METABOLISM OF CHOLESTEROL IN THE CHICK EMBRYO*

II. ISOLATION AND CHEMICAL NATURE OF TWO COMPANION STEROLS

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Previous work (1–9) has shown that, shortly after administration of acetate-1-C\(^{14}\), the non-saponifiable fraction of intact animals, perfused organs, and yeast contains highly labeled substances. These have been implicated as direct precursors of cholesterol since efficient conversion of some of these substances to cholesterol has been demonstrated \textit{in vivo} (5, 6). One of these substances has been identified as squalene (5, 10); another fraction closely resembles cholesterol in that it is precipitated together with cholesterol digitonide and is difficult to separate from cholesterol by physical means.

The present authors found that the digitonide fraction of chick embryos from eggs injected up to 32 hours previously with labeled sodium acetate contained similar substances (11). This paper describes the isolation and characterization of two new sterols from the sterol mixture of 14 day-old chick embryos. The high specific activities and the structures of these companions of cholesterol make them plausible cholesterol precursors and intermediates in sterol metabolism.

\textit{Methods}

\textit{Injection with Sodium Acetate-1-C\(^{14}\)}—Sodium acetate-1-C\(^{14}\) was prepared by the method of Lemmon (12). Fertile eggs were injected, as previously described (11), with 5 mg. of acetate per egg.

\textit{Hydrolysis of Tissue}—The pooled embryos and membranes (amnion and chorio-allantois) were hydrolyzed by adding a kilo of KOH pellets and a liter of 95 per cent ethanol for each kilo of tissue and refluxing under nitrogen on a steam bath for 2 hours. The cooled hydrolysate was first extracted with 600 ml. of diethyl ether for each kilo of tissue. The aqueous

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phase was then extracted twice with 300 ml. of ether, and the dark colored pooled ether extract was washed repeatedly with small portions of water until light yellow, then allowed to stand over Drierite overnight, and filtered through glass wool. The ether was taken off under nitrogen on a steam bath, leaving a mixture of crystals and yellow oil.

Formation and Cleavage of Digitonides—Digitonides were prepared by the method of Windaus (13) and dried to constant weight in a vacuum oven at 60°. The factor, 0.25, was used whenever necessary to convert digitonide weight to sterol weight (14). Large digitonide fractions were cleaved by the method of Bergmann (15), with the modification that the sterol was dissolved by adding dry ether to the flask and triturating the residue. Small digitonide fractions were cleaved by the method of Schoenheimer and Dam (16). The ether was removed under nitrogen, and the sterol was dried to constant weight in vacuo at 60°.

Preparation of Steryl p-Iodobenzoates-$^{1131}$—A 20 per cent molar excess of $p$-iodobenzyol chloride-$^{1131}$ was added to the dried sterol (taken as cholesterol), and the mixture was dissolved with warming in dry pyridine and left overnight at 55° (17). The esters were recovered by adding an excess of water to the reaction mixture, filtering out the crystals, washing them with water, cold 10 per cent Na$_2$CO$_3$ solution, and again water, and drying in vacuo to constant weight at 60°.

Chromatography—Unless otherwise specified, the steryl $p$-iodobenzoate-$^{1131}$ mixtures were chromatographed on columns of 2:1 silicic acid-Celite 503 (18), pretreated with 1 hold-up volume of 1:10 benzene-Skellysolve C. The developer was Skellysolve C, treated to remove unsaturated hydrocarbons (19). It was drawn through the column by reducing the pressure in the receiver to 120 mm. of Hg. The progress of development was visualized by repeated surveys down the length of the column with a scintillation counter traveling at approximately 1 cm. per minute, the counting rate at each level being recorded by a strip chart recorder (18).

The ester zones were located and cut from the column with a hot wire. The adsorbent was packed into small columns, and the esters were eluted with benzene or benzene-ethanol until samples of the eluates showed only traces of $^{1131}$ activity when tested in a well type $\gamma$-ray counter.

