to attack either corticotropin-A or hemoglobin at *p*H 7.5 makes it unlikely that it is related to the liver enzyme of Tomizawa and Williams, which has been shown recently to have an effect on the corticotropin of sheep.[4]

CHICAGO 9, ILLINOIS

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

In Vitro Conversion of Zymosterol and Dihydrozymosterol to Cholesterol

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C^{14}-Zymosterol, prepared by incubation of yeast with labeled acetate, is shown to be converted to cholesterol by homogenates of rat liver. The process requires aerobic conditions and both the particulate fraction and the supernatant of liver homogenates. 24,25-Dihydrozymosterol can be transformed to cholesterol, but this reaction does not appear to occur normally in cholesterol biogenesis.

Introduction

In the biogenesis of the steroids, lanosterol is the first recognizable cyclic intermediate.[1] It is known to be metabolized further to cholesterol[2] by a process involving an oxidative removal of the three methyl substituents,[3] the saturation of the isooctenyl side chain and a shift[4] of the 8,9-double bond to the 5,6-position. It is not clear as yet in what order these transformations occur. One clue to the sequence of steps is provided by the structure of zymosterol ($\Delta^{8,24}$-cholestadienol) which may be regarded as a 4,4,14-trisnorlanostadienol. It is worth noting that zymosterol is a constituent of the lipids of yeast but has not so far been encountered in animal sources. However, the possibility that zymosterol is more widely distributed cannot be excluded since experience has shown that many of the intermediates in steroid biogenesis ordinarily occur in amounts too small to be detected by conventional methods. It therefore seemed worth investigating whether zymosterol, though hitherto considered to be a mycosterol, takes part in cholesterol biogenesis. For this purpose, we have employed the same procedures which have previously led to the recognition of squalene[5] and lanosterol[2] as cholesterol precursors. C^{14}-Zymosterol was prepared by biosynthesis and shown to yield cholesterol on incubation with liver homogenates. This transformation occurs also with the 24,25-dihydro derivative of zymosterol. The zymosterol–cholesterol conversion requires the particulate fraction as well as the soluble portion of liver homogenates for catalysis. In view of the fact that the net chemical changes involved are a reduction and the shift[4] of a double bond, it is of particular interest that aerobic conditions are essential for the over-all process.

Schwenk, *et al.*, have described the isolation of C^{14}-zymosterol[6] from yeast and have reported briefly[7] on the conversion of zymosterol to cholesterol in the whole animal.

(1) T. T. Tchen and K. Bloch, THIS JOURNAL, **78**, 1516 (1956).
(2) R. B. Clayton and K. Bloch, *J. Biol. Chem.*, **218**, 319 (1956).
(3) J. A. Olson and K. Bloch, *Federation Proc.*, **15**, 323 (1956).
(4) The term shift is used here to indicate the net change without reference to the mechanism involved.
(5) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 135 (1953).
(6) E. Schwenk, G. J. Alexander, T. H. Stoudt and C. A. Fish, *Arch. Biochem. Biophys.*, **55**, 274 (1955).
(7) E. Schwenk, G. J. Alexander, C. A. Fish and T. H. Stoudt, *Federation Proc.*, **14**, 752 (1955).

Preparation of C^{14}-Zymosterol.—According to the observations of Klein,[8] the synthesis of sterols from acetate by baker's yeast is slow under anaerobic conditions. However, on exposure of anaerobically grown yeast to air or oxygen, non-saponifiable materials including ergosterol accumulate in considerable quantity. In the present experiments, anaerobically grown baker's yeast was briefly exposed to air, transferred to a nitrogen-free medium and incubated under oxygen. When the cells were harvested after three hours and extracted, squalene was the principal radioactive constituent of the lipid fraction. In contrast, after incubation for 34 hours, the yeast cells contained, apart from C^{14}-squalene, labeled zymosterol and ergosterol in considerable quantity (Fig. 1). The appropriate radioactive column fraction (III, Fig. 1), was

