Conversion of Cholesterol to 5α-Cholestan-3β-ol in Germfree Guinea Pigs*

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Germfree guinea pigs were fed for 4 days a diet containing [4β-^{3}H]cholesterol and [4-^{14}C]-cholesterol. Cholesterol and 5α-cholestan-3β-ol (DHC) were then isolated from their adrenal glands, livers, and intestines. The values for the ratio ^{3}H/^{14}C of the cholesterols isolated from these tissues were very close to those of the cholesterols ingested. Hence, during its absorption and deposition in tissues, the ^{3}H of the [4β-^{3}H]cholesterol was not displaced, nor was discrimination discernible in the animal's use of the two labeled cholesterols. Both the ^{3}H-labeled and the ^{14}C-labeled cholesterol contained minute amounts of labeled DHC as a radiochemical contaminant that could not be removed by repeated purification. A comparison of the values for the ratio ^{3}H/^{14}C of this DHC in the diet with those for the DHC isolated from the tissues demonstrated that DHC must have been synthesized from cholesterol in the germfree guinea pigs. The additional finding that the ^{14}C content of the DHC isolated from three tissues, adrenal glands, livers, and intestines, exceeded that of the DHC ingested as a radiochemical impurity supported the conclusion that the conversion took place in the tissues. A study of the distribution of the ^{3}H in the DHC isolated from the tissues of the germfree animals showed that 54–64% was at carbon atoms 3 and 4. A comparison of these values with the percentages of ^{3}H found at these same carbon atoms in (a) dietary DHC (radiochemical impurity in the diet) and (b) the fed [4β-^{3}H]cholesterol suggests that the biosynthesis of [^{3}H]DHC from [4β-^{3}H]cholesterol involved a partial loss of ^{3}H.

Recently we presented evidence showing that cholesterol is converted to DHC[1] in the guinea pig (Werbin *et al.*, 1962), but we were unable to decide whether synthesis of the stanol occurred in the animal's tissues or was carried out by microorganisms in its intestinal tract. This led us to study the conversion in germfree guinea pigs, and the results of such studies are reported here. This approach, though seemingly simple, was beset by the handicap that all synthetic labeled cholesterols that we have examined contain minute amounts of DHC. Furthermore, extensive purification of the labeled cholesterols, though reducing considerably the extent of the contamination, did not free them completely of the labeled DHC. Thus the feeding of a purified preparation of labeled cholesterol would be expected to give rise to labeled DHC in the tissues of an animal, even though there were no biosynthesis of the stanol. However, we were able to take advantage of the stanol contamination in determining whether conversion of cholesterol to DHC actually occurred in animal tissues. It is highly improbable that two labeled cholesterols, one with ^{3}H and the other with ^{14}C, synthesized by widely differing procedures would be contaminated to the same degree by the labeled DHC. A diet containing a mixture of purified [4-^{14}C]cholesterol and [4β-^{3}H]cholesterol, the latter recently synthesized in this laboratory (Werbin and Chaikoff, 1964), was prepared, and the value for the ^{3}H/^{14}C ratio of the DHC present in that diet as a radiochemical impurity was determined. The diet was then fed to germfree guinea pigs, and DHC was isolated from their tissues by the reverse isotope dilution procedure. A comparison of the values for the ^{3}H/^{14}C ratio of DHC in tissues with that in the diet established that DHC was formed from cholesterol in animal tissues. Although any cholesterol labeled with stably bound ^{3}H could have been used in these experiments, we chose [4β-^{3}H]-cholesterol because, by following the course of the tritium during conversion of the sterol to DHC, we could gain insight concerning the nature of the enzymatic pathway involved in DHC synthesis.

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5α-Cholestan-3β-ol is often referred to as dihydrocholesterol and 3β-cholestanol.

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[1] Abbreviations used in this work: DHC, 5α-cholestan-3β-ol; [4β-^{3}H-4-^{14}C]cholesterol, a mixture of [4β-^{3}H]-cholesterol and [4-^{14}C]cholesterol.

EXPERIMENTAL

Preparation of the [4β-^{3}H-4-^{14}C]Cholesterol-containing Diet.—The synthesis of [4β-^{3}H]cholesterol and the determination of its tritium distribution have been reported (Werbin and Chaikoff, 1964). [4-^{14}C]Cholesterol was purchased from the New England Nuclear Corp., Boston, and was purified by bromination and debromination (Fieser, 1953). To 120 g of ground Purina laboratory chow was added an ether solution containing 248 μc (14.8 mg) of [4β-^{3}H]cholesterol and 120 μc (48.3 mg) of [4-^{14}C]cholesterol. The mixture was heated on a steam bath and stirred until the ether had evaporated. One hundred g of diet was shipped to Bethesda for incorporation into the diet of germfree guinea pigs.