Equivalent Weight Determination—The $^{1131}$ specific activities of crystalline compounds recovered from the column are inversely proportional to their equivalent weights. The specific activities of samples of unknown esters and of cholesteryl $p$-iodobenzoate-$^{1131}$, prepared from the same labeled $p$-iodobenzyol chloride-$^{1131}$, were determined by precise weighing and $\gamma$-ray counting (20). The equivalent weights of unknown compounds were then calculated by a comparison of the ratios of their specific activities to that of cholesteryl $p$-iodobenzoate-$^{1131}$, the equivalent weight of which is 616.65.
**Recovery of Sterols and Counting of Sterol Digitonides**—Hydrolyses of the steryl p-iodobenzoates were carried out under the conditions recommended by Idler and Baumann for steryl p-phenylazobenzoates (21). The benzene or benzene-ether extracts of these hydrolysates were allowed to stand for 1 hour over Drierite, were filtered, and the solvent was evaporated, and the residues converted to digitonides. After standing overnight, the digitonides were filtered out on fritted glass disks by the method described by Van Slyke et al. (22), washed, without being allowed to run dry, with 5 ml. of 90 per cent ethanol and 5 to 10 ml. of 1:1 ether-acetone, and oven-dried at 110°. The dried filter cake (1.505 sq. cm. in area, weighing 2 to 20 mg.) was generally non-adherent to the fritted glass and could be transferred quantitatively to weighed aluminum planchets for weighing and counting. An Amperex model G-60 thin window counter was used, and the counting period was of a duration sufficient to give duplicate counts agreeing within 2 per cent. Counts were corrected to infinite thinness by the method of Gora and Hickey (23).

**EXPERIMENTAL**

*Isolation and Preliminary Study of High Counting Fraction*

*Isolation*—Preliminary examination of the labeled sterols by twenty tube counter-current distribution between petroleum ether and aqueous ethanol showed that the digitonin-precipitable high counting companions of cholesterol were concentrated in the slower moving (more polar) fraction. Subsequently, chromatography of the p-iodobenzoates-I^{131} was employed to isolate the high counting fraction (HCF).

Ten 11 day-old embryos were injected with acetate having a specific activity of 4.9 × 10⁶ c.p.m. per mg. They were sacrificed 16 hours later and pooled with twenty-four untreated embryos. The non-saponifiable fraction was isolated as described above, except that 20° petroleum ether was employed. 1.7986 gm. of digitonide were obtained, which yielded 0.4427 gm. of sterol with a specific activity of 57.5 c.p.m. per mg. of digitonide. 0.4336 gm. of the sterol was esterified to yield 0.6873 gm. of ester, which was chromatographed on a 92.5 cm. column.

On the developed chromatogram appeared a major zone and a minor slow moving zone. The major zone was cholesteryl p-iodobenzoate with some cholestanyl p-iodobenzoate in the leading portion. Its maximum was 66 cm. from the top of the column and its I^{131} activity content was 98.1 per cent of the total in the two zones, as estimated from the net areas on the chart. The minor zone (HCF) had its maximum at 15.6 cm., and its I^{131} activity content was 1.9 per cent. The two zones were cut out in

1 Unless otherwise noted, C^{14} counts were made on samples of sterol digitonide, and specific activities are reported as counts per minute per mg. of digitonide.
four sections and eluted with benzene-ethanol, and digitonides of aliquots were prepared (Table I).

Calculations from the data in Table I indicate an 88 per cent recovery of material and an 81 per cent recovery of C14 activity. Although the HCF zone accounts for only 2.46 per cent of the total weight of material chromatographed, it contains 26 per cent of the total C14 activity.

Differentiation of HCF from \( \Delta^7 \)-Cholesterol—Since \( \Delta^7 \)-cholestenol (lathosterol) is found in animal sterol (24) and is converted into cholesterol \textit{in vivo} by rabbits (25), and since its \( p \)-iodobenzoate separates from that of cholesterol upon chromatography as a slow moving zone (18), the possible identity of the active material with \( \Delta^7 \)-cholestenol was investigated by mixed chromatography. When carbon-labeled HCF material (1250 c.p.m., total) was esterified with cholesterol and \( \Delta^7 \)-cholestenol and the mixture

<table>
<thead>
<tr>
<th>Zone</th>
<th>Column section, distance from top cm.</th>
<th>Total digitonide content mg.</th>
<th>Specific activity digitonide c.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High counting companions</td>
<td>9.6-22.5</td>
<td>42.6</td>
<td>615.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>28.0-57.8</td>
<td>500.0</td>
<td>37.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>57.8-67.2</td>
<td>492.5</td>
<td>35.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>67.2-87.6</td>
<td>429.5</td>
<td>33.3</td>
</tr>
</tbody>
</table>

chromatographed, neither the recovered cholesterol nor \( \Delta^7 \)-cholestenol digitonide displayed significant activity. Subsequently 50 per cent of the added activity was recovered from the column above the \( \Delta^7 \)-cholestenol zone.

Chromatography of Egg Sterol—To determine whether HCF compounds are also found in the digitonin-precipitable fraction of hydrolyzed egg yolk, an infertile egg which had been incubated for 14 days was hydrolyzed. The 0.7617 gm. of digitonide was cleaved to give 0.1679 gm. of sterol, which yielded 0.2821 gm. of labeled ester. This was chromatographed in the usual manner and gave, after 24 hours, a single zone (cholesterol) but no detectable HCF zone.