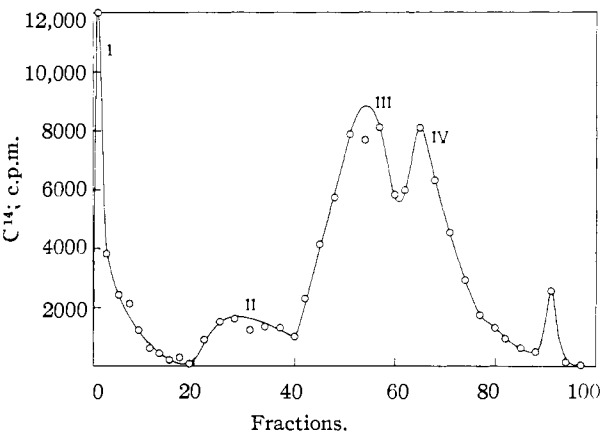

Fig. 1.—Chromatographic separation of non-saponifiable materials from yeast incubated with C^{14}-acetate: I, squalene; II, lanosterol; III, zymosterol; IV, ergosterol.

mixed with authentic zymosterol, crystallized and the specific activity of crystals and mother liquor material determined. These crystallizations were repeated with the acetyl and benzoyl derivatives. In all cases, the radioactivity remained associated with the crystalline carrier. Moreover, the elution diagram obtained by chromatography of the radioactive yeast sterol with carrier zymosterol showed satisfactory coincidence between radioactivity and the weight of solid material. These criteria furnish adequate proof for the identity of

(8) H. P. Klein, *J. Bacteriology*, **69**, 620 (1955).

the yeast sterol with zymosterol and demonstrate that the material is homogeneous.

It was noted upon chromatography of the non-saponifiable fraction on alumina that approximately two-thirds of the radioactivity remained adsorbed even after leaching of the column with methanol, indicating the presence of highly polar substances. Such losses did not occur when silica gel was the adsorbent. The material in question has been obtained in crystalline form (m.p. 54–57°). On the basis of preliminary evidence it is believed to be an acid though it is extractable by ether from alkaline solution. Since the acid is not an intermediate in steroid biogenesis, its isolation and properties will be described in a separate publication.

Metabolic Studies.—Zymosterol and cholesterol are closely related in physical properties and this presents a considerable problem in the assay of the enzymatic conversions. The methods employed here (chromatography and a chemical method) are relatively cumbersome but are effective in separating the radioactive substrate from the reaction products. Though the difference in polarity between cholesterol and zymosterol is slight, a mixture of the two can be resolved by chromatography of the steryl acetates on alumina, Woelm Grade II (Fig. 2). Under the conditions used, cholesteryl

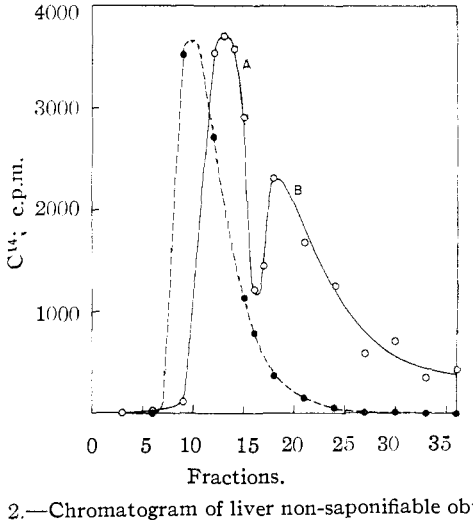

Fig. 2.—Chromatogram of liver non-saponifiable obtained after incubation with C^{14}-zymosterol. Solid line, first chromatogram, A, cholesterol, B, zymosterol. Dotted line, peak A rechromatographed. In the latter case the C^{14} values were multiplied by 2 for comparison of the two elution diagrams.

acetate is the first to emerge. The absence of contaminating zymosterol in the cholesterol fraction was established by a method used earlier to remove $\Delta^{8,9}$-stenols from cholesterol.[2] It utilizes the fact that in a neutral solvent Δ^5-stenols are reduced by platinum–hydrogen, whereas $\Delta^{8,9}$-stenols resist hydrogenation or isomerization under these conditions.[9] After catalytic reduction, the mixture of cholestanyl acetate and 24,25-dihydrozymosteryl acetate is treated with monoperphthalic acid to transform any $\Delta^{8,9}$-stenols to the corresponding epoxides. These are subsequently reduced to diols with lithium aluminum hydride. As a result of these operations, any contaminating zymosterol is transformed into a highly polar product, which is readily removed from cholestanol by chromatography. The efficiency of this separation was established with a synthetic mixture of non-radioactive cholesterol and C^{14}-zymosterol. For the purpose of assaying the cholesterol formed biologically, it is possible, therefore, to dispense with the rather tedious initial chromatography of the steryl acetates. Cholesterol derivatives free of contaminating substrate are prepared by direct hydrogenation of the total non-saponifiable fraction, as isolated from the enzymatic digests, and subsequent transformation of the Δ^8 stenols into diols.

