Biochemical Pharmacology, 1965, Vol. 14, pp. 445–456. Pergamon Press Ltd., Printed in Great Britain.

THE COMMON MODE OF ACTION OF THREE NEW CLASSES OF INHIBITORS OF CHOLESTEROL BIOSYNTHESIS

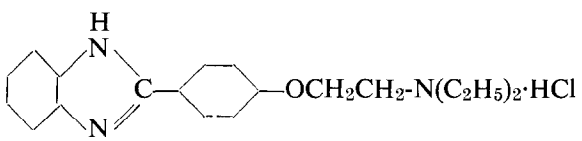
GERTRUDE RODNEY, MARTIN L. BLACK and ORSON D. BIRD

Research Laboratories, Parke, Davis & Company, Ann Arbor, Mich., U.S.A.

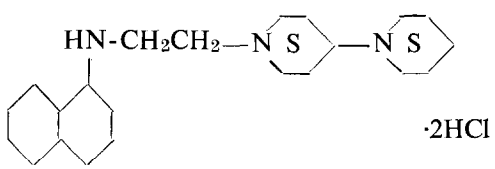
(*Received* 4 *September* 1964; *accepted* 16 *November* 1964)

Abstract—Rats and monkeys treated orally with three new compounds showed decreased plasma and tissue cholesterol, but accumulation of a "fast-acting" sterol which was identified by u.v. absorption maximum. thin-layer chromatography, and gas chromatography as 7-dehydrocholesterol. The implications of this finding relative to the sequence of events in cholesterol synthesis are discussed.

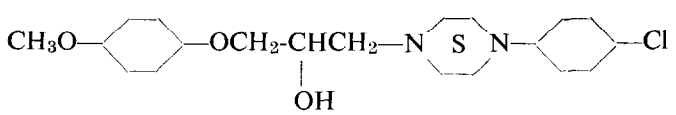
A RECENT report from the Ayerst Laboratories[1] described the properties of an agent (AY-9944) that inhibits the biosynthesis of cholesterol, resulting in the accumulation of 7-dehydrocholesterol. We have been engaged in similar studies of three different classes of hypocholesteremic agents typified by the following structures:

A. 

2[*p*-(2-diethylaminoethoxy)phenyl]benzimidazole monohydrochloride

B. 

1′-[2-(1-napthylamino)ethyl]-1,4′-bipiperidine dihydrochloride

C. 

4-(2-chlorophenyl)-α-(*p*-methoxyphenoxymethyl)-1-piperazine ethanol

Comparative studies in these laboratories have established that these compounds act in a qualitatively identical manner as *in-vivo* and *in-vitro* inhibitors of cholesterol synthesis, also resulting in an accumulation of 7-dehydrocholesterol.

The present report describes the work on which these conclusions are based and supports the interpretation of the intermediary role of 7-dehydrocholesterol in cholesterol biosynthesis offered by several recent studies.[1–4]

MATERIALS AND METHODS

Sterols used as reference standards were: cholesterol (Difco) purified by recrystallization from acetic acid and from ethanol;[5] desmosterol* from General Biochemicals Co.; Δ^7-cholestenol† from Calbiochem Co.; and 7-dehydrocholesterol‡ from Nutritional Biochemicals Co. Methostenol§ was kindly supplied by Dr. W. W. Wells; 4α-methyl-Δ^8-cholesten-3β-ol by Dr. A. Kandutsch; and zymosterol¶ by Dr. O. N. Breivik. We are grateful to Dr. D. Dvornik of the Ayerst Laboratories for a supply of AY-9944. We are indebted to Drs. D. B. Capps and E. F. Elslager of these laboratories for generous supplies of compounds A and B respectively. Compound C is a member of a series of related compounds made available to us by the late Professor Cash B. Pollard.