Treatment of Germfree Guinea Pigs.—Four Hartley-strain germfree guinea pigs, which had been delivered by Caesarean section, were maintained in a steel unit of the Reynier's germfree system on dietary regimen L-445 (Phillips *et al.*, 1959), and their microbiological status was ascertained by methods already described (Phillips *et al.*, 1959). The animals were housed in individual cages and were 81 days old when the experiment was begun. The doubly labeled diet was added to diet L-445, and the mixture was autoclaved into the germfree rearing unit. The mixture was fed to the guinea pigs for 4 days and during this period the animals ingested a total of 92 g of the Purina chow. Thus each guinea pig ingested daily an average of 3.02 mg labeled cholesterol containing 5.75 μc of ^{14}C and 11.85 μc of ^{3}H.

The animals were killed within the germfree isolator approximately 7 hours after consuming all of the labeled diet. Their livers, adrenal glands, and intestines (partially freed of fecal material) were placed in

sealed sterile jars containing 95% ethanol. The jars were shipped to Berkeley, where, upon their arrival, a loopful of material from each jar was streaked on Difco nutrient agar in Petri dishes. The latter were placed in an incubator for 15 days and maintained at 37°. No growth of microorganisms was noted during this time. This test was performed through the courtesy of Dr. Vivian Moses of the Biodynamics Laboratory, Berkeley.

Isolation of Total Crystalline Digitonin-precipitable Sterols from Diet and Tissues.—The sterols were isolated from the diet by the reverse isotope-dilution procedure already described (Werbin and Chaikoff, 1961). To obtain total crystalline sterols from each of the four pooled livers, the eight pooled adrenal glands, and the four pooled intestines, they were refluxed for 3 hours with 15 volumes of 20% KOH in alcohol. After the mixture had been cooled and an equal volume of water had been added, the lipids were extracted 3–4 times with petroleum ether. The extracts were washed with water until neutral and were dried over anhydrous sodium sulfate. The combined petroleum ether extracts were filtered and distilled *in vacuo*, and the sterols in the residue were precipitated with digitonin. The washed and dried digitonides were split with pyridine and the sterols were then isolated in the usual manner.

Isolation of Crystalline DHC from the Crystalline Sterol Mixture Recovered from the Diet, Livers, and Adrenal Glands.—To about 20–30 mg of labeled crystalline-tissue sterols in a screw-capped vial were added 125 mg of unlabeled DHC (a gift of the Schering Corp.), that had been purified as described by Kuroda *et al.* (1964), and 4.0 ml of a chloroform solution containing 2.0 mmoles (a 5-fold excess) of peroxybenzoic acid (Braun, 1941). The latter converted all digitonin-precipitable sterols with double bonds to polar-epoxide derivatives from which the less polar DHC was readily separated by chromatography on aluminum oxide. The solution was shaken well and allowed to stand in the dark for 2 hours. It was then transferred, with the aid of 4 volumes of ethyl ether, to a separatory funnel and extracted 4–6 times with 0.1 volume of 2 N NaOH. The ether-chloroform solution thus obtained was washed with water and dried over anhydrous sodium sulfate. After filtering off the drying agent, the solvents were distilled under reduced pressure. The sterol residue was dissolved in benzene, and the solution was transferred to a 60-g column of aluminum oxide (Merck Sharp and Dohme, acid-washed) packed in benzene. The column was eluted, first with 400 ml of benzene and next with 1200 ml of benzene containing 4% by volume of ethyl ether. DHC was recovered from the last liter of the benzene-ether eluate after the solvents had been removed *in vacuo*. About 15 mg of unlabeled cholesterol were added to the DHC, and the mixture was again treated with peroxybenzoic acid. The subsequent routine was the same as that described above. After its second chromatographic separation the DHC was recrystallized from methanol. The crystalline material melted at 141.5–142° (reported 141.5–142° by Willstätter and Mayer, 1908). It gave a negative Lieberman-Burchard test.