Resolution of HCF Esters into Two Components

Resolution of Non-Active HCF—The heterogeneity of the high counting zone was established by rechromatography. The esters from 2.313 gm. of sterol derived from 85 13 day old chick embryos were chromatographed on a 3.5 \( \times \) 40 cm. column. The HCF zone was cut from the column, and
eluted esters were rechromatographed on a 1.8 × 50 cm. column. After 32 hours, the HCF zone was resolved into two definitely distinguishable components, termed Components A and B, and a possible third component. The major (and fastest moving) component, B, contained between 75 and 80 per cent of the iodine activity of the HCF zone. On elution, it weighed 55.5 mg., equivalent to 1.4 per cent of the original sterol weight. Component B was recrystallized from methanol-acetone. The colorless crystal plates shrunk\(^2\) at 160°, fusion was marked by the appearance of an iridescent green spot at 166°, and the entire mass became a brilliant green cholesteric melt at 167°. Recrystallization from methanol-acetone did not raise the melting point. The equivalent weight of this material, compared with cholesteryl \(p\)-iodobenzoate from the same run, was 613.09. The material showed absorption between 225 and 300 nm, identical with that of cholesteryl \(p\)-iodobenzoate (m.p. 184.5°) and definitely different from that of 7-dehydrocholesteryl \(p\)-iodobenzoate (m.p. 178.5°).

Resolution of Carbon-Labeled HCF—Thirty-five embryos from eggs injected on the 13th day of incubation with 5 mg. of sodium acetate-\(^1\)\(^-\)C\(^{14}\) (specific activity 5.7 × 10^5 c.p.m. per mg.) were sacrificed 16 hours after injection. The pooled embryos and membranes weighed 442 gm. The crude digitonin-precipitable fraction had a specific activity of 120.1 and weighed 4.1153 gm. It yielded 0.9662 gm. of sterol, which was converted to 1.5362 gm. of esters. After a preliminary separation of the HCF zone by chromatography, this zone and a portion of the adjacent cholesterol zone were eluted with benzene, yielding 42.0 mg. of ester. This material was rechromatographed twice to yield 34.8 mg. of purified HCF ester. This material was rechromatographed on a column 1.8 × 42 cm., prewashed with 1 hold-up volume of 2 per cent benzene in Skellysolve C. The esters were applied with 5 ml., and the column was developed with 2 liters of the same solvent. At this time a distinct resolution into Components A and B was achieved, but there was considerable overlapping. The column was sectioned as presented in Table II, and the esters were converted to digitonides and counted. The specific activity of the digitonide of cholesterol separated from HCF was 66.2. Hence the specific activity of Component B is approximately 20 times that of the accompanying cholesterol.

Preparative Isolation of High Counting Fraction

Isolation—Further study of the components of the HCF required the isolation of larger quantities of material. Fertile eggs from Rhode Island red hens, incubated for 14 days, were obtained from a commercial hatch-

\(^2\) Melting points were determined with short stemmed Anschütz thermometers and are uncorrected.
ery. 569 viable embryos and their membranes (6.256 kilos of tissue) were pooled in lots of 45 to 85 for hydrolysis. The sterol content of this crude non-saponifiable fraction was estimated to be 0.027 gm. per embryo. When this crude sterol fraction is crystallized from aqueous ethanol, the companions of cholesterol are concentrated in the mother liquor. Thus 80 ml. of 95 per cent ethanol and 40 ml. of water were added for every estimated gm. of sterol, the mixture was heated to dissolve all solids, and the sterols crystallized overnight at room temperature. The HCF was concentrated in the mother liquor, which was filtered off, leaving 13.304 gm. of sterol crystals. Ethanol was added to the mother liquor to raise the ethanol content to 80 per cent, and the digitonin-precipitable content of an aliquot was determined. The remainder was precipitated with a calculated 10 per cent excess of digitonin, yielding 7.813 gm. of digitonide.