Male Holtzman rats (200-250 g), on a Rockland mouse–rat pellet diet, were given daily oral doses of A, B, C, or AY-9944 at 1·5 to 50 mg/kg body weight for 7, 14, or 21 days. Groups of ten animals were used for each dose level or time interval, with an equal number of control rats in each case. In addition, compound A was given orally to one male and one female rhesus monkey on a normal diet in daily doses of 10 mg/kg for 6 weeks. Blood was collected for analysis of total plasma cholesterol[6, 7] and "fast-acting" sterols.[8] Several tissues were taken for sterol analysis from the groups treated for 21 days, and total livers were pooled for isolation and identification of individual sterols. Digitonin-precipitated sterols from the nonsaponifiable fraction[9] were regenerated with pyridine.[10] All procedures during the isolation were carried out in an atmosphere of nitrogen, in absence of direct light. The recovered sterols were stored at $-12°$ under nitrogen until analyses were complete. Cholesterol, "fast-acting" sterols, and substances showing u.v. absorption with a maximum at 281·5 mμ[11] were determined. Aliquots of the regenerated sterols dissolved in ethanol were chromatographed on thin-layer silica gel G plates, essentially by the methods of Avigan *et al.*[12] For visualization of sterol zones, spraying with water,[13] exposure to iodine vapor, or u.v. fluorescence at 360 mμ allowed differentiation of the sterols present.

For studies of the inhibition of cholesterol synthesis, rats treated with A or B for one week were given 1–5 μc sodium acetate-1-^{14}C by i.p. injection 3 hr before sacrifice. The livers were saponified, and the nonsaponifiable material was precipitated with digitonin. Specific activities of the digitonides from control and treated livers, before and after purification of the cholesterol as the dibromide,[14] were determined with the Packard liquid scintillation counter.

The effect of the compound *in vitro* on the enzymatic conversion of 7-dehydrocholesterol to cholesterol was determined by Kandutsch's procedure.[15]

The final identification of the liver sterols was carried out by a previously unreported and more specific gas chromatographic technique. A liver sterol sample (1–2 mg) was dissolved in 1 drop of dry tetrahydrofuran. Trifluoroacetic anhydride (6–8 drops) was added, the resulting solution swept briefly with dry nitrogen, and then stoppered and kept at room temperature for at least 20 min before being injected onto the column.

* $\Delta^{5,24}$-Cholestadien-3β-ol.
† Δ^7-Cholesten-3β-ol.
‡ $\Delta^{5,7}$-Cholestadien-3β-ol.
§ 4 α-Methyl-Δ^7-cholesten-3β-ol.
¶ $\Delta^{8,24}$-Cholestadien-3β-ol.

The solution (0·1–0·2 μliter) was injected from a Hamilton μliter syringe directly onto the glass column of a Research Specialties Co. series 600 gas chromatograph fitted with a hydrogen flame ionization detector and a Brown Electronik 5-mV recorder.

The separations reported here were rather sensitively dependent on operating conditions. Those found most useful follow.

Column temp., 185–190°; flash-heater temp., 230°; detector temp., 200°.

Gas flow rates: N_2, 26–30 ml/min; H_2, 35 ml/min; air, 230 ml/min.

Column size, 6 ft × 2·4 mm (i.d.).

Column packing (purchased from Applied Science Laboratories, State College, Pa., and silanized *in situ*[16] with hexamethyldisilazane): support, Gaschrom P, 80/100 mesh; coating, ECNSS-M, 1%, lot no. 542-3.

Attenuation, 30–100×.

The success of the gas chromatographic method was largely dependent on the combined use of the trifluoroacetate (TFA) derivatives, the dilute ECNSS-M coating, and the 2·4-mm (i.d.) column. Many variations were tried, but until this combination was found, three of the four sterols that were of most interest emerged as a single, unresolved peak.

The gas chromatographic peaks were identified by comparison of retention times with those of authentic specimen TFA's, both singly and in artificial admixture. Added confirmation of peak identities was provided by adding successively the reference sterols to the liver isolates, converting to TFA's, and noting the unchanged shape of the accentuated peak. With experience, the chromatographic patterns become recognizable at a glance, despite some variation in retention times owing to slight changes in day-to-day operating conditions. Quantitation of the drug-induced changes in chromatographic pattern was considered unnecessary for the present purpose, especially since only the cholesterol/7-dehydrocholesterol ratio seems to be affected, and this to an unmistakable degree.

The small column diameter and the low concentration of coating found necessary for the separations reported here occasionally led to problems which were due to column overloading, with attendant loss in resolution, skewness of peaks, and variability in retention times. It was therefore often necessary to chromatograph several samples of the same material before arriving at a combination of attenuation and sample size that gave acceptable chromatograms while, at the same time, utilizing most of the scale of the recorder.

