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THE EFFECT OF α-METHYL γ-PHENYL BUTYRIC ACID ON STEROL BIOSYNTHESIS *IN VITRO*

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Abstract—Cholesterol synthesis from acetate-2-^{14}C and mevalonate-2-^{14}C has been examined in rat liver slices, in the presence of α-methyl γ-phenyl butyric acid, a potential hypocholesterolemic drug. The results show that cholesterol synthesis from labelled acetate is inhibited by final concentrations of the drug as small as 1μM. When acetate is replaced by mevalonate, inhibition occurs, though less strongly pronounced. Syntheses of squalene, lanosterol and cholesterol from mevalonate are inhibited by the same degree, although differences in the rate of labelling are found at high concentration of the drug (1 mM and 10 mM). The results indicate that α-methyl γ-phenyl butyric acid probably affects some of the enzymic reactions which convert acetate into mevalonate and mevalonate into squalene, without influencing successive metabolic steps.

PHENYL derivatives of 5-carbon branched chain acids have been reported to be hypocholesterolemic agents in various animals and man.[1] The most active compounds in this series appeared to be β-benzal butyric acid and α-methyl γ-phenyl butyric acid (inhibition of cholesterol biosynthesis *in vivo* and Triton hyperlipidemia test). Apart from some preliminary results,[2] the phenyl derivatives of the 5-carbon chain acids have not been tested however as *in vitro* inhibitors of sterol synthesis nor has their mechanism of action been elucidated. The suggestion that the site of action of α-phenyl butyric acid is at the first steps of cholesterol biosynthesis,[3, 4] i.e. between acetate and mevalonate needs to be substantiated.

The main purpose of the present paper is to find out whether the synthesis of cholesterol and squalene, in the presence of α-methyl γ-phenyl butyric acid (MPBA), is inhibited *in vitro* only from acetate or also from mevalonate. The labelling of the principal sterol intermediates, i.e. of squalene, lanosterol and cholesterol, has been therefore examined in rat liver slices. Experimental evidence will be given which shows that MPBA acts as a noticeable inhibitory agent of liver cholesterol synthesis, and that the inhibition is efficient both from labelled acetate and mevalonate.

EXPERIMENTAL

Chemicals and labelled substrates

Benzene, chloroform, diethyl ether, pyridine, hexane, acetone, methyl alcohol and petroleum ether (b.p. 60–80°) were freshly distilled over calcium chloride and stored under nitrogen. All the other solvents were not further purified.

MPBA was kindly provided by Istituto Biochimico Italiano (Milan, Italy). The compound (sodium salt), with an estimated equivalent molecular weight of 197·54 (based on its chemical composition), possessed three u.v. absorption maxima at 206, 251 and 294 mμ, with corresponding values of ϵ equal to 519, 152 and 6·8. The free

acid form had infrared absorption maxima at 2950, 1700, 1600, 1500, 1450, 1400 1300, 1240, 1030, 750 and 705 cm^{-1}, which were very close to the predicted values.

Cholesterol and dihydrocholesterol were from B.D.H. (London); the first compound was crystallized via the dibromide,[5] recrystallized with absolute ethanol and dried to constant weight under vacuum. 7-Dehydrocholesterol and Δ_7-cholestenol were from Nutritional Biochemical Corp., Cleveland, Ohio. Methostenol (4-methyl-5α-cholest-7-en-3β-ol), lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol, contaminated with dihydrolanosterol), and squalene were products of Mann Res. Labs., Inc. Other products were of reagent grade.

2-^{14}C-acetate (26·3 mc/m-mole) and 2-^{14}C-mevalonate (3·45 mc/m-mole) were commercial products, which were diluted with inert substrates to a suitable specific activity. Before diluting, the *N,N'*-dibenzylethylenediamine salt of the mevalonic acid was converted to the potassium salt.