TABLE II
Chromatographic Separation of High Counting Companion p-Iodobenzoate Zone into Carbon-Labeled Compound A and Compound B (Desmosterol)

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Column section, distance from top</th>
<th>Description</th>
<th>Digitonide weight</th>
<th>Specific activity digitonide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0-9.0</td>
<td>Compound A</td>
<td>11.95</td>
<td>889</td>
</tr>
<tr>
<td>2</td>
<td>9.0-12.3</td>
<td>Compounds A and B</td>
<td>7.03</td>
<td>1388</td>
</tr>
<tr>
<td>3</td>
<td>12.3-15.4</td>
<td>Compound B</td>
<td>14.93</td>
<td>1405</td>
</tr>
<tr>
<td>4</td>
<td>15.4-17.7</td>
<td>&quot; &quot;</td>
<td>21.80</td>
<td>1338</td>
</tr>
<tr>
<td>5</td>
<td>17.7-27.4</td>
<td>&quot; &quot;</td>
<td>19.60</td>
<td>1063</td>
</tr>
</tbody>
</table>

The 13.304 gm. of sterol were recrystallized at room temperature from 480 ml. of 90 per cent ethanol, and the crystals were filtered and washed with 100 ml. of 90 per cent ethanol. The recrystallized sterol weighed 9.568 gm.; the filtrate, taken to dryness under nitrogen, left 3.909 gm. of residue. The 9.568 gm. of sterol were recrystallized from 350 ml. of 90 per cent ethanol, and the crystals washed with 50 ml. of the same solvent, yielding 7.264 gm. of recrystallized sterol and 2.175 gm. of dry residue.

The HCF compounds were then concentrated in the 7.813 gm. of digitonide from the first mother liquor and in the two mother liquor residues. The latter were now converted to digitonides, and all three digitonide fractions cleaved in batches, beginning with the digitonides of the first mother liquor. A total of 6.998 gm. of sterol were recovered and esterified with p-iodobenzoyl-chloride-1131 in seven batches of 0.38 to 1.39 gm. Yields of ester, based on cholesterol, were 96.4 to 98.7 per cent.

According to subsequent chromatography, the esters obtained from the first mother liquor contained up to 7.6 per cent HCF compounds, those
from the second mother liquor 2.8 per cent, while those from the third mother liquor less than 1 per cent. Hence it was impractical to take more crops by further recrystallization.

The seven columns were sectioned, and the esters of the HCF zone were eluted and taken to dryness under nitrogen, yielding 470 mg. of material. A 0.01 aliquot was treated with digitonin, but only a trace (less than 0.02 mg.) of free sterol was present. Hydrolysis gave 294.7 mg. of white crystals.

**Homogeneity of Component B**—It was still possible that the high carbon specific activities found above in Component B were due to contamination of the digitonides by a trace of highly active material. Hence some of the carbon-labeled Component B was rechromatographed with crude carrier HCF to determine whether the C¹⁴ activity would be recovered and whether it would label the Component B zone uniformly.

C¹⁴-labeled Component B was prepared by dissolving 5.68 mg. of digitonide with a specific activity of 1338 (Fraction 4, Table II) in 1 ml. of dry pyridine, and by adding 60 ml. of dry ether, filtering through a small column of Celite, removing the ether and pyridine under nitrogen, and drying the residue in vacuo. The labeled Component B was added in ether to 74.52 mg. of the crude HCF derived from the preparative chromatographic separation. The ether was removed under nitrogen, and the residue dried to constant weight (76.85 mg.) and esterified with p-iodobenzoyl chloride. The total esters (125.3 mg.) were dissolved with warming in 25 ml. of Skellysolve C and chromatographed on a 1.8 X 65 cm. column. A fast moving fraction (cholesteryl p-iodobenzoate), representing 15 per cent of the ester I¹³¹ activity, passed out of the column. From the areas under the curves of the iodine activity, and with the assumption that the equivalent weights were the same as the molecular weights, it was estimated that the esterifiable content of the crude HCF material was made up of 15 mole per cent cholesterol, 7 mole per cent Component A, and 78 mole per cent Component B.

After 74 hours of development the column was sectioned, and digitonides of aliquots were prepared and counted. The Component B zone was divided into six sections. The carbon specific activity of the digitonides prepared from the top five sections was constant at 28.6 ± 1.5. The specific activity (23.5) of the leading portion of Component B zone was somewhat low. This was also observed in the previous experiment and probably represents between 2 and 3 per cent of non-radioactive contaminant.

The equivalent weights of the p-iodobenzoates of the cholesterol and Component B zones were 616.6 and 613.1, respectively, and that of the
Component A ester was subsequently found to be the same as that of Component B. From the above information it can be computed that the Component B content of the 76.9 mg. of starting material is equivalent to 240 mg. of digitonide (with a specific activity of 28.6). Hence 90 per cent of the total added carbon activity reappeared in Component B. This high recovery indicates that the carbon activity does not reside in a "weightless" impurity, while the chromatographic homogeneity, as demonstrated by the uniform carbon labeling throughout the zone of Component B, indicates that it is a single chemical species.