RESULTS

Plasma and tissue sterols

After preliminary determination of the hypocholesteremic effect of these compounds by means of the Zak color reagent, quite different results were obtained with Liebermann-Burchard reagent, indicating the presence of "fast-acting" sterols. The nonsaponifiable material also showed the presence of compounds with u.v. absorption maximum at 281·5 mμ, characteristic of $\Delta^{5,7}$-dienes (Table 1.) Analyses of sterols in five tissues from rats given A, B, or C showed striking accumulation of "fast-acting" sterols in all cases (Table 2).

Effect on acetate-^{14}C incorporation into liver sterols

As anticipated after discovery of sterols other than cholesterol in plasma and tissues of rats treated with the compounds, very little cholesterol was synthesized from acetate-^{14}C, but other sterol intermediates were present in the original digitonides. These were removed by purification of the cholesterol as the dibromide, as is apparent in Table 3.

TABLE 1. PLASMA STEROL LEVELS

Compound	No. of Animals	Duration (weeks)	Dose (mg/kg)	Cholesterol (mg/100 ml)	Fast-acting sterols (mg/100 ml)	$\Delta^{5,7}$ Sterols at 281·5 mμ (mg/100 ml)
Rats						
Controls	10 ♂	1		74 ± 9	0	0
A	10 ♂	1	15	16 ± 4	9 ± 2	9 ± 1
B	10 ♂	1	20	27 ± 7	21 ± 2	24 ± 3
C	10 ♂	1	50	49 ± 2	13 ± 4	20 ± 3
AY-9944	10 ♂	1	8	20 ± 5	16 ± 2	20 ± 2
Monkeys						
Controls	10 ♂♀	6		206 ± 19	0	
A	1 ♂	6	10	38	12	
A	1 ♀	6	10	68	22	

Sterols isolated from livers

Analyses of the isolated sterols for cholesterol, "fast-acting" sterols, and material absorbing at 281·5 mμ are shown in Table 4. The sterol absorbing at 281·5 mμ, which correlated well with the "fast-acting" sterol, was present in amounts varying from 30% to 80% of the total, depending on the compound, dose, and length of time of administration. It was shown by thin-layer chromatography that liver sterols contained material that resembled $\Delta^{5,7}$-sterols in showing fluorescence in u.v. light (360 λ), in giving a deep brown color with iodine vapor, and in having an R_f value characteristic of 7-dehydrocholesterol separated from those for Δ^7-cholestenol or cholesterol on plates sprayed with $AgNO_3$ before development.

Because of the adsorption at 281·5 mμ, the "fast-acting" properties with Liebermann-Burchard reagent, and the resolution with thin-layer chromatography, it seemed probable that 7-dehydrocholesterol was the sterol accumulating in the livers of rats treated with A, B, or C. The difficulty of excluding the possible presence of traces of other sterols by use of these techniques was overcome by gas chromatography of the liver sterol isolates.

Gas chromatography

The clarity with which the necessary separations can be accomplished by the gas chromatographic method described is illustrated for an artificial mixture of sterol

TABLE 2. CHOLESTEROL AND "FAST-ACTING" (FA) STEROLS IN RAT TISSUES

Compound	Dose (mg/kg)	Liver		Aorta		Heart		Kidney		Adrenal	
		Chol. (mg/g)	FA	Chol. (mg/g)	FA	Chol. (mg/g)	FA	Chol. (mg/g)	FA	Chol. (mg/g)	FA
Controls		2·64 ± 0·2	0	2·08 ± 0·3	0	1·56 ± 0·1	0	5·13 ± 0·2	0	37·3 ± 6	0
A	15	1·72 ± 0·2	1·62 ± 0·2	1·68 ± 0·2	1·26 ± 0·07	1·02 ± 0·1	1·07 ± 0·07	4·53 ± 0·3		5·16 ± 2	2·46 ± 0·6
B	20	1·43 ± 0·2	2·50 ± 0·5	1·56 ± 0·3	1·12 ± 0·1	0·77 ± 0·1	1·66 ± 0·2	3·54 ± 0·3	3·03 ± 0·3	6·6 ± 2	9·5 ± 2
C	50	1·98 ± 0·1	1·12 ± 0·1	1·56 ± 0·3	0·52 ± 0·08	1·17 ± 0·08	0·74 ± 0·04	4·30 ± 0·4	0·97 ± 0·09	19·4 ± 3	8·4 ± 2

TFA's in Fig. 1. It would have sufficed for the present purpose to separate only cholesterol and 7-dehydrocholesterol, since only these sterols are affected by the drugs under study.