Tissue preparation and incubation

Male Sprague–Dawley rats (150 ± 25 g), fed with standard rat cubes, were fasted for 6 hr and sacrificed in pairs. Liver slices (400 μ of thickness, and weighing about 400 mg) were quickly prepared in the cold with a Stadie–Riggs microtome (A. H. Thomas, U.S.A.). Only the slices removed from the same separate liver block were distributed among the flasks of the same experiment (about 20 slices per flask). They possessed a constant weight/surface ratio value of 0·40–0·44 mg/mm^2 for all the experiments; this result indicates therefore a uniform value of the exchange surface

The slices were incubated in Warburg flasks containing 118 mM NaCl, 4·7 mM KCl, 1 mM KH_2PO_4, 1·14 mM $MgSO_4$, 3·56 mM $NaHCO_3$, 3·05 mM $MgCl_2$ and 9·2 mM Na_2HPO_4-HCl buffer (pH 7·40). The amounts of the precursors were as follows: 2-^{14}C-acetate, 9·20 μc, 0·58 mM (sp. act., S.A., 3·96) or 2-^{14}C-mevalonate, 2·48 μc, 0·32 mM (S.A., 1·94). The Ringer solution was added before the slices and precursors, while MPBA was added last. The total volume was of 4 ml. The flasks were flushed with O_2 and then shaken at 37° for 2 hr at about 80 strokes/min.

Separation and determination

Digestion of lipid and extraction of the free sterols and squalene were carried out as described previously.[6, 7] The subsequent precipitation with digitonin and further purification of the digitonides were performed by standard techniques.

Prior to purification by means of digitonin, a small aliquot of the acetone-ethanol (1:1, v/v) solution[6, 7] was chromatographed in quadruplicate on 40 cm-long silica gel G plates pre-stained with 2,7-dichlorofluorescein, with benzene-ethyl acetate (20:1, v/v) as the solvent (24 hr in the dark under nitrogen).[8] The u.v.-absorbing or fluorescent zones were, (1) scraped from the plate for radioactivity assay, (2) eluted for sterol determination, (3) radioautographed, and (4) further rechromatographed, as explained later.

The above reported chromatographic method clearly evidentiates labelled cholesterol and small amounts of radioactive squalene, lanosterol and methostenol, after 2-^{14}C-acetate incorporation. Traces of labelled 7-dehydrocholesterol were sometimes present. When 2-^{14}C-mevalonate was the precursor, the products were radioactive Δ_7-cholestenol, large quantities of cholesterol (presumably contaminated with traces of cholestanol), 7-dehydrocholesterol, methostenol, lanosterol and large amounts of

squalene. Some overlap was present however in this case between cholesterol, Δ_7-cholestenol and 7-dehydrocholesterol.

Rechromatography was therefore carried out on the three zones of cholesterol, Δ_7-cholestenol and 7-dehydrocholesterol, collected together. All the other spots were found in fact to be homogeneous on further separation, and therefore were not rechromatographed. For rechromatography, the zones were accurately scraped from the plates, eluted into small sintered-glass columns twice with chloroform (6 ml) and then with 3 ml of chloroform–methanol (98:2 v/v), evaporated under nitrogen and quantitatively rechromatographed on silver nitrate-impregnated silica gel plates,[9] with chloroform–acetone (95:5 v/v) as the solvent. The plates were run in the dark under nitrogen for 90–100 min at 4°, and then lightly sprayed with 0·2% 2,7-dichlorofluorescein in absolute ethanol. A clear separation of the labelled 7-dehydrocholesterol, cholesterol and Δ_7-cholestenol was obtained. Rechromatography was always carried out in triplicate, and the spots were, (1) removed for sterol determination, (2) scraped off for radioactivity estimation, and (3) radioautographed. The recovery of each of the sterols after rechromatography was over 85 per cent. Chromatography on the silver nitrate-plates has been also carried out on the free sterols regenerated from the purified digitonide precipitate. As a result, the values of the labelling rates of the radioactive sterols were very close either after rechromatography on the silver nitrate-plates of the initially separated sterol spots or after decomposition of the digitonide precipitate by heating in pyridine and successive argentation chromatography of the free sterols. For this reason, we have mainly adopted, for sterol purification, the technique of the rechromatography on the silver nitrate-plates of the sterols initially separated on the long 40 cm-2,7-dichlorofluorescein-silica gel G-plates.

The amount of sterol was measured both on the purified digitonides and on the eluted chromatographic spots.[10] The method of Zlatkis *et al.*[11] was used. The recovery of standards from the plates approached 100 per cent. When estimation was carried out on the $AgNO_3$-plates, the Zak *et al.*[12] procedure was used, by replacing the ferric chloride reagent by ferric nitrate, to avoid cloudiness due to the silver chloride. Recovery was between 90–95 per cent.