Isolation of DHC by Reverse-Isotope Dilution from Intestinal Sterols.—To 172 mg of the total crystalline intestinal sterols isolated, 10.23 mg of unlabeled DHC was added, and the mixture was dissolved in 10 ml of peroxybenzoic acid solution. The mixture was treated as outlined in the previous section, except that the aluminum oxide column was eluted consecutively with 500-ml amounts of benzene containing 0.5, 1, 2, 3, and 4% by volume of ethyl ether. DHC was identified in the benzene eluates that contained 1% ethyl ether. The stanol residue (16 mg) that remained after the solvents were evaporated *in vacuo* was subjected to a second perbenzoylation and chromatographic separation. About 12 mg of a crystalline steroid with a mp of 140.5–141.5° was obtained. Its infrared spectrum was the same as that of authentic DHC. It was diluted with 113 mg of unlabeled DHC to prepare the derivatives described.

Oxidation of Crystalline-Tissue DHC to 5α-Cholestan-3-one.—The oxidation of labeled DHC was carried out as follows: The stanol, 100 mg, was dissolved in 0.8 ml of warm benzene. Then 1.0 ml of a solution containing sodium dichromate, acetic acid, and sulfuric acid, prepared as described by Bruce (1943), was added. For the oxidation of smaller DHC samples, the amounts of benzene and oxidizing agent were varied proportionately. The mixture was vigorously shaken for 3 hours at room temperature, and was then transferred to a separatory funnel with 10–15 ml. of a 4:1 ether-chloroform mixture. The mixture was washed, first with 0.1 volume of 2 N NaOH until the washings were colorless, and then with water. The ether-chloroform solution was dried over anhydrous sodium sulfate. After the drying agent was filtered off and the solvents were removed *in vacuo*, the 5α-cholestan-3-one was dissolved in methanol containing 2.5% KOH, and the solution was refluxed for 3 hours to remove enolizable ^3H (Fukushima and Gallagher, 1952). The 5α-cholestan-3-one was isolated in the usual manner and purified by chromatography on aluminum oxide by procedures described previously (Werbin *et al.*, 1962). After its recrystallization from alcohol, the steroid melted at 129.5–130.5° (reported 129°, Diels and Abderhalden, 1906).

Conversion of 5α-Cholestan-3-one Derived from Tissue DHC to Cholest-4-en-3-one.—The labeled 5α-cholestan-3-one prepared from tissue DHC was converted to 2α,4α-dibromocholestan-3-one as described by Wilds and Djerassi (1946). By refluxing of the dibromoketone with sodium iodide and acetone, 2-iodocholest-4-en-3-one was formed (Rosenkranz *et al.*, 1950). This was dehalogenated with chromous chloride to cholest-4-en-3-one (Rosenkranz *et al.*, 1950). The crude cholest-4-en-3-one was refluxed for 3 hours with 0.5 N KOH in 90% aqueous methanol to remove enolizable ^3H (Rosenfeld *et al.*, 1956). It was isolated in the usual manner and was purified by chromatography on a 2-g column of aluminum oxide. After its recrystallization from methanol it melted at 84–87° (reported 82° and 88°, Barton and Jones, 1943). The sample in ethanol had a maximum absorption at 242 mμ. Its molar absorptivity at this wavelength, 15,500, indicated that it was at least 97% pure.

Radioactivity Measurements.—All samples were dissolved in a mixture of 13.0 ml of toluene and 2.0 ml of dioxane (Distillation Products Industries, No. 2144) containing 45 mg of 2,5-diphenyloxazole. The counting data obtained from both the upper and lower channels of a Packard liquid scintillation spectrometer, Model 314E, were converted to dpm by the discriminator-ratio method (Okita *et al.*, 1957). The values for all ratios represent dpm of ^3H divided by dpm for ^{14}C.

Infrared Measurements and Melting Points.—A Perkin-Elmer Model 237 spectrometer was used to obtain infrared spectra. Uncorrected melting points were determined on a Fisher-Johns melting-point apparatus.

Purification of Solvents.—Benzene and diethyl ether were distilled over sodium.

Results

Four germfree guinea pigs were fed the sterilized diet containing the mixture of ^{14}C- and ^{3}H-labeled cholesterols ($^{3}H/^{14}C$ = 2.32, Table I) for 4 days.