Some raggedness of the chromatograms was always encountered in the 7-dehydrocholesterol region, even with a pure, authentic sample of this material alone. This

TABLE 3. ACETATE-1-^{14}C INCORPORATION INTO LIVER STEROLS

Group	Digitonides (dpm/mg sterol)	Recovered from dibromide (dpm/mg sterol)
1. Controls*	54·4 ± 7·6	64·1 ± 12·8
A, 15 mg/kg	85·4 ± 39·5	7·4 ± 0·6
2. Controls†	430 ± 136	416 ± 100
B, 20 mg/kg	431 ± 93	123 ± 34

* Injected with 1 μc acetate-1-^{14}C.
† Injected with 5 μc acetate-1-^{14}C.

TABLE 4. COMPOSITION OF STEROLS ISOLATED FROM LIVERS

Compound	Dose (mg/kg)	Duration (weeks)	Cholesterol %	Fast-acting sterol %	281·5 mμ sterol %
Rats					
Controls			100	0	0
A	15	3	34	75	76
B	20	3	17	83	82
AY-9944	5	2	50	38	36
Monkeys					
Controls			99	0	
A ♂	10	6	58	17	
A ♀	10	6	75	12	

feature, appearing as one or more shoulders or secondary peaks, is considered to be artifactual because of the known[17] incomplete stability of 7-dehydrocholesterol under gas chromatographic conditions approximating those reported here. The presence of these secondary peaks in the isolates from the control animals is taken as evidence for the presence of a steady-state concentration of 7-dehydrocholesterol too small to survive intact at the operating temperature.

The presence of large amounts of 7-dehydrocholesterol in the livers of rats treated with compound B is clearly evident from the chromatogram in Fig. 2. Qualitatively identical patterns were shown by the gas chromatograms derived from livers of animals treated with compounds A and C, including the two monkeys treated with compound A. Quantitation of the gas chromatographic responses was not attempted, but visual estimation of relative peak areas ranked the activities of compounds A, B, and C as 7-dehydrocholesterol reductase inhibitors in the same order suggested by the "Liver, FA" column in Table 2. Comparison of these chromatograms, typified by

Fig. 2, with the chromatographic pattern shown by isolates from control animals (Fig. 3) indicated that, except for a compensatory decrease in cholesterol concentration, the rest of the liver sterol pattern was essentially unaffected by these drugs. For this reason it was considered unnecessary to operate at the lower carrier gas-flow rate required to produce the degree of separation shown in Fig. 1. As a result, the small

