Localization and biosynthesis of 7-dehydrocholesterol in rat skin

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SUMMARY By differential assay, the concentration of 7-dehydrocholesterol in the dead keratin layer, the epidermal mucosa, the sebaceous glands and associated appendages of the epidermis, and the dermis of rat skin was determined; 85% of the 7-dehydrocholesterol was isolated from slices of skin that contained surface keratin and sebaceous glands.

Slices of skin were incubated with labeled acetate in vitro. Labeled 7-dehydrocholesterol was separated from other labeled skin sterols as the hydrolyzed 5α, 8α Diels-Alder adduct. Approximately 80% of the labeled 7-dehydrocholesterol was isolated from slices of rat skin that contained sebaceous glands.

The formation of cutaneous 7-dehydrocholesterol was studied in vivo by the injection of labeled acetate. More than 80% of the labeled cutaneous 7-dehydrocholesterol was isolated with the sebaceous tissue. Time-course studies suggested that Δ^7-cholestenol may be a precursor of cutaneous 7-dehydrocholesterol.

WINDAUS AND BOCK (1) isolated 7-dehydrocholesterol[1] from skin. Miller and Baumann (2) reported that 7-dehydrocholesterol is present in the skin of many mammals. As early as 1938, Bills (3) attached considerable nutritional significance to the possible actinic conversion of cutaneous 7-dehydrocholesterol into vitamin D_3. Subsequently, several investigators have suggested that clinical and experimental rickets may be cured by irradiation of the 7-dehydrocholesterol of skin (4).

Wheatly and Reinertson (5) concluded that cutaneous 7-dehydrocholesterol must be concentrated on or near the surface of the skin for effective irradiation. They reported that 7-dehydrocholesterol is localized in the Malpighian layer of human skin and little was associated with sebaceous glands. Wells and Baumann (6) reported that 7-dehydrocholesterol is localized similarly in the epidermis of rodent skin; however, the amount of 7-dehydrocholesterol that is associated with either the appendages (sebaceous glands) or the surface epithelium (Malpighian layer) of the epidermis was not determined. Brooks, Lalich, and Baumann (7) observed with histological techniques that most of the Δ^7-sterols of rat skin are stored in the sebaceous glands; the localization of the individual Δ^7-sterols (e.g. 7-dehydrocholesterol, Δ^7-cholestenol, methostenol) was not studied. This report describes studies on the concentration of 7-dehydrocholesterol in the dead keratin layer, the epidermal mucosa,[2] the sebaceous glands and associated appendages of the epidermis, and the dermis of rat skin.

Brooks and Baumann (8) reported that 7-dehydrocholesterol is biosynthesized by rat skin in vitro. The amount of radioactivity associated with 7-dehydrocholesterol was determined by the difference between the counting rate of C^{14} activity in a fraction of crude sterols before and after 7-dehydrocholesterol was removed from the mixture as the Diels-Alder adduct (9). Similarly, Kandutsch and Russell (10) removed labeled 7-dehydrocholesterol as the adduct from chromatographic fractions containing labeled 7-dehydrocholesterol and Δ^7-cholestenol, but conditions for the recovery of the labeled adduct were not obtained. Gaylor and Baumann (11) investigated several methods for separating 7-dehydrocholesterol from mixtures of the two sterols,

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[1] Common names of sterols are used in this report. Δ^7-Cholestenol is 5α-cholest-7-en-3β-ol; cholesterol is cholest-5-en-3β-ol; 7-dehydrocholesterol is cholesta-5,7-dien-3β-ol; methostenol is 