Table I

$^{3}H/^{14}C$ Ratios for Crystalline Sterol Mixtures and Cholesterol Isolated from the Diet and Tissues of Germfree Guinea Pigs Fed [4β-^{3}H-4-^{14}C]Cholesterol

Compound	Diet	Livers	Adrenal Glands	Intestines
Sterol mixture	2.37	2.24	2.39[a]	2.39
Cholesterol[b]	2.32		2.36[a]	2.40

[a] The close agreement between the values for the sterol mixture and the cholesterol isolated from it indicates that the mixture could not have contained 16% DHC as we previously reported for normal guinea pig adrenal glands (Werbin *et al.*, 1962). If the DHC ($^{3}H/^{14}C$ of 0.93, Table II) had been present to such an extent, its elimination by debromination during conversion of the sterol mixture to pure cholesterol would have resulted in the latter having a value for the ratio significantly greater than 2.36. [b] Prepared from sterol mixture by bromination and debromination (Fieser, 1953).

Crystalline, digitonin-precipitable sterols were isolated from the pooled livers, pooled adrenal glands, and pooled intestines. Portions of some of the sterols were converted to pure cholesterol by bromination and debromination (Fieser, 1953). The values for the $^{3}H/^{14}C$ ratio of the tissue sterols and the cholesterols prepared from them are given in Table I. They are almost identical. These findings therefore demonstrate: (a) that the tritium at the 4β position of [4β-^{3}H]cholesterol remained stably bound in the animals, since absorption and circulation of the sterol failed to displace the ^{3}H; (b) that there was no appreciable discrimination in the absorption and tissue disposition of the ^{14}C-labeled cholesterol over that of the ^{3}H-labeled one.

All ^{14}C- and ^{3}H-labeled cholesterols examined, even after their purification by bromination, contained minute quantities of labeled DHC. For example, the purified [4β-^{3}H]cholesterol and [4 ^{14}C]cholesterol fed the germfree animals had 0.03 and 0.10% of their radioactivity, respectively, as DHC. We have assumed that the conclusions drawn above regarding the stability of ^{3}H in [4β-^{3}H]cholesterol and the lack of isotope discrimination in absorption and tissue disposition of [4β-^{3}H]cholesterol and [4-^{14}C]cholesterol in the guinea pig also apply to the two DHC's present in the diet as radiochemical impurities. Evidence supporting this assumption has appeared (Werbin *et al.*, 1962).

In Table II values are given for the $^{3}H/^{14}C$ ratio of the DHC isolated by reverse-isotope dilution from the diet and from tissue sterols of the germfree animals. The values for the tissue stanols, all of which were in close agreement, were higher than that for dietary DHC. These data in are accord with the idea that synthesis of DHC from cholesterol took place in the tissues

Table II

$^{3}H/^{14}C$ Ratios for (a) DHC Present in Dietary [4β-^{3}H-4-^{14}C]Cholesterol as a Radiochemical Impurity and (b) DHC Isolated from Tissue Sterols of Germfree Guinea Pigs

Diet (a)	Livers (b)	Adrenal Glands (b)	Intestines (b)
0.34	0.85	0.93	0.85

Table III

Comparison between Total ^{14}C in DHC Ingested as a Radiochemical Impurity and Total ^{14}C in DHC Isolated from Three Tissues of Germfree Guinea Pigs

Ingested		
[4-^{14}C]-Cholesterol (dpm)	^{14}C of DHC as Impurity in [4-^{14}C]-Cholesterol (%)	Total dpm in DHC
202.4 × 10^{6}	0.1	202,400

Recoveries in Tissues			
Tissue	^{14}C of DHC per mg Sterol (dpm)	Total Sterol (mg)	Total ^{14}C in DHC (dpm)
Adrenal glands	425	39.6	16,750
Livers	1230	90.1	110,800
Intestines	1110	179	199,400
			326,950

of the germfree animals. Had this conversion not occurred, the values for the ratio in the diet and animal tissues would have been the same.

The data in Table III provide confirmation of DHC synthesis from cholesterol in tissues of the germfree guinea pigs: the amounts of ^{14}C of DHC isolated from *only* three tissues (adrenal glands, liver, and intestines) exceeded the total ^{14}C in the DHC ingested by the animals as a radiochemical impurity.

The fate of tritium of [4β-^{3}H]cholesterol during its reduction in the animal body was investigated by determining the distribution of ^{3}H in rings A and B of (a) the DHC present as a radiochemical impurity in the dietary [4β-^{3}H]cholesterol, and (b) the DHC isolated from the tissues.