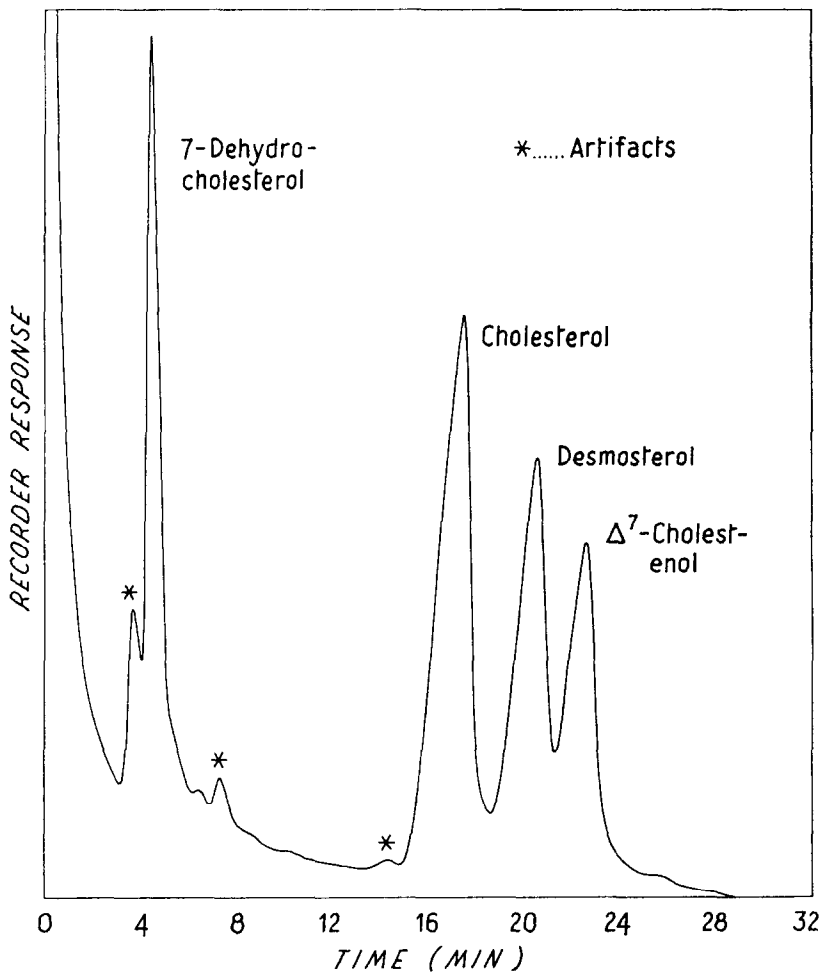


FIG. 1. Gas chromatogram: trifluoroacetates of cholesterol, 7-dehydrocholesterol, Δ^7-cholestenol and desmosterol in artificial admixture.

steady-state concentration of desmosterol presumably present in liver at any given mement is discernible only when the compensatory decrease in cholesterol concentration induced by the drug is large enough to permit the emergence of a postcholesterol shoulder, as in Fig. 2.

Figure 4, a chromatogram of TFA's of a liver sterol isolate from rats treated in an identical manner with AY-9944, is included for comparison, since this compound is known[1] to owe its hypocholesteremic effect to the inhibition of 7-dehydrocholesterol

reductase. Quantitative differences may exist, but the similarity of Fig. 4 to Fig. 2 suggests, as does the rest of the present work, that AY-9944 and compounds A, B, and C inhibit the biosynthesis of cholesterol in qualitatively identical manners.

The small precholesterol peak appearing in Figs. 2–4 is considered adventitious, since the material responsible is sometimes present and sometimes not, even in the