Radioactivity was determined by liquid scintillation counting on the purified digitonides or after elution from the Kieselgel with methanol.[13] The dichlorofluorescein dye and the $AgNO_3$ exerted a moderate effect by quenching the samples, which, however, was properly corrected with calibration experiments carried out with standard radioactive lipids. In addition, silver nitrate concentration was scaled down in the plates to 4 per cent ,and this correction has avoided a stronger interference with the countings. The ^{14}C-counting efficiency was over 78 per cent, and the recovery of label from the plates approached 100 per cent.

RESULTS AND DISCUSSION

In all the series of experiments, labelling has been determined only for those components which were detected in measurable amounts, were homogeneous and definitely identified. Identity and radiopurity of the final spots of the ^{14}C-lipids has been confirmed by cochromatography with authentic lipid and cocrystallization, lanosterol and cholesterol as acetate derivatives, and squalene as the hexahydrochloride. Other techniques have been adopted, in order to check the radiopurity of the final sterol spots, as described elsewhere.[7] As a result, the final sterol spots,

separated by the techniques reported in the Experimental Section, turned out to be reasonably and sufficiently pure to allow accurate results.

Obviously, the radioactivity and the sterol content of the chromatographic fractions have always been corrected, according to the recovery values obtained by cochromatography with authentic material. This was important when rechromatography or elution for sterol assay were to be carried out, the recovery values of the applied sterols being in these two cases of the order of 85 per cent and 90–95 per cent, respectively (see Experimental Section).

After acetate incorporation, determinations were carried out only on the cholesterol spot isolated on the 40 cm-long plates, and on the purified labelled digitonide precipitate. The two procedures gave always comparable results. Radioactivity was occasionally assayed on the squalene spot. In the case of mevalonate incorporation, countings were carried out on the squalene and lanosterol spots, both isolated on the 40 cm-long plates, and on the cholesterol, isolated by the silver nitrate impregnation method. Additional estimations were performed also on the cholesterol digitonide precipitate. When available in sufficient amounts, Δ_7-cholestenol and 7-dehydrocholesterol were also examined.

The results of Figs. 1 and 2 clearly indicate that cholesterol biosynthesis is reduced, when incubation of the liver slices with labelled acetate or mevalonate is carried out in the presence of various concentrations of MPBA. MPBA is an efficient inhibitor of cholesterol synthesis from acetate *in vitro* (Fig. 1), and 50 per cent inhibition is obtained with a final concentration of 7×10^{-5} M. According to our result, MPBA also inhibits the incorporation of 2-^{14}C-mevalonate into cholesterol (Fig. 2), although not to

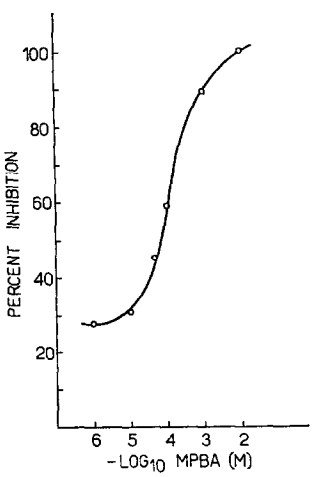

FIG. 1. The inhibition by α-methyl γ-phenyl butyric acid of liver cholesterol synthesis from 2-^{14}C-acetate. Incubation of the liver slices and isolation of cholesterol were carried out as described in the text. The results, expressed as per cent inhibition values (0 = control levels), were calculated as mμmoles of cholesterol formed per hr per mg of liver cholesterol (mean control value $\pm$ S.E.M. of 0·78 $\pm$ 0·13, in 9 experiments). For calculation, the millimicrocuries of ^{14}C of the isolated cholesterol are divided by the specific activity of the precursor (3·96) $\times$ mg of the tissue free cholesterol $\times$ hr of incubation $\times$ C. C represents the number of the labelled 2-^{14}C-acetate molecules, which are comprised in 1 molecule of ^{14}C-labelled cholesterol. As known, for 2-^{14}C-acetate, i.e. for the acetate labelled in the methyl position, C = 15.
Eight experiments were carried out for each value of α-methyl γ-phenyl butyric acid concentration.