The [^{3}H]DHC present as a contaminant in the dietary cholesterol was isolated by the reverse isotope-dilution procedure, and its specific activity was determined. It was then oxidized to 5α-cholestan-3-one, and the specific activity of the steroid formed was determined after its equilibration with alkali. The decrease in specific activity measured the amount of ^{3}H

Table IV

Per Cent of ^{3}H Bound to Carbon Atoms 3+4 and 5+6 of [^{3}H]DHC Present as a Radiochemical Impurity in Fed [4β-^{3}H]Cholesterol[a]

Steroid	Amount Counted (mg)	Specific Activity (dpm ^{3}H/mg)	^{3}H Bound at Carbon Atoms: 3+4 (%)	5+6 (%)
DHC, after perbenzoylation and chromatography	15.1	213		
5α-Cholestan-3-one, after alkali equilibration	12.5	21	90.2	
Cholest-4-en-3-one, after alkali equilibration	11.8	3		8.3

[a] To 33.9 μc [4β-^{3}H]cholesterol were added 125 mg of nonlabeled DHC, and the mixture was treated with peroxybenzoic acid. The methods for isolating the DHC and converting it to 5α-cholestan-3-one and cholest-4-en-3-one are given in the experimental section.

bound at positions 3 and 4, since there was little likelihood of the ^3H being bound at position 2. The findings in Table IV show that about 90% of the ^3H was bound to carbon atoms 3 and 4. The amount of ^3H bound to carbon atoms 5 and 6 was determined by converting the 5α-cholestan-3-one to cholest-4-en-3-one. The latter was equilibrated with alkali and reisolated. A comparison of its specific activity with those of the 5α-cholestan-3-one and DHC from which the cholest-4-en-3-one was derived provided an estimate of the ^3H bound at positions 5 and 6 of DHC. Table IV shows that about 8% of the ^3H was in these positions.

Identical procedures were then used to determine the amount of ^3H at carbon atoms 3–6 of the DHC's that were isolated from the tissues of the germfree guinea pigs. The values in Table VI were calculated from the $^3H/^{14}C$ ratios of the 5α-cholestan-3-ones and cholest-4-en-3-ones given in Table V. For all tissue DHC's,

TABLE V

$^3H/^{14}C$ RATIOS FOR 5α-CHOLESTAN-3-ONE AND CHOLEST-4-EN-3-ONE PREPARED FROM DHC'S ISOLATED FROM GERMFREE GUINEA PIG TISSUES[a]

Crystalline Sterol Isolated after Alkali Equilibration	Livers	Adrenal Glands	Intestines
5α-Cholestan-3-one	0.37	0.43	0.31
Cholest-4-en-3-one[b]	0.34	0.30	0.34

[a] After unlabeled DHC was added to the tissue DHC, no further dilutions with unlabeled carrier were made to prepare these derivatives. [b] The calculated dpm of ^3H/mg of cholest-4-en-3-one was corrected as follows: the value was plotted in Fig. 1 and a line parallel to that shown was drawn through the point intersecting the ordinate. The dpm for ^3H/mg at the point of intersection was taken as the true value.

TABLE VI

PER CENT OF ^3H BOUND AT CARBON ATOMS 3–6 IN DHC ISOLATED FROM TISSUES OF GERMFREE GUINEA PIGS[a]

Livers			Adrenal Glands			Intestines		
Carbon Atoms			Carbon Atoms			Carbon Atoms		
3+4 (%)	5+6 (%)	Unknown Positions (%)	3+4 (%)	5+6 (%)	Unknown Positions (%)	3+4 (%)	5+6 (%)	Unknown Positions (%)
57	0.8	43	54	14	32	64	0	36

[a] The calculations were made as follows: Let: A = % ^3H at carbon atoms 3 + 4; B = % ^3H at carbon atoms 5 + 6; R_1 = $^3H/^{14}C$ for DHC, R_2 = $^3H/^{14}C$ for 5α-cholestan-3-one; R_3 = $^3H/^{14}C$ for cholest-4-en-3-one. Then A = 100 − (R_2/R_1) × 100, and B = 100 − (R_3/R_1) × 100 − A.

the highest percentages of ^3H, 54–64%, were bonded to carbon atoms 3 and 4. It is noteworthy that, in the DHC's isolated from livers and intestines, practically no ^3H was bonded to carbon atoms 5 and 6. The DHC from adrenal glands contained 14% at these positions. All tissue DHC's had a significant proportion of ^3H, between 32 and 43%, bound to carbon atoms other than 3–6.