### Table III

*Preparative Chromatographic Separation of Compound A and Compound B (Desmosteryl) p-Iodobenzoates-\(^{113}I\)*

<table>
<thead>
<tr>
<th>Fraction No</th>
<th>Column section, distance from top</th>
<th>Description</th>
<th>Benzene eluate mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0-1.2</td>
<td>Some activity adsorbed at column top</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>1.2-13.7</td>
<td>Large slow moving peak</td>
<td>44.1</td>
</tr>
<tr>
<td>3</td>
<td>13.7-27.4</td>
<td>Compound A peak</td>
<td>29.0</td>
</tr>
<tr>
<td>4</td>
<td>27.4-30.1</td>
<td>Compounds A and B</td>
<td>7.2</td>
</tr>
<tr>
<td>5</td>
<td>30.1-42.1</td>
<td>Compound B</td>
<td>60.1</td>
</tr>
<tr>
<td>6</td>
<td>42.1-53.4</td>
<td>&quot; &quot;</td>
<td>112.6</td>
</tr>
<tr>
<td>7</td>
<td>53.4-67.0</td>
<td>&quot; &quot;</td>
<td>57.9</td>
</tr>
</tbody>
</table>

**Characterization of High Counting Companions of Cholesterol**

As previously described, resolution of the slow moving, high counting zone yields at least two fractions, a major one (Component B) and a slower moving minor one (Component A). This resolution was now repeated on a larger scale with the object of isolating these components in amounts sufficient for characterization.

219.9 mg. of the crude HCF material were esterified with p-iodobenzoyl chloride-\(^{113}I\) dissolved in dry pyridine as a 6 per cent solution, and 70 mg. of cholesterol, purified through the dibromide, were esterified separately with the same solution. The cholesteryl p-iodobenzoate, after chromatography and recrystallization, served as the standard in equivalent weight determinations. The yield of crude HCF esters was 387.9 mg. They were chromatographed as before on a 1.8 \(\times\) 85 cm. column. After 144 hours the column was allowed to run dry and was sectioned into seven fractions, and these were eluted with benzene and then separately with acetone (Table III). Five of the seven benzene eluates were processed as described below. The acetone eluates were slightly colored and were of too small quantity for further study.
Fraction 2 had an equivalent weight of 258 and a melting point of 233°. It is apparently impure p-iodobenzoic acid, equivalent weight 248, m.p. 269–270°.

Fraction 3 comprises the Component A ester zone. The crystals were dissolved in acetone and filtered through a plug of glass wool into a centrifuge tube. The acetone was reduced to a small volume, and the contents were allowed to crystallize finally at 0°. 18.2 mg. of foliated crystals were obtained, which sintered at 130° and melted at 140.5° to a cloudy liquid which cleared near 149°. The equivalent weight of a sample weighing 11.198 mg. was 615.64.

Recrystallization from "hexane"-acetone yielded crystals shrinking at 140° and becoming a cloudy melt at 143.8°. This last crop of crystals was pooled with the 11.2 mg. used in the equivalent weight determination, and an analytical sample (m.p. 143.8°) was obtained by recrystallization from acetone.

Found. " 66.52, " 7.87, " 20.71
This ester is hereafter referred to as Compound A p-iodobenzoate.

Fraction 5 was the upper quarter of the Component B ester zone. It was crystallized from acetone to yield 50.4 mg. of foliated crystals, which shrunk at 164.5° with the appearance of a characteristic color play and completely lost their structure at 166°. The equivalent weight of a 32.001 mg. sample was 607.5.

Fraction 6 was the middle half of the Component B ester zone. When crystallized as above, it yielded 96.9 mg. of crystals (m.p. 166–167.5°). Duplicate samples of approximately 26 mg. had an equivalent weight of 611.1 ± 1.5. An analysis of this compound gave the following values:

Found. " 66.53, " 7.83, " 20.74
This ester is hereafter referred to as Compound B p-iodobenzoate.

Fraction 7 (m.p. 162.5–164.5°) was the lower quarter of the Component B ester zone. Its equivalent weight was 607.5. Since this portion of the zone may contain a few per cent impurity, it was not pooled with the other fractions.

Compound A—3.8 mg. of Compound A p-iodobenzoate, m.p. 140–143°, were hydrolyzed. The infra-red spectrum of the crude product shows the band near 1040 cm⁻¹ which, taken with the digitonin precipitability, is diagnostic of a 3β-hydroxy sterol with a trans A/B ring fusion (26).

All elementary analyses were made by the Schwarzkopf Microanalytical Laboratory, Woodside, New York.
Compound B—154.2 mg. of Compound B p-iodobenzoate, m.p. 166-167.5°, were hydrolyzed under nitrogen to yield 97.55 mg. of Compound B. This was recrystallized from methanol at 0°, yielding 75.1 mg. of plates, m.p. 120.5-121.0°; [α]_D~27~ 40.2°, 1 per cent in chloroform. Two recrystallizations of a portion from low boiling petroleum ether gave a product with a melting point at 120.2-120.9°. 35.5 mg. were recrystallized from methanol-acetone to yield 23.6 mg. of crystals dried in vacuo at 70°, m.p. 120.8-121.2°, which were analyzed.