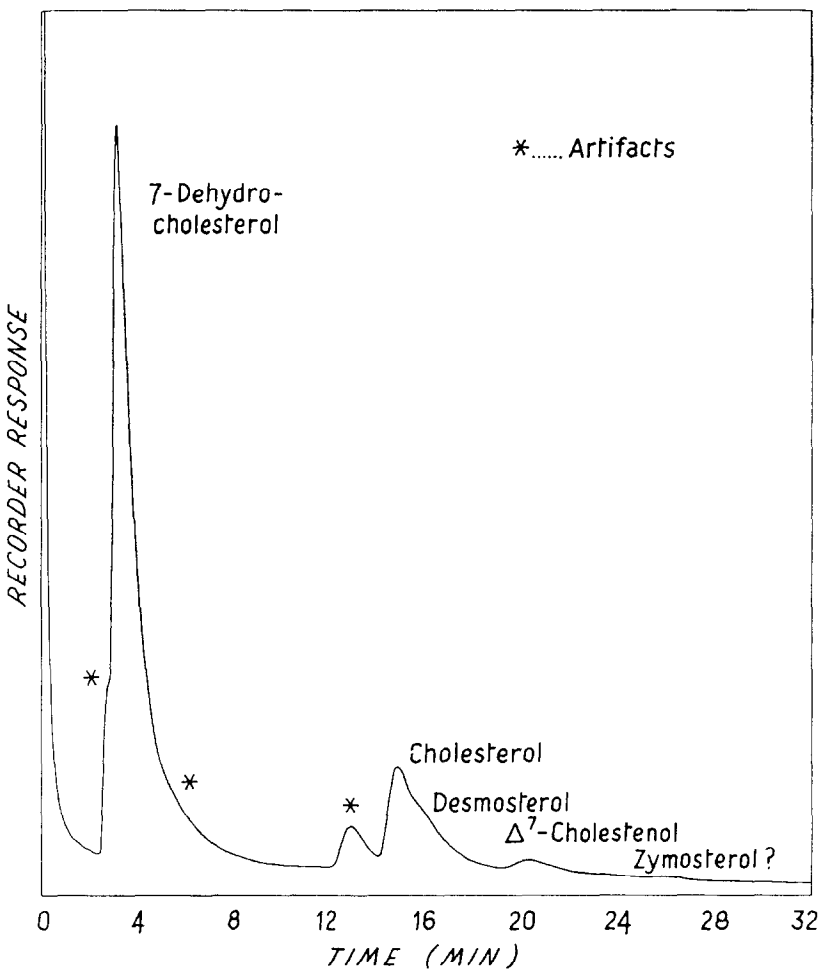


FIG. 2. Gas chromatogram: trifluoroacetates of sterols from livers of rats treated with compound B.

control animals. It is present in greater amounts before digitonin precipitation and, therefore, probably represents occluded nonsteroidal matter.

The zymosterol reference sample (m.p. 107°–110°, $[\alpha]_D$ = +42° at 1% in $CHCl_3$) gave two peaks when chromatographed as the TFA. One of these coincided with the tentatively identified zymosterol peaks in Figs. 2–4. The validity of this identification rests, of course, on the assumption that the reference peak that corresponds does in fact represent intact zymosterol, while its companion represents either an impurity

or a pyrolytic artifact. This point could not be explored adequately because of the infinitesimal amount of reference material available.

Enzymatic reduction of 7-dehydrocholesterol

As confirmation that the compounds were inhibiting the reduction of 7-dehydrocholesterol, it was desirable to test the effects of A and B *in vitro* on the enzyme

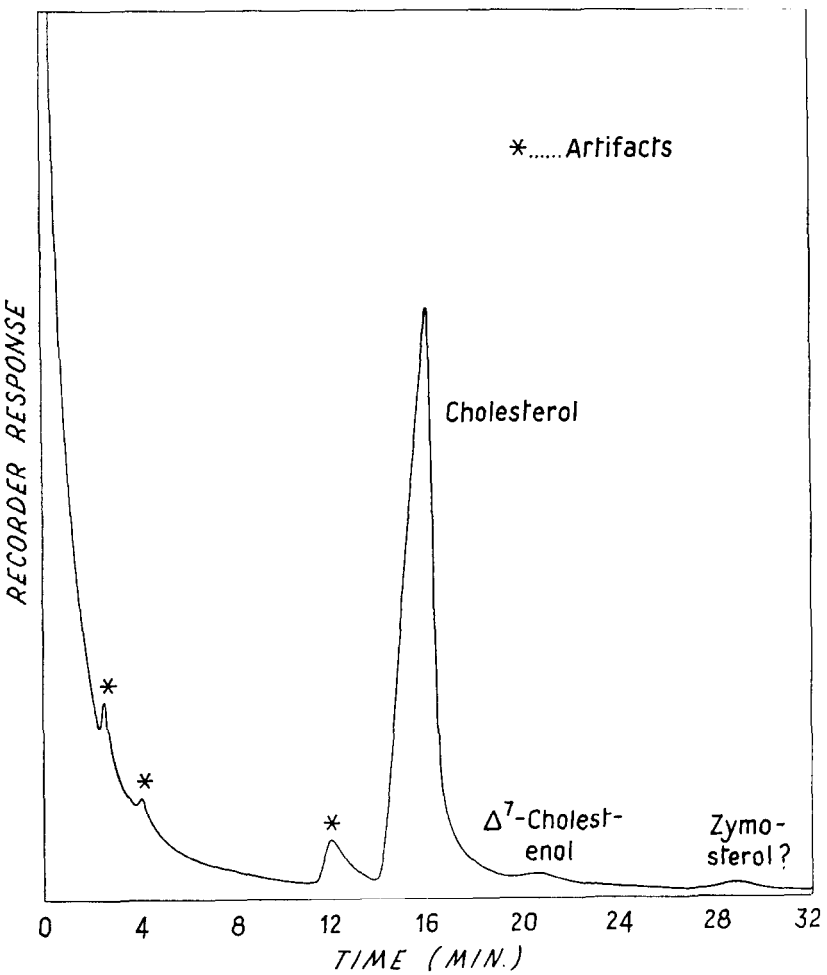


FIG. 3. Gas chromatogram: trifluoroacetates of sterols from livers of control rats.

system involved. Some difficulty was experienced in producing the extent of enzymatic conversion of 7-dehydrocholesterol reported by Kandutsch.[15] However, both A and B at 1×10^{-3} M concentration inhibited the observed conversion (Table 5). Presumably, the reduction of 7-dehydrocholesterol proceeded to form cholesterol, as proved by Kandutsch, although the procedure followed measured only the decrease in sterol absorbing at 281·5 mμ.