The values given in parentheses (*vide infra*) for the percentages of ^3H bound at carbon atoms 3 and 4 of (a) [4β-^3H]cholesterol (96) (Werbin and Chaikoff, 1964), (b) the [^3H]DHC impurity in it (90) (Table IV), and (c) the tissue DHC's (54–64) (Table VI) support our other findings that DHC is derived from cholesterol and, in addition, show that this conversion occurs by an enzymatic process involving partial loss of ^3H. Were this not so, the values for the percentages of ^3H at carbon atoms 3 and 4 of the tissue DHC would lie between 90 and 96%. The synthesis of [^3H]DHC in tissues with partial loss of ^3H also explains why the values of the $^3H/^{14}C$ ratios of the tissue DHC's are between that for the [^3H]DHC impurity in the diet, 0.34, and that for [4β-^3H]cholesterol, 2.32 (Table I). While the partial loss of ^3H during [^3H]DHC synthesis in tissues may have been induced by a single enzymatic step, it can also be accounted for by the simultaneous occurrence of two enzymatic reactions, one involving no ^3H loss, the other inducing a total loss of ^3H. Our findings do not permit a decision as to which of the alternative enzymatic processes may be operative.

While the values for the $^3H/^{14}C$ ratio of labeled 5α-cholestan-3-one, obtained by oxidation of tissue DHC, is unaffected by the weight of the sample assayed (Werbin and Chaikoff, 1964), this was not the case for the labeled cholest-4-en-3-one prepared from it. Figure 1 shows that the calculated values for dpm of ^3H per mg of cholest-4-en-3-one increased linearly with the weight of sample counted—in contrast to that found for the calculated ^{14}C values. The $^3H/^{14}C$ ratios given in Table V have been corrected for this effect (see footnote *b* in Table V).

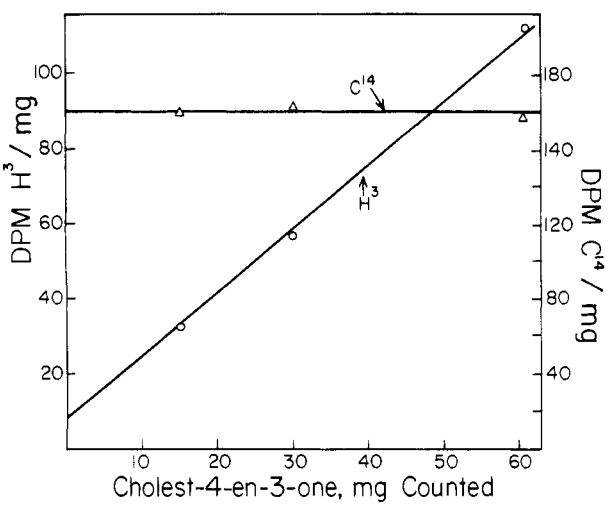

FIG. 1.—Relation of dpm of ^3H and of ^{14}C per mg [^3H-^{14}C]-cholest-4-en-3-one to mg counted.

We reported finding ^3H-labeled water in the blood and urine of guinea pigs that were fed [4β-^3H-4-^{14}C]-cholesterol for 21 days (Werbin and Chaikoff, 1964). Although we did not analyze the blood and urine of the germfree animals for [^3H]water, it is reasonable to assume its presence in these fluids. Then, because guinea pig tissue DHC can in part be derived from fed [^3H]water (Werbin *et al.*, 1962), it is necessary to determine whether an appreciable amount of ^3H in the tissue DHC's isolated from the germfree animals could have been derived from the ^3H in their body water.

In guinea pigs whose body water contained 1.78 μc of ^3H/ml, liver DHC had 6.3 dpm of ^3H/mg (Werbin *et al.*, 1962). In contrast, liver DHC had 3180 dpm of ^3H/mg in guinea pigs fed [4β-^3H]cholesterol and whose body water had only 3.9 × 10^{-3} μc of ^3H/ml (Werbin and Chaikoff, 1964). Obviously, such data allow us to ignore any contribution of ^3H from body water to tissue DHC in the germfree animals fed the [4β-^3H]cholesterol.

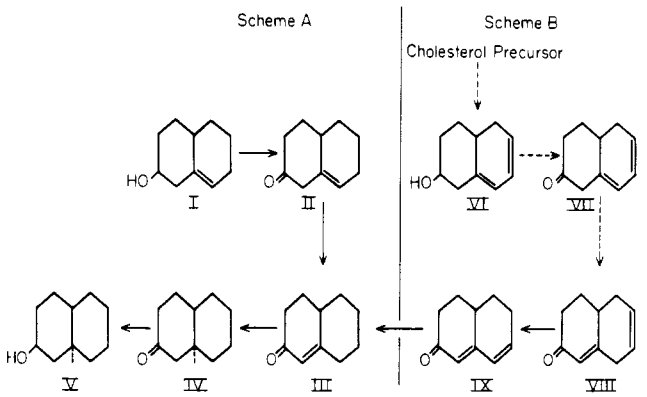

FIG. 2.—Proposed pathways for biogenesis of DHC. In scheme A cholesterol is an obligatory intermediate. In scheme B cholesterol is not a precursor of DHC. Established reactions are shown by ——→, those not established by · · · ·→.