C_{24}H_{44}O (384.62). Calculated, C 84.31, H 11.53; found, C 84.25, H 11.48

Compound B shows no significant absorption above 225 mμ. The sulfuric acid chromogen spectrum of a 7 × 10⁻⁷ m solution in 97 per cent sulfuric acid (27) contained a minimum at 256 mμ (E₁₀₀,₉₃~93~), a maximum at 320 mμ (240), a minimum at 393 mμ (109), a maximum at 433 mμ (154), and an inflection at 496 mμ (84). The infra-red spectrum is presented in Fig. 1.

Bromination of Compound B p-Iodobenzoate—By analogy with the reported bromination of cholesteryl benzoate (28) and zymosterol benzoate (29), it seemed feasible to brominate steryl p-iodobenzoates. To check the procedure, 25 mg. of cholesteryl p-iodobenzoate-I^131 were brominated at 0° with bromine in chloroform to an orange end-point. The product was precipitated and washed with 95 per cent ethanol and dried in vacuo at 60°. The equivalent weight of the white granular product (m.p. 134.8°) was 776.9; calculated, for C_{34}H_{49}O_{2}Br_{2}, 776.5.

Since it was subsequently necessary to determine the radiometric equivalent weight (20) of steryl p-iodobenzoates-I^131 recovered from debromination with NaI in ethanol-benzene (30), it was necessary to check the possibility of loss of I^131 activity by exchange with NaI, catalyzed by the I₂ formed in the reaction. 100.8 mg. of cholesteryl p-iodobenzoate-I^131 dibromide were dissolved in 2.4 ml. of dry benzene, and a solution of 120 mg. of NaI in 1.6 ml. of absolute ethanol was added. The solution was allowed to stand at room temperature in the dark for 24 hours. It was then transferred to a separatory funnel with benzene, washed with Na₂SO₃ solution and with water, filtered through anhydrous Na₂SO₄, evaporated to dryness, and the residue recrystallized from acetone. The recovered cholesteryl p-iodobenzoate-I^131, m.p. 184.5°, had a specific activity 1.8 per cent higher than the starting material. Hence it can be concluded that no appreciable exchange occurred.

35.2 mg. of Compound B p-iodobenzoate-I^131, m.p. 164.5°, were dissolved at 0° in 1 ml. of chloroform, and 0.1 ml. of a 1:10 (volume per volume) solution of bromine in chloroform was added dropwise with shaking. 5 ml. of cold 95 per cent ethanol were then added, and the resulting precip-
itate was centrifuged, washed successively with 2 ml. of ethanol and 3 ml. of acetone, and dried in vacuo at 60°. 37.6 mg. of product were obtained. The equivalent weight of a 29.790 mg. sample of the powdery white product was 939.4; calculated for C_{34}H_{47}O_{2}Br_{4}, 936.5.

It has been demonstrated (30, 31) that under the mild conditions described above it is possible to debrominate the sterol ring system without removing side chain bromine. Accordingly, 29.8 mg. of Compound B p-iodobenzoate-I^{131} tetrabromide were debrominated by the above method. Recrystallization of the product from acetone-ether at 0° yielded fine needles (m.p. 181–182°, with decomposition). The equivalent weight of a 12.126 mg. sample was 786.3; calculated for C_{34}H_{47}O_{2}Br_{2}, 776.4.

Hydrogenation of Compound B—If, as the empirical formula and the infra-red spectrum indicate, Compound B is a dehydrocholesterol, it
should be possible to hydrogenate it to cholestanol. Owing to the small amount of material available, a carrier technique was employed. 2.41 mg. of Compound B digitonide with a specific activity of 1372 were cleaved, and the ether solution of the recovered sterol was taken to dryness with 98.3 mg. of purified cholesterol. The mixture was hydrogenated with Adams' catalyst in acetic acid-cyclohexane and worked up according to Nace (32). The slightly yellow product was recrystallized from methanol to yield 75.1 mg. of white crystals, which gave a weak Liebermann-Burchard reaction. A portion of the crystals was converted to digitonide with a specific activity of 7.1, while its mother liquor gave 91.6 mg. of digitonide with a