The compounds had no effect on another NADPH-requiring system; at 1×10^{-3} M concentration of A or B, the reduction of oxidized glutathione by purified glutathione reductase (Calbiochem Co.), in the presence 1×10^{-3} NADPH, proceeded at the maximal rate. This finding suggests that the inhibition of the 7-dehydrocholesterol reductase did not involve inactivation of NADPH.

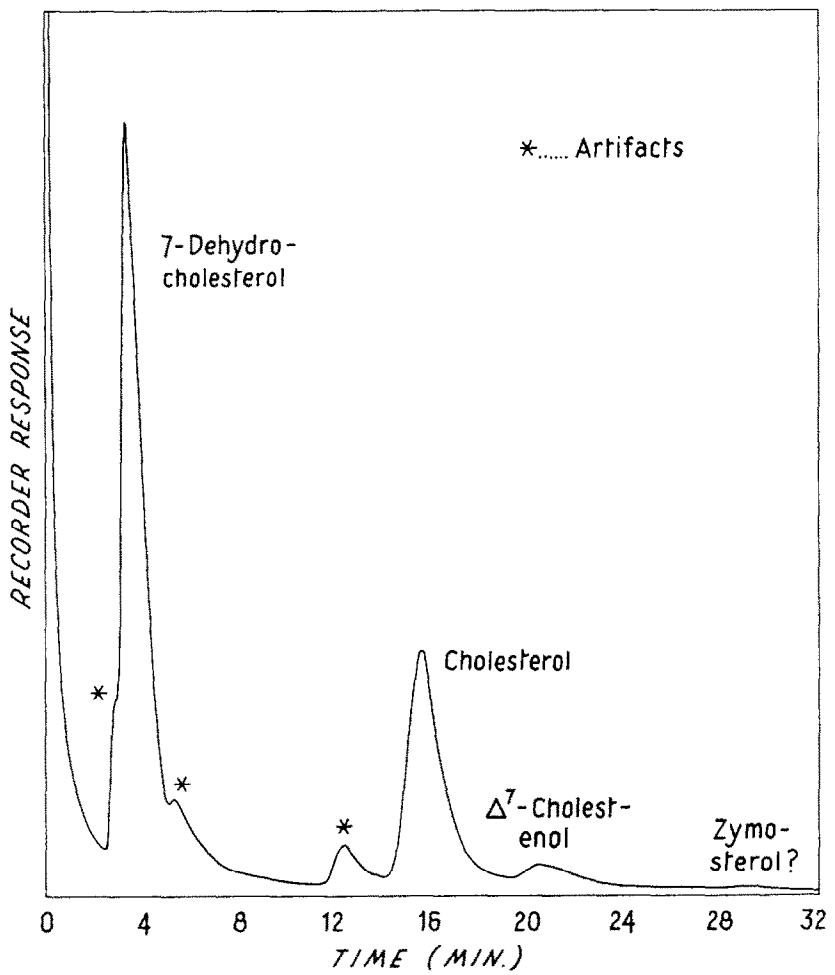


FIG. 4. Gas chromatogram: trifluoroacetates of sterols from livers of rats treated with AY-9944.

DISCUSSION

The recent studies of Goodman *et al.*[2] on the time-course of distribution of radioactivity in rat liver sterols, after the injection of 2-^{14}C mevalonic acid, have indicated that zymosterol and desmosterol do not lie on the main pathway of normal cholesterol biosynthesis, and that the early appearance of Δ^7-cholestenols, leading later to formation of cholesterol, shows that reduction of the lanosterol side chain occurs at a

fairly early intermediate stage. The observations of Dempsey *et al.*[4] on the transformation of Δ^7-cholestenol via 7-dehydrocholesterol to cholesterol by rat liver homogenates are pertinent, as are those of Kandutsch and Russell[3] working with sterols from a preputial gland tumor, in which a complete sequence from lanosterol to cholesterol was proposed, including 7-dehydrocholesterol as an intermediate.