DISCUSSION

Despite the fact that the close structural relation between cholesterol and DHC was established very early (Diels and Abderhalden, 1904; Willstätter and Mayer, 1908), and even though it was known that DHC is present as a constant companion of cholesterol to the extent of 1–3% in normal tissues (Schönheimer *et al.*, 1930), it was only recently that cholesterol was shown to be a precursor of DHC in the normal animal (Werbin *et al.*, 1962). The results of the present experiments now leave no doubt that the conversion of cholesterol to DHC actually takes place in the tissues of animals. That Scheme A of Figure 2 depicts a probable sequence of reactions for the conversion, is supported by the following observations: Yamasaki *et al.* (1959) found enzymes in the supernatant fraction of rat liver homogenates that converted cholesterol (I) to cholest-4-en-3-one (III), nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate being involved here as cofactors. We have prepared fractions of beef adrenal tissue that isomerized cholest-5-en-3-one (II) to III (Ewald, unpublished observations). The formation of DHC (V) from compound III has been demonstrated repeatedly both *in vivo* (Anker and Bloch, 1949; Stokes *et al.*, 1955; Harold *et al.*, 1956; Chapman and Chaikoff, 1959) and *in vitro* (Harold *et al.*, 1956). The *in vitro* conversion was also observed in microsomal and mitochondrial preparations of rat liver (Tombropoulos, *et al.*, 1962) and in guinea pig adrenal gland homogenates (Werbin and Chaikoff, unpublished observations). Finally, Harold *et al.* (1956) isolated compound V from rat liver homogenates incubated with 5α-cholestan-3-one (IV).

Recently an alternative pathway (scheme B, Fig. 2) for the formation of DHC, one that does not involve cholesterol as an obligatory intermediate, was proposed by Kandutsch (1963). In this scheme 7-dehydrocholesterol (VI), a precursor of cholesterol, is converted to DHC through the intermediates cholesta-5,7-dien-3-one (VII), cholesta-4,7-dien-3-one (VIII), cholesta-4,6-dien-3-one (IX), and compound III. With the formation of intermediate III, scheme B merges with scheme A (Fig. 2). Although the reactions involved in conversion of compound VI to VIII could not be demonstrated by Kandutsch (1963) in mouse liver homogenates, the microsomal component of the homogenates did convert compound VIII to IX and IX to III. The increase in "other sterols," presumably DHC, that Cook *et al.* (1954) found in the liver of rabbits fed 7-dehydrocholesterol makes alternative pathway B for DHC biogenesis worthy of consideration.

According to Kritchevsky (1958), the cholesterol content of human tissues is 300–600 mg/100 g. It can therefore be calculated that the human body contains from 5 to 10 g of DHC. The physiological significance of this seemingly large amount of DHC in mammals is not understood at present.

In several insects and some microorganisms that require cholesterol for growth, this requirement can be met or spared by DHC (Clayton, 1964; Smith, 1964). Although cockroaches dehydrogenate DHC to 5α-cholest-7-en-3β-ol (Louloudes *et al.*, 1962; Clayton and Edwards, 1963), the bulk of the DHC incorporated into insects remains unmetabolized (Clark and Bloch, 1959) and is believed to occupy the structural spaces in the cell normally taken up by cholesterol (Clayton and Bloch, 1963). In mammalian tissues cholesterol is also believed to have a structural function in cell membranes (Davson, 1962; Finean, 1962), mitochondria (Green and Fleischer, 1963) and in the endoplasmic reticulum (Ernster *et al.*, 1962). Whether DHC can mimic this role of cholesterol in membranous substructures is not known at present. It is of interest in this connection that microsomal- and mitochondrial-containing fractions of rat liver do convert cholest-4-en-3-one to DHC (Tombropoulos *et al.*, 1962).