| Table IV |

**Chromatography of Carbon-Labeled Hydrogenated Compound B (Desmosteryl) p-Iodobenzoate with Carrier Cholesteryl and Cholestanyl p-Iodobenzoates**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Column section, distance from top</th>
<th>Total digitonide</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Steryl p-iodobenzoate</th>
<th>M.p. of ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm.</td>
<td>mg.</td>
<td>c.p.m.</td>
<td>mg. per mg.</td>
<td>c.p.m.</td>
<td></td>
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<td>32.60</td>
<td>0.2</td>
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<td>Cholesteryl*</td>
<td>183.5-184.5</td>
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<td>129.70</td>
<td>0.5</td>
<td>64.8</td>
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<td>&quot;</td>
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<td>3</td>
<td>41.1-49.4</td>
<td>101.28</td>
<td>3.1</td>
<td>314.0</td>
<td>Mixture</td>
<td>184.0-185.8</td>
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<tr>
<td>4</td>
<td>49.4-52.7</td>
<td>46.00</td>
<td>6.1</td>
<td>280.6</td>
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<tr>
<td>5</td>
<td>52.7-56.0</td>
<td>55.92</td>
<td>6.7</td>
<td>374.7</td>
<td>Cholestanyl*</td>
<td>184.0-186.0</td>
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<tr>
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<td>56.0-59.3</td>
<td>50.45</td>
<td>7.5</td>
<td>378.4</td>
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<tr>
<td>7</td>
<td>59.3-62.5</td>
<td>33.95</td>
<td>7.3</td>
<td>247.8</td>
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<td>&quot;</td>
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<tr>
<td>8</td>
<td>62.5-65.8</td>
<td>26.27</td>
<td>7.3</td>
<td>191.8</td>
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<tr>
<td></td>
<td>Total</td>
<td>467.17†</td>
<td></td>
<td>1858.6††</td>
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</table>

* Identified by characteristic melting behavior and crystal habit.
† Recovery. 98 per cent of sterol; 92.5 per cent of activity.

specific activity of 8.3. 70.7 mg. of the recrystallized hydrogenated sterol mixture and 50.4 mg. of carrier cholesterol were esterified to give 192.0 mg. of [I]-labeled p-iodobenzoates, which were chromatographed on a 1.6 X 77 cm. column. After 44 hours the cholestanol and cholesterol zones were resolved, with some overlapping. The column was sectioned, with the results which appear in Table IV. It is evident that the hydrogenated sterol yields an ester chromatographically indistinguishable from that of cholestanol.

**Comparison of Compound B with 25-Dehydrocholesterol**—The melting point and optical rotation of Compound B (m.p. 120.8-121.2°, [α]_D^25<sup>-40.2°</sup>) do not differ from those reported for 25-dehydrocholesterol (m.p. 121.2-122.2°; [α]_D^25<sup>-43.0°</sup>; m.p. 120.5-121.5°; [α]_D^25<sup>-40.2°</sup>) (33, 34). Samples of authentic 25-dehydrocholesterol were obtained from three sources. The
two older samples had deteriorated. One of them, provided by Professor W. G. Dauben, was converted to the p-iodobenzoate-I\(^{131}\), and the portion soluble in ether and Skellysolve C was chromatographed. The material from the "slow moving ester zone" was recrystallized from acetone to give a 24 per cent yield of crystals melting (like Compound B p-iodobenzoate) at 166.0-167.6\(^o\), with a cholesteric color play. Hydrolysis of this ester gave sterol, m.p. 120.5-121.5\(^o\). The 25-dehydrocholesterol provided by Dr. D. S. Fredrickson, melted at 122.0-123.5\(^o\). The sulfuric acid chromogen spectrum of this specimen was substantially identical with that of Compound B. Nevertheless both samples of 25-dehydrocholesterol were clearly distinguished from Compound B by their infra-red spectra, which showed a strong band at 887 cm.\(^{-1}\). This band is characteristic of the isopropenyl group (35) and occupies a region in which Compound B has only a weak band (Fig. 1). The close correspondence of the other observed properties indicates that Compound B and 25-dehydrocholesterol are very closely related isomers.

DISCUSSION

The chromatography of the p-iodobenzoates-I\(^{131}\) of the sterol fraction of the 12 to 14 day-old chick embryos yields a slow moving zone containing 2.0 to 2.5 per cent of the total sterol. This zone cannot be detected in sterol of the infertile egg. When 12 day-old embryos were harvested 16 hours after injection of acetate-1-C\(^{14}\), 26 per cent of the digitonin-precipitable carbon activity was isolated from this zone, while an estimated 60 per cent was present in cholesterol. Hence the slow moving zone contains the bulk of digitonin-precipitable components that Schwenk (1-6) has termed the higher counting companions of cholesterol. Rechromatography of this zone yields two isomeric p-iodobenzoates (of Compounds A and B).