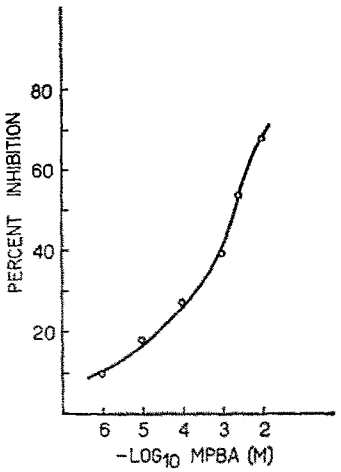

FIG. 2. The inhibition by α-methyl γ-phenyl butyric acid of liver cholesterol synthesis from 2-^{14}C-mevalonate. Incubation of the liver slices and isolation of cholesterol were carried out as described in the text. The results are expressed as outlined in Fig. 1. For calculation, the millimicrocuries of ^{14}C of the isolated cholesterol are divided by the specific activity of the precursor (1·94) × mg of the tissue free cholesterol × hr of incubation × C. C represents the number of the 2-^{14}C atoms of the labelled 2-^{14}C-mevalonate molecules which are comprised in 1 molecule of the ^{14}C-labelled cholesterol. Owing to the mechanisms of squalene cyclisation and lanosterol transformation into cholesterol, C = 5. As known, six molecules of mevalonate enter in fact the final squalene structure, but one originary 2-^{14}C atom is lost as a 4-methyl group, after lanosterol formation.

Six experiments were carried out for each value of the inhibitor concentration.

the same extent as it occurs when acetate is the sterol precursor. At a 10^{-6} M concentration, MPBA has in fact no effect on the conversion of mevalonate into cholesterol, and a 50 per cent inhibition is obtained at a final concentration of about 4×10^{-3} M. The experiments reported in Figs. 1 and 2 were carried out by measuring the rate of labelling of the cholesterol spot isolated through thin-layer procedures. No significant differences were observed, at any value of MPBA concentration, between these results and those obtained by carrying out estimations on the purified labelled digitonide precipitate.

According to the degrees of inhibition of cholesterol synthesis, at the different levels of MPBA, we may postulate, although not conclusively, that the drug inhibits more actively some of the enzymes which convert acetate into mevalonate rather than those which carry out the conversion of mevalonate into cholesterol. This consideration is particularly valuable at the high levels of MPBA (Figs. 1 and 2). Two sites of inhibition at least can therefore be visualized along the entire pathway. If we postulate in fact tentatively that the inhibition from acetate takes place only after mevalonate, then we cannot explain, at least for the higher values of MPBA concentrations, the experimental data of Figs. 1 and 2.

The inhibition brought about by MPBA along the steps which lie between mevalonate and sterols seems to be of noticeable interest, since our data indicate (Table 1) that the enzymic reactions which are affected along this pathway are very likely to be those connected with the conversion of mevalonate into squalene, and not with the subsequent steps. Table 1 shows indeed that the degree of inhibition of cholesterol and

TABLE 1. THE EFFECT OF α-METHYL γ-PHENYL BUTYRIC ACID (MPBA) ON STEROL SYNTHESIS FROM 2-^{14}C-MEVALONATE IN RAT LIVER SLICES*

MPBA(mM)†	Rate of labelling in Squalene‡	P	Lanosterol‡	P	Cholesterol‡	P
— (7)	4·43 ± 0·30		2·00 ± 0·32		24·5 ± 1·31	
0·001 (6)	4·14 (7)	< 0·1	—		22·1 ± 1·12 (10)	< 0·01
0·01 (6)	3·81 ± 0·36 (14)	< 0·1	1·71 (15)	> 0·1	19·9 ± 1·07 (18)	< 0·1
0·1 (7)	—		1·45 (27)	> 0·1	17·9 ± 0·96 (27)	< 0·001
1·0 (6)	1·95 ± 0·14 (56)	< 0·001	0·97 ± 0·11 (52)	< 0·01	15·1 ± 0·91 (39)	< 0·001
10·0 (6)	0·49 ± 0·06 (89)	< 0·01	0·43 ± 0·05 (78)	< 0·01	7·8 ± 0·69 (68)	< 0·001

* Incubation was carried out as described in the text. The squalene and lanosterol labelling was determined after isolation of the compounds by thin-layer procedures on the 40 cm-long plates, and that of cholesterol after rechromatography and isolation on $AgNO_3$-impregnated plates. Owing to the faintness of the area and to the poor homogeneity of the isolated material, values for 7-dehydrocholesterol and Δ_7-cholestenol had a large scatter and results were unreliable.