For determining the conversion of 24,25-dihydrozymosterol to cholesterol, either one of the two following methods is suitable. As outlined above, the non-saponifiable fraction can be reduced catalytically and then taken through the peracid procedure. The resulting cholestanol is separated by chromatography from the diol derived from dihydrozymosterol. More conveniently the non-saponifiable fraction is mixed with carrier cholesterol, brominated and cholesterol dibromide separated from the unreacted dihydrozymosterol. When tested with an artificial mixture, this procedure yielded pure cholesterol dibromide after one recrystallization.

The C^{14}-zymosterol used as the substrate was isolated from relatively small quantities of yeast. Since zymosterol is known to be a minor constituent of the yeast lipids,[9] the *weight* associated with the radioactive fractions, as obtained by chromatography, must have been negligible. For this reason the conversions reported here refer in the most part to experimental conditions involving trace amounts of zymosterol. In one series of experiments with dihydrozymosterol, the radioactive substrate was mixed with graded quantities of non-isotopic carrier in order to determine the capacity of the liver system for metabolizing this type of sterol. The maximum yield corresponded to 4.5 μg. of cholesterol formed per ml. of homogenate in a 4-hr. period (Table V).

In the majority of incubation experiments, the liver tissue was homogenized according to Bucher ("loose pestle" homogenates).[10] These preparations converted both zymosterol and dihydrozymosterol to cholesterol with a radiochemical yield of approximately 20%. For testing the distribution of the respective enzymes in the cell, the liver homogenates were fractionated by centrifugation. Separately, neither the particulate portion including mitochondria and microsomes, nor the soluble supernatant catalyzed the metabolism of zymosterol. On the other hand, the two fractions when combined approached the activity of unfractionated homogenates. The reactions under study require aerobic conditions; incubations carried out in an atmosphere of helium yielded cholesterol containing only insignificant amounts of C^{14} (Table III).

(9) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publ. Corp., New York, N. Y., 1944, pp. 241–244.

(10) N. L. R. Bucher, THIS JOURNAL, **75**, 498 (1953).

For converting zymosterol to cholesterol, liver homogenates prepared in a Waring Blendor were found to be as active as the "loose pestle" homogenates prepared according to Bucher.[10] In contrast, dihydrozymosterol was not metabolized significantly by Waring Blendor homogenates (Table V).

Discussion

The facile conversion of zymosterol to cholesterol in liver homogenates (Table II) suggests that these reactions are part of the normal reaction sequence in cholesterol biogenesis. This assumption appears reasonable on structural grounds because the two double bonds of zymosterol occupy the same sites in the steroid skeleton as in lanosterol, which in turn has been well established as an obligatory intermediate in the squalene–cholesterol transformation.[1] The type of evidence presented here for the sequence lanosterol → zymosterol → cholesterol, *i.e.*, the enzymatic conversion of one compound into another, though consistent with a precursor–product relationship, does not prove it to be obligatory. Apart from zymosterol, a number of C_{27} sterols have in recent years been considered as cholesterol precursors,[11] but so far no consistent scheme can be devised which will accommodate these sterols in a single scheme for cholesterol biogenesis. A case in point are the present experiments showing that 24,25-dihydrozymosterol is as readily converted to cholesterol as zymosterol itself, at least in one type of homogenate. To conclude from this result that in the further metabolism of zymosterol, saturation of the side chain is the first step would create the following conflict. Good evidence has been presented that a cholesterol precursor isolated from egg yolk and termed desmosterol is $\Delta^{5,24}$-cholestadienol.[12] If the reasonable assumption is made that desmosterol is the immediate progenitor of cholesterol, it would follow that the shift of the 8,9-double bond in zymosterol precedes the saturation of the side-chain double bond and in this event dihydrozymosterol would be eliminated as an obligatory intermediate. Similar arguments apply to the possible role of Δ^7-cholestenol (lathosterol) and 7-dehydrocholesterol as precursors. Though there is evidence that these two sterols are convertible to cholesterol,[13–15] it is doubtful that this occurs normally since these substances have saturated side chains. To account for the fact that lathosterol and 7-dehydrocholesterol are constituents of animal tissues,[16,17] it is worth considering the possibility that they are products of cholesterol metabolism rather than precursors or, alternatively, that they are formed as side products from more unsaturated intermediates, *viz.*, $\Delta^{7,24}$-cholestadienol and $\Delta^{5,7,24}$-cholestatrienol. It is clear from these arguments that not all the structures of naturally occurring C_{27} stenols and their metabolic conversions can be fitted into a single biogenetic reaction sequence.