TABLE 5. EFFECT OF COMPOUNDS ON ENZYMATIC CONVERSION OF 7-DEHYDROCHOLESTEROL

Treatment	Δ O.D.$_{281\cdot5}$	7-Dehydrocholesterol converted (μmoles $\times$ 10^3/2 hr)	Reduction (% inhibition)
1. Controls	0·100	65·0	
A, 1×10^{-3} M	0·00	0	100
2. Controls	0·113	65·1	
B, 1×10^{-3} M	0·065	32·5	50

Incubation mixtures contained 150 μmoles Tris–maleate buffer (pH 7·4), 60 μmoles nicotinamide, 2·5 μmoles NADPH, 0·52 μmole 7-dehydrocholesterol, and 600-*g* supernatant fraction of rat liver homogenate equivalent to 300 mg liver, in a total volume of 2 ml. Incubation was for 2 hr at 37°, under nitrogen.

In the present studies with three new types of cholesterol synthesis inhibitors, as well as with AY-9944, 7-dehydrocholesterol has been shown to accumulate in large amounts in plasma and tissues of treated rats, by spectrophotometric and chemical analysis, thin-layer chromatography, and gas chromatography. The presence of this sterol in skin and intestinal mucosa has previously been noted.[18] It would appear that it is intimately involved in cholesterol synthesis, with a rapid turnover in the normal animals, but subject to inhibition of further conversion to cholesterol by various hypocholesteremic agents.

Acknowledgements—The authors wish to thank Paul D. Uhlendorf, Claude H. Spencer, and John W. Vaitkus for their able assistance during the course of these investigations. We are indebted to Dr. Donald H. Kaump and his staff for making available the monkey plasma and livers.

REFERENCES

1. D. DVORNIK, M. KRAML, J. DUBUC, M. GIVNER and R. GAUDRY, *J. Amer. chem. Soc.* **85**, 3309 (1963).
2. DEW. S. GOODMAN, J. AVIGAN and D. STEINBERG, *J. biol. Chem.* **238**, 1287 (1963).
3. A. A. KANDUTSCH and A. E. RUSSELL, *J. biol. Chem.* **235**, 2256 (1960).
4. M. E. DEMPSEY, J. D. SEATON, G. J. SCHROEPFER and R. W. TROCKMAN, *J. biol. Chem.* **239**, 1381 (1964).
5. L. F. FIESER, *J. Amer. chem. Soc.* **75**, 4395 (1953).
6. L. ABELL, B. B. LEVY, B. B. BRODIE and F. E. KENDALL, *J. biol. chem.* **195**, 357 (1952).
7. H. L. ROSENTHAL, M. L. PELUKE and S. BUSCAGLIA, *J. Lab. clin. Med.* **50**, 318 (1957).
8. R. P. COOK, in *Cholesterol*, R. P. COOK, ed., p. 489. Academic Press, New York (1958).
9. T. R. BLOHM and R. D. MACKENZIE, *Arch. Biochem.* **85**, 245 (1959).
10. R. SCHOENHEIMER and H. DAM, *Hoppe-Seyler's Z. physiol. Chem.* **215**, 59 (1933).
11. E. I. MERCER and I. GLOVER, *Biochem. J.* **80**, 552 (1961).

12. J. AVIGAN, DEW. S. GOODMAN and D. STEINBERG, *J. Lipid Res.* **4**, 100 (1963).
13. R. J. GRITTER and R. J. ALBERS, *J. Chromatog.* **9**, 392 (1962).
14. J. J. KABARA and J. T. MCLAUGHLIN, *J. Lipid Res.* **2**, 283 (1961).
15. A. A. KANDUTSCH, *J. biol. Chem.* **237**, 358 (1962).
16. E. P. ATKINSON and G. A. P. TUEY, *Nature (Lond.)* **199**, 482 (1963).
17. S. R. LIPSKY and R. A. LANDOWNE, *Analyt. Chem.* **33**, 828 (1961).
18. P. R. MOORE and C. A. BAUMANN, *J. biol. Chem.* **195**, 615 (1951).