ADDED IN PROOF

Shefer *et al.* (1964) recently isolated [^{14}C +] labeled DHC from the adrenal glands of rabbits that had been injected intravenously with [2-^{14}C]mevalonate. They reported, however, that they could not detect the labeled stanol in the livers, adrenal glands, and intestines of rabbits 24 hours after feeding them [4-^{14}C]cholesterol. This is a surprising finding because all commercial preparations of [4-^{14}C]cholesterol that we have examined were contaminated with [^{14}C]DHC to the extent of 0.1–0.2% of the total ^{14}C. The dilution of this [^{14}C]DHC (radiochemical impurity) with a considerable endogenous pool of DHC in the intact rabbit may have made its detection difficult.

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Acyl-Carrier Protein. II. Intermediary Reactions of Fatty Acid Synthesis

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The fatty acid synthetase of *Escherichia coli* has been fractionated and several enzymatic activities have been partially purified. Two of these, acetyl transacylase and malonyl transacylase, catalyze the transfer of acetyl and malonyl groups, respectively, from CoA (coenzyme A) to ACP (the acyl-carrier protein). The condensation between acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP is catalyzed by an enzyme which purifies along with the malonyl transacylase fraction. The reduction of acetoacetyl-ACP to form β-hydroxybutyryl-ACP is catalyzed by β-ketoacyl-ACP reductase. This enzyme is relatively specific for acyl-ACP compounds, but it also catalyzes the reduction of acetoacetyl-CoA and acetoacetyl pantetheine. The reaction is readily reversible, and study of the oxidation of stereoisomers of β-hydroxybutyryl thiolesters has established that the enzyme specifically reacts with the D(−) isomer. The conversion of crotonyl-ACP to a product tentatively identified as β-hydroxybutyryl-ACP is catalyzed by enoyl-ACP hydrase which has been partially purified from the *E. coli* system. Two mammalian enzyme systems, fatty acid synthetase of rat adipose tissue and pig heart β-hydroxyacyl-CoA dehydrogenase, catalyze the reduction of *E. coli* acetoacetyl-ACP.

The discovery by Lynen of protein-bound acetoacetate in a yeast fatty acid–synthesizing system led to the proposal that all the intermediates in long-chain fatty acid biosynthesis might be similarly protein bound (Lynen, 1961, 1962). Fractionation of an *Escherichia coli* fatty acid synthetase has allowed the demonstration of a unique acyl-carrier protein (ACP),[1] formerly designated as Enzyme II (Alberts *et al.*, 1963; Goldman *et al.*, 1963a,b). Acyl compounds which are involved in fatty acid biosynthesis are bound through thiolester linkage to the sulfhydryl group of ACP. ACP has been isolated and has been shown to contain a single sulfhydryl group (Majerus *et al.*, 1964). Acetyl-ACP, malonyl-ACP (Majerus *et al.*, 1964), acetoacetyl-ACP (Goldman *et al.*, 1963b; Goldman, 1964), and butyryl-ACP (Goldman, 1964) have been isolated, and the latter two have been shown to be intermediates in fatty acid synthesis (Goldman *et al.*, 1963b; Goldman, 1964). Chemical synthesis of various acyl-ACP derivatives has allowed study of the intermediate steps in fatty acid biosynthesis. The following reactions have been proposed:

$$\text{acetyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{acetyl-S-ACP} + \text{CoA-SH} \quad (1)$$

$$\text{malonyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{malonyl-S-ACP} + \text{CoA-SH} \quad (2)$$

$$\text{acetyl-S-ACP} + \text{malonyl-S-ACP} \rightleftharpoons \text{acetoacetyl-S-ACP} + \text{ACP-SH} + CO_2 \quad (3)$$

$$\text{acetoacetyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{D(}-\text{)-}\beta\text{-hydroxybutyryl-S-ACP} + \text{NADP}^+ \quad (4)$$

$$\text{D(}-\text{)-}\beta\text{-hydroxybutyryl-S-ACP} \rightleftharpoons \text{crotonyl-S-ACP} + H_2O \quad (5)$$

$$\text{crotonyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{butyryl-S-ACP} + \text{NADP}^+ \quad (6)$$

The purification of acetyl transacylase, malonyl transacylase, condensing enzyme, and β-ketoacyl-ACP reductase, which catalyze reactions 1, 2, 3, and 4, respectively, in the *E. coli* system, will be reported in this paper. Evidence that reaction 5 is catalyzed by a partially purified protein fraction of *E. coli* will be presented. In addition, experiments which show that *E. coli* acyl-ACP derivatives are metabolized by mammalian enzyme systems will be reported.

[1] Abbreviations used in this work: ACP, acyl-carrier protein; this name has been agreed upon by Drs. K. Bloch, P. Stumpf, and S. J. Wakil.