The observations that both zymosterol and its 24,25-dihydro derivative are transformed to cholesterol has a counterpart in the behavior of the corresponding 4,4,14-trimethylsterols. Lanosterol and 24,25-dihydrolanosterol are converted to cholesterol at comparable rates,[18] a finding consistent with the assumption that saturation of the isoöctenyl side chain might occur prior to demethylation. This is contradicted, on the other hand, by evidence indicating that dihydrolanosterol is not synthesized from acetate in isolated liver[19] and, furthermore, that the side chain double bond is still retained[20] in a recently described partial demethylation product of lanosterol, 14-norlanostadienol. We therefore conclude that the conversion of the dihydro derivative of lanosterol is not a normal or obligatory event in cholesterol formation, but rather reflects the inability of the enzymes involved to distinguish between the dienol and the dihydro derivative.

With regard to the role of dihydrozymosterol in cholesterol biogenesis, it appears to us significant that the conversion takes place in "Bucher" homogenates but not in Waring Blendor preparations of rat liver, whereas zymosterol forms cholesterol in both systems. Although no rational explanation can be offered for the phenomenon,[21] it is reasonable to postulate that dihydrozymosterol, were it an intermediate in the zymosterol–cholesterol conversion, should be metabolized by both liver preparations which are active with zymosterol. Since this is not the case, the reaction dihydrozymosterol → cholesterol is considered to be irrelevant to the process of normal sterol biogenesis. The examples given serve to emphasize the difficulty in establishing a biogenetic reaction sequence using as a sole basis the enzymatic convertibility of one metabolite into another.

The net structural changes associated with the zymosterol–cholesterol transformation are a reductive step and a shift of the nuclear double bond. As the present experiments have shown, both the particulate fraction and the supernatant of liver homogenates are required for the catalysis of these relatively trivial reactions and, moreover, these occur only in an atmosphere of oxygen. Since the side-chain reduction should proceed under anaerobic conditions, it follows that the double bond shift from 8,9 to 5,6 is not a simple intramolecular change, but is associated with oxidative steps. Conceivably, a hydroxyl group is introduced into ring B of

(11) M. Friedman, S. O. Byers and S. St. George, *Ann. Rev. Biochem.*, **25**, 613 (1956).

(12) W. M. Stokes, W. A. Fish and F. C. Hickey, *J. Biol. Chem.*, **220**, 415 (1956).

(13) M. W. Biggs, R. M. Lemmon and F. T. Pierce, *Arch. Biochem. Biophys.*, **51**, 155 (1954).

(14) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **202**, 77 (1953).

(15) G. M. Tomkins, W. G. Dauben, H. Sheppard and I. L. Chaikoff, *ibid.*, **202**, 487 (1953).

(16) L. F. Fieser, THIS JOURNAL, **75**, 4395 (1953).

(17) G. N. Festenstein and R. A. Morton, *Biochem. J.* (*London*), **60**, 22 (1955).

(18) H. C. Rilling, P. B. Schneider and K. Bloch, unpublished.

(19) R. B. Clayton and K. Bloch, *J. Biol. Chem.*, **218**, 305 (1956).

(20) F. Gautschi and K. Bloch, THIS JOURNAL, **79**, 684 (1957).

(21) Waring Blendor homogenates catalyze the conversion of squalene to cholesterol (T. T. Tchen and K. Bloch, *ibid.*, **77**, 6085 (1955)), but fail to incorporate acetate into steroids. The available evidence suggests that in such preparations diphosphopyridine nucleotide, whether endogenous or added, is more rapidly degraded by a DPN-ase released from red cells than in "Bucher" homogenates. It is difficult to see, however, in what respect DPN levels could have a differential effect on the metabolism of zymosterol and dihydrozymosterol.

the steroid skeleton and the 5,6-double bond established by subsequent elimination of water.