† Number in parentheses indicate number of experiments in each group. Owing to the small number of samples examined in some squalene and lanosterol experiments, only the mean values are shown, where indicated.

‡ mμmoles formed per hr per g of fresh tissue. For calculation, the mμcuries of the isolated compound are divided by the specific activity of the mevalonate (1·94) × g of fresh tissue × hr of incubation × C (for mevalonate-2-^{14}C, C = 5). Per cent of inhibition is shown in parentheses. For the significance of C, see Fig. 2.

lanosterol formation produced by MPBA is sufficiently similar to that of squalene biosynthesis, some differences being observed only at very high concentration values.* The estimations of the labelling rates of 7-dehydrocholesterol and Δ_7-cholestenol (not shown in the Table and occasionally carried out in the same incubation system under the same experimental condition) have given strictly comparable results. These findings may signify, although not necessarily, that no further effect of MPBA occurs along the metabolic steps between squalene and cholesterol.

Few inhibitors acting between mevalonic acid and squalene have been described, and their mechanism of action is largely unsolved.[14] According to our results, MPBA may be considered an example of inhibitory compound which acts on two desirable sites at which to attempt a specific inhibition of cholesterol synthesis, i.e. those laying between acetate and mevalonate and between mevalonate and squalene. It will be useful to compare the present results with experiments to be carried out on the inhibition of sterol synthesis in animal species treated with MPBA, as well as to study the labelling rates of all the possible intermediates between squalene and cholesterol. Clearly, the mechanism of action of the drug and the specific sites of inhibition need to be completely elucidated with future work.

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REFERENCES

1. L. Canonica, R. Santi and V. Scarselli, *Drugs affecting Lipid Metabolism* (Ed. S. Garattini and R. Paoletti), pp. 328–335. Elsevier, Amsterdam (1961).
2. C. S. Rossi, *Bull. Soc. Ital. Biol. Sper.* **36**, 1914 (1960).
3. D. Steinberg and D. S. Fredrickson, *Proc. Soc. exp. Biol. Med.* **90**, 232 (1955).
4. D. Steinberg and D. S. Fredrickson, *Ann. N.Y. Acad. Sci.* **64**, 579 (1956).
5. L. F. Fieser, *J. Am. Chem. Soc.* **75**, 5421 (1953).
6. M. L. Speranza, G. Porcellati and M. C. Zapponi, *Giorn. Arter.* **5**, 292 (1967).
7. G. Porcellati, D. Giorgini and E. Toja, *Lipids* in press.
8. J. Avigan, S. DeWitt Goodman and D. Steinberg, *J. Lipid. Res.* **4**, 100 (1963).
9. N. W. DiTullio, C. S. Jacobs and W. L. Holmes, *J. Chromat.* **20**, 354 (1965).
10. B. B. Zeitman, *J. Lipid. Res.* **6**, 579 (1965).
11. A. Zlatkis, B. Zak and A. J. Boyle, *J. Lab. Clin. Med.* **41**, 486 (1953).
12. B. Zak, R. C. Dickenman, E. G. White, H. Burnett and P. J. Chernej, *Am. J. Clin. Path.* **24**, 1307 (1954).
13. G. Porcellati, F. di Jeso, M. Malcovati and M. G. Biasion, *Life Sci.* **5**, 1791 (1966).
14. R. Hess, *Adv. Lipid. Res.* **2**, 295 (1964).

* The results of Table 1 are expressed as mμ moles/hr/g of fresh tissue. It is pertinent to note in this connection that the levels of the liver sterol (cholesterol, lanosterol, 7-dehydrocholesterol and Δ_7-cholestenol) and squalene did not change to any appreciable extent, at any value of MPBA concentration, according to the procedures adopted in the present work.[11, 12]