Compound B, to which the authors have applied the trivial name desmosterol,\(^4\) comprises 1.8 to 2.2 per cent of the total sterol. The equivalent weight of its p-iodobenzoate, the elementary analyses, its conversion to a p-iodobenzoate tetrabromide, and its hydrogenation to a compound which yields a p-iodobenzoate chromatographically indistinguishable from cholestany1 p-iodobenzoate all indicate that it is a cholestadienol. The p-iodobenzoate tetrabromide is converted to a dibromide by selective debromination, indicating a side chain double bond. The nuclear double bond is located at 5,6 by the infra-red spectrum. In its melting point, optical rotation, sulfuric acid chromogen spectrum, and melting point of its p-iodobenzoate, desmosterol agrees with synthetic \(\Delta^5,\, \Delta^5\)-cholestadiene-3\(^\beta\)-ol. The infra-red spectra of the two preparations, like zymosterol, lack the

\(^4\) From the Greek word, desmos, link.
band near 1368 cm$^{-1}$, associated with the isopropyl group present in many sterols; e.g., cholesterol (36). However, at 887 cm$^{-1}$, desmosterol has only a very weak band, whereas 25-dehydrocholesterol has a strong one. The latter is characteristic of the isopropenyl group (35).

On the basis of the above evidence, it is attractive to assume that desmosterol is $\Delta^\beta_{5,24}$-cholestadiene-3$\beta$-ol. This formulation is also plausible on biogenetic grounds, since a compound of the $\Delta^\beta_{5}$, $24$-lanostadiene-3$\beta$-ol type is a precursor of cholesterol in the rat liver (37), and $\Delta^\beta_{5}$, $24$-cholestadiene-3$\beta$-ol appears to lie close to the path of ergosterol biosynthesis in yeast (6). A compound of this type also provides a route to the 24-hydroxy, methyl, and ethyl derivatives of cholesterol found in nature.

Compound A represents only 0.2 to 0.3 per cent of the total sterol weight. It is isomeric with desmosterol and its chromatographic similarity and the infra-red spectrum suggest that it is a structurally similar cholestadienol without a 5,6 double bond.

When desmosterol and Compound A were isolated from 14 day-old embryos of eggs injected 16 hours previously with labeled acetate, the specific activity of the desmosterol was 20 to 21 times, and that of Compound A containing some desmosterol was approximately 13 times, that of the accompanying cholesterol. The high specific activity, the structure, and recent preliminary experiments on the conversion in vivo of desmosterol to cholesterol in rats, make it probable that desmosterol is a specific cholesterol precursor.

Other evidence exists for the occurrence of side chain saturation-desaturation in cholesterol metabolism. Clayton and Bloch (37) report that the conversion in vitro of lanosterol to cholesterol also produces more polar sterols. Fredrickson, Horning, and Anfinsen (38) suggest, on the basis of the oxidation of the 25-dehydrocholesterol side chain by rat liver homogenates, that desaturation of cholesterol is an early step in side chain degradation.

We are indebted to Professor H. H. Wotiz and Mrs. A. Smakula of the Boston University School of Medicine for the infra-red spectra illustrated, and to Professor H. R. Nace of Brown University for the preliminary infra-red spectra. For sterol samples we thank Professor C. A. Baumann of the University of Wisconsin, Professor W. G. Dauben of the University of California at Berkeley, Dr. D. S. Fredrickson of the National Heart Institute, and Dr. A. I. Ryer of the Chemical Development Division of the Schering Corporation.

**SUMMARY**

Two compounds have been isolated from the sterol mixture of 12 to 14 day-old chick embryos by chromatography of the steryl p-iodobenzoates.
One of the compounds (desmosterol, m.p. 121.2°, $[\alpha]_D^{27}$ -40°; p-iodobenzoate, m.p. 167.5°) constitutes about 2 per cent of the total sterol and has been tentatively assigned the structure of $\Delta^5$, $\alpha^2$-cholestadiene-3$\beta$-ol. The other compound (p-iodobenzoate, m.p. 143.8°) represents only 0.2 to 0.3 per cent of the total sterol and is an isomer of desmosterol.

When 12 day-old embryos are harvested 16 hours after injection with sodium acetate-1-C$^{14}$, 26 per cent of the digitonin-precipitable carbon activity is recovered in these two compounds, with an estimated 60 per cent in cholesterol. The structures and high specific activities of these compounds suggest that they are involved in cholesterol biosynthesis.

**BIBLIOGRAPHY**

METABOLISM OF CHOLESTEROL IN THE CHICK EMBRYO: II. ISOLATION AND CHEMICAL NATURE OF TWO COMPANION STEROLS
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