Experimental

Preparation of C^{14}-Zymosterol.—Fleischman's baker's yeast was grown anaerobically at 32° according to the procedure of Klein.[8] After 60 hours the cells were separated by centrifugation at 0°, washed twice with cold distilled water and resuspended in a solution containing 1% glucose in 0.1 *M* phosphate buffer, *p*H 7.4, to give a density of 20 mg. of cells per ml. This suspension was vigorously aerated for 1.5 hr. and the yeast cells again collected by centrifugation. Two grams of anaerobically grown and subsequently aerated yeast was suspended in 12 ml. of Bucher's medium[10] containing 1% glucose and 0.1 ml. of a 0.1 *M* solution of sodium acetate-1-C^{14} (1×10^7 c.p.m.) and incubated in an oxygen stream at room temperature for 34 hr. The cells were again collected by centrifugation, washed with water and digested by heating in 10% methanolic KOH for 4 hr. in a nitrogen stream. After isolation in the usual manner, the non-saponifiable fraction contained 5–10% of the radioactivity originally added.

For the isolation of zymosterol, a solution of the non-saponifiable material in Skellysolve B, containing 8.3×10^5 c.p.m. was chromatographed on 3 g. of alumina which had been deactivated by treatment with 7% aqueous acetic acid. After elution of squalene with petroleum ether (b.p. 67–75°) and of a lanosterol-containing fraction with 5% benzene in petroleum ether, zymosterol emerged with benzene–petroleum ether 1:10 as the eluent, separated from ergosterol, which is eluted on raising the concentration of benzene to 15% (Fig. 1). The radioactivities of the various column fractions are listed in Table I.

TABLE I

DISTRIBUTION OF C^{14} IN NON-SAPONIFIABLE MATTER OF YEAST, AFTER INCUBATION WITH 1-C^{14}-ACETATE

Eluent	Column fraction	Total c.p.m.
Skellysolve	I; 1–20; squalene	38000
5% Benzene–petroleum ether	II; 21–40; lanosterol[a]	26000
10% Benzene–petroleum ether	III; 41–60; zymosterol	120000
15% Benzene–petroleum ether	IV; 61–80; ergosterol[b]	110000
20% Benzene–petroleum ether	81–90; ?	2000
Ether	91–100 ?	2700
Methanol	.... ?	2500
		301200
	Total non-saponifiable	830000
	Not eluted	528800

[a] Identity determined by cocrystallization with authentic lanosterol. [b] Characterized by its typical ultraviolet spectrum with maxima at 294, 282 and 272 mμ; the weight of this fraction (15 mg.) accounted for the bulk of the solids in the non-saponifiable matter.

The more polar solvents, ether and methanol, eluted no significant quantities of C^{14}.

Characterization of C^{14}-Zymosterol.—The above zymosterol fraction was rechromatographed on 2 g. of deactivated alumina and thereafter a portion of the radioactive material cocrystallized with authentic zymosterol (kindly supplied by Dr. C. N. Breivik, Fleischmann Laboratories, New York, N. Y.). All of the radioactivity crystallized with the carrier. The same was true when the zymosterol fraction was benzoylated and recrystallized with authentic zymosteryl benzoate. As a further test, the mixture of C^{14}-zymosterol and carrier was acetylated and chromatographed on alumina, Woelm, grade II with petroleum ether as the solvent and eluent. The elution diagram showed satisfactory coincidence for the two curves representing weight and radioactivity, respectively. The specific activities (c.p.m./mg.) of 7 successive column fractions were: 53, 57, 50, 49, 45, 56 and 52. On mixing the same column fraction with non-isotopic ergosterol, radioactivity completely separated from the solid. These data are summarized in Table II.

Separation of Zymosterol and Cholesterol.—Cholesterol was separated from the radioactive zymosterol remaining after incubation, either by chromatography or by the peracid procedure described further below. The crude non-saponifiable fraction isolated from the liver tissue after incubation was acetylated with acetic anhydride in the presence of pyridine in the conventional manner. A solution of the acetylated mixture (24000 c.p.m.) in petroleum ether was placed on a column of alumina, Woelm grade II (4 g. for 1–10 mg. of non-saponifiables) and the eluted fractions collected with an automatic fraction collector. The elution diagram (solid curve, Fig. 2) showed 2 radioactive materials, the first of which (A, fractions 9–16, 11000 c.p.m.) yielded only a single peak (dotted curve) on rechromatography under the same conditions. This less polar fraction was identified as cholesteryl acetate and shown to be free of contaminating zymosterol by catalytic reduction to cholestanyl acetate and recovery of material with an unchanged specific activity after treatment with monoperphthalic acid and lithium aluminum hydride.[8] Fractions 6–16 (dotted curve, Fig. 2) containing a total of 10000 c.p.m. were added to 30 mg. of non-isotopic carrier cholesterol. The mixture was acetylated in the usual manner with acetic anhydride in pyridine, and reduced with Pt–H_2 in acetic acid solution. The reduction products were taken up in benzene and the solution filtered through a column of 3 g. of deactivated alumina. An aliquot was evaporated and found to have a S.A. (specific activity, c.p.m./mg.) of 241. For the oxidation of any contaminating Δ^8-stenols, the solution containing cholestanyl acetate was treated with a freshly prepared ethereal solution of monoperphthalic acid and the mixture allowed to stand at room temperature for 4 days. The reaction products were processed in the usual manner and then reduced with lithium aluminum hydride in order to convert any epoxides formed from the Δ^8-stenols into diols. The final products, when chromatographed on alumina, yielded 32.8 mg. of cholestanyl acetate. After one crystallization from methanol, this had a S.A. of 252. The unchanged S.A. after exposure to peracid confirms the absence of contaminating C^{14}-zymosterol in the cholesterol separated initially by chromatography (Fig. 2). For further characterization the above cholestanyl acetate (m.p. 143°) was saponified, diluted with carrier cholestanol to a S.A. of 33 c.p.m. and 10.9 mg. of the mixture oxidized to cholestanone.[22] The product after chromatography on deactivated alumina, yielded after recrystallization 6 mg. of material, m.p. 124–126° (reported[22] 128–129°); S.A. 36 c.p.m. Although these results demonstrate that the initial chromatography of the non-saponifiable fraction from the incubation experiments affords cholesterol free of contaminating zymosterol, this step was subsequently discarded as unsuitable for routine assay. For determining cholesterol formation from zymosterol in the liver homogenates, the crude non-saponifiable fraction was catalytically reduced and then directly subjected to the treatment with peracid. When checked with an artificial mixture of non-isotopic cholesterol and C^{14}-zymosterol, this procedure yielded cholestanyl acetate free of significant amounts of C^{14}. In 6 such experiments the cholestanol retained 1.6 to 4.4% of the C^{14}, with an average value of 2.8%. Therefore, in most of the experiments reported, the sterols isolated from the tissue were mixed with carrier cholesterol (20 mg.), acetylated, reduced catalytically and treated with peracid followed by lithium aluminum hydride as described. The conversion figures listed in Table III are based on the S.A. of the resulting cholestanyl acetate.

TABLE II

CHARACTERIZATION OF C^{14}-ZYMOSTEROL BY CRYSTALLIZATION WITH CARRIER[a]

Crystallizations	1		2		3	
	Crystals	M. l.	Crystals	M. l.	Crystals	M. l.
A. Yeast fr. + zymosterol	440	400	400	420	..	..
B. Benzoylated yeast fr. + zymosterol benzoate	300	208	294	344	310	310
C. Yeast fr. + ergosterol	50	350	19	77	0	35

[a] The values given are the specific activities (c.p.m./mg.) of crystals and of mother liquor solids (m.l.), respectively.

(22) O. Diels and E. Abderhalden, *Ber.*, **39**, 884 (1906).

TABLE III

CONVERSION OF ZYMOSTEROL TO CHOLESTEROL

		Vol., ml.	C^{14} Zymosterol added, c.p.m.	Cholesterol, c.p.m.	% conversion
(1)	Whole homogenates (Bucher)				
	(a) Aerobic	15	24000	6560	27.3
	Aerobic	15	12000	2400	28.4
	(b) Anaerobic	15	24000	780	3.2
	Anaerobic	15	12000	140	1.7
(2)	Particles, aerobic	7	4000	60	1.5
(3)	Supernatant, aerobic	7	4000	100	2.5
(4)	Particles (3.5 ml.) + supernatant (3.5 ml.) aerobic	7	4000	680	17.0
(5)	Whole homogenates, Waring Blendor, aerobic	10	5160	920	18.0

The method described above was used in a single experiment for assaying cholesterol formed from C^{14}-dihydrozymosterol (Table V, expt. A1). In all others, cholesterol was separated from the mixture of sterols as the 5,6-dibromide. In a blank experiment, 20 mg. of cholesterol and C^{14}-dihydrozymosterol (4000 c.p.m.) were brominated in ether in the usual manner. The precipitated cholesterol dibromide, after one recrystallization from chloroform–methanol, was free of C^{14}. The non-saponifiable fractions from the biological experiments were mixed with 10 mg. of carrier cholesterol before bromination. The data obtained with this procedure are illustrated below. The S.A. of the dibromide remains constant after the first crystallization.

