, by 98 per cent, the conversion of sterol intermediates to cholesterol in liver homogenate [3]. The action of AY-9944 on the appearance of fast-acting sterols is fairly rapid; fast-acting sterols can be detected in the perfusate 20 min after the addition of AY-9944. These features and the solubility of AY-9944 in water make AY-9944 an attractive alternative to the use of triparanol as a tool in studying the cholesterogenesis of the perfused liver, by measuring the accumulation of sterol intermediates.

On the basis of the mechanism of action of AY-9944 [3], the fast-acting sterols would represent 7-dehydrocholesterol, which accumulates in the presence of the inhibitors. The amount of fast-acting sterols observed, however, may be an over-estimation for the amount of 7-dehydrocholesterol, a Δ^7 sterol, especially in the liver. In addition to 7-dehydrocholesterol, other Δ^7, $\Delta^{5,7}$ sterols and peroxides of sterols may react similarly with Liebermann–Burchard reagent [6, 15, 16]. The amount of sterols other than 7-dehydrocholesterol measured as fast-acting sterols may be small since the amount of fast-acting sterol in the rat liver before perfusion is 0.07 ± 0.01 μmole/g of liver (n = 6) as compared to 0.32 ± 0.29 and 0.61 ± 0.50 μmole/g of

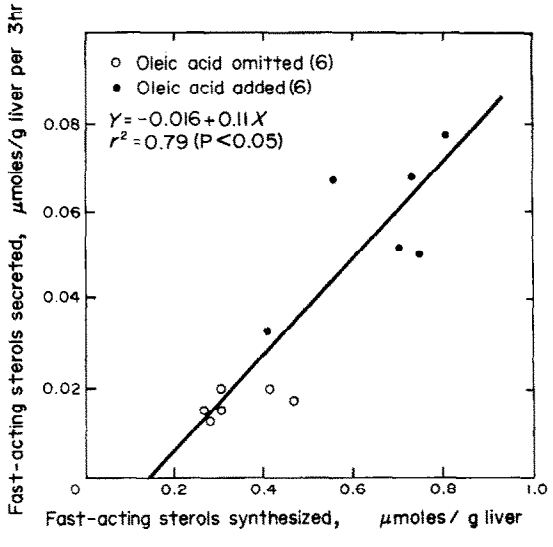

Fig. 3. Correlation between synthesis and secretion of fast-acting sterols in perfused livers treated with AY-9944. The data shown are the individual experiments reported in this manuscript. The amount of fast-acting sterols secreted is the cumulative output of the liver after 3 hr of perfusion (Fig. 1). The amount of fast-acting sterols synthesized is the sum of the cumulative output after 3 hr of perfusion (Fig. 1) and the amount found in the liver (Table 1, A) after perfusion. Correlation is determined by linear regression analysis.

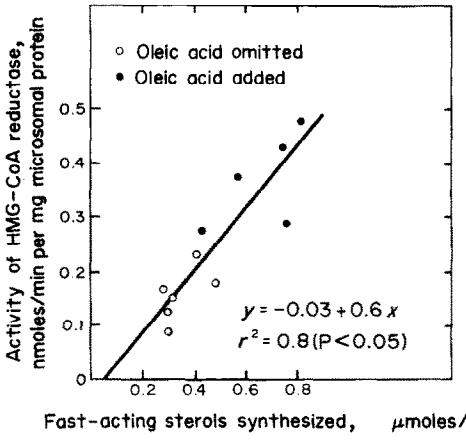

Fig. 4. Correlation between the synthesis of fast-acting sterols and the activity of microsomal HMG-CoA reductase. The data shown are the individual experiments reported in the manuscript. The calculation for the synthesis of fast-acting sterols is given in the legend of Fig. 3. Correlation is determined by linear regression analysis.

liver after perfusion in the presence of AY-9944 without or with oleic acid respectively (Table 1). Despite this, the increase in the secretion and in the synthesis of fast-acting sterols in the perfused liver by oleic acid is in agreement with the reported increases in the secretion and synthesis of cholesterol estimated by isotopic methods [10, 11].

In the perfused liver, the appearance of fast-acting sterols would represent newly synthesized cholesterol. The slow-acting sterol, cholesterol, would represent cholesterol which is in the liver before the addition of AY-9944. Assuming the metabolism of fast-acting sterols, or 7-dehydrocholesterol, is similar to that of cholesterol, then the level of fast-acting sterols can be used to indicate this distribution of newly synthesized cholesterol and the total mass of fast-acting sterols, as an estimate of cholesterogenesis. Consequently, oleic acid stimulated the secretion of both newly synthesized and preformed cholesterol (Fig. 1) and the proportion of the preformed cholesterol was reduced as perfusion continued (Fig. 2). The total amount of fast-acting sterols, the sum of liver plus perfusate after 3 hr of perfusion, is 0.33 ± 0.03 and 0.66 ± 0.06 μmole/g of liver ($P < 0.005$) for experiments in the absence or presence of oleic acid respectively. Assuming that the total amount of fast-acting sterols, in these experiments, represents cholesterogenesis by the perfused liver, oleic acid stimulated hepatic cholesterogenesis by 100 per cent after 3 hr of perfusion. This is comparable to a 150 per cent increase in cholesterogenesis measured by the incorporation of tritium (^3H_2O) into cholesterol [10] and, separately [11], by a 140 per cent increase in cholesterogenesis measured by the activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterogenesis. In both of these experiments, livers were perfused for 4 hr with the same amount of oleic acid added. In the present experiments, the activity of HMG-CoA reductase of microsomes isolated from the same liver also showed a 110 per cent increase in activity which correlates with the synthesis of fast-acting sterols (Fig. 4). Apparently, the level of fast-acting sterols and the activity of microsomal HMG-CoA reductase can be used concurrently to estimate cholesterogenesis in the same perfused liver.

Currently the most widely used method to measure the activity of HMG-CoA reductase is the isotopic method employing [^{14}C]HMG-CoA as substrate and [^{3}H]mevalonic acid as internal standard [17]. While there are spectrometric methods of measuring the activity of the enzyme [18, 19], the sensitivity of these methods is low [18]. The isotopic method of measuring the activity of the enzyme makes it difficult to use, concurrently, the incorporation of another isotope, such as [^{3}H]water or [^{14}C]octanoate [20], into cholesterol, as an additional estimate of cholesterogenesis. Cholesterogenesis, when estimated by the isotopic measure of the activity of the enzyme and the incorporation of radioactive precursors into cholesterol, has been determined in duplicate samples of the tissue [21, 22]. The spectrometric analysis of sterol intermediates after treatment with AY-9944 may be a useful alternative in cases where two different isotopic methods were used to estimate cholesterogenesis [21, 22], in cases where radioactive measurement of the activity of the enzyme disassociates from the incorporation of an isotope into cholesterol [21, 22], and where the activity of the enzyme may be altered in the isolation of microsomes [13].

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