TABLE IV

C^{14}; C.P.M./MG. IN CHOLESTEROL DIBROMIDE OBTAINED FROM INCUBATION EXPERIMENTS WITH C^{14}-DIHYDROZYMOSTEROL

Expt.	1	2	3	4	5
1st crystn.	39	35	38	20	16
M.l.	59	58	59	38	42
2nd crystn.	42	37	37	26	18
M.l.	48	34	42	25	20

Incubation Experiments.—Rat liver homogenates were prepared according to Bucher.[10] C^{14}-Zymosterol or C^{14}-dihydrozymosterol were added as aqueous suspensions prepared with 3 mg. of Tween 80 in 1 ml. of distilled water. Incubations were carried out in open erlenmeyer flasks in a Dubnoff shaker at 37° for 4 hr. For the anaerobic experiments, the homogenates were placed in the main compartment of Thunberg flasks and the suspensions of C^{14}-sterol placed into the side arm. The flasks were cooled in ice and alternately evacuated and filled with helium six times. The radioactive substrate was then tipped into the homogenate, the flasks warmed to 37° and incubated. In order to test whether enzyme inactivation occurs as a result of these manipulations, one of the "anaerobic" flasks was opened to the air just prior to incubation. The conversion of zymosterol to cholesterol proceeded at the normal aerobic rate. Waring Blendor homogenates were prepared by suspending rat liver in Bucher's medium (1 g. per 2 ml.) and homogenizing for 20 sec. at 0°. The preparation was centrifuged for 10 min. at 3000 r.p.m. and diphosphopyridine nucleotide added to the supernatant fluid (0.4 mg. per ml.) immediately before incubation. After incubation, 5 g. of KOH and 25 ml. of methanol were added to each flask and the contents heated under reflux for 1 hr. in a N_2 stream. The non-saponifiable fractions were extracted with ether and the washed extracts dried over anhydrous Na_2SO_4. The separation of cholesterol from remaining zymosterol or dihydrozymosterol was performed as described above.

TABLE V

CONVERSION OF 24,25-DIHYDROZYMOSTEROL TO CHOLESTEROL

		C^{14}-Dihydrozymosterol added, c.p.m.	Cholesterol, c.p.m., total	Conversion %	Conversion μg. of steroid
A.	Homogenates (Bucher, 6 ml. aerobic)				
(1)	Carrier-free	10000	2920	29.2	..
(2)	Carrier-free	5100	672	13.2	..
(3)	20 μg.	5100	600	10.9	2.2
(4)	50 μg.	5100	610	11.0	5.5
(5)	100 μg.	5100	416	8.2	8.2
(6)	500 μg.	5100	210	5.7	28.5
B.	Homogenates, Waring Blendor (10 ml.) aerobic				
(1)	500 μg.	8700	61	0.7	..
(2)	Carrier-free	9000	162	1.8	..

For testing the activity of various cellular fractions 25 ml. of rat liver homogenate prepared as described by Bucher[10] was centrifuged in the Spinco Preparative Ultracentrifuge for 45 min. at 105000 × *g*. The supernatant fluid was decanted and the sedimented particles suspended in 0.1 *M* phosphate buffer *p*H 7.2. The suspension was recentrifuged for 45 min. at 105000 × *g* and the washings discarded. The washed particles were finally suspended in 12 ml. of the medium described by Bucher,[10] and 1 ml. of an aqueous suspension of C^{14}-zymosterol in Tween 80 added. The mixture was incubated in air at 38° for 3.5 hr. The same quantity of C^{14}-zymosterol was also incubated separately with either 7 ml. of the supernatant fraction or with a mixture of 3.5 ml. of washed particles and 3.5 ml. of supernatant. The results are shown in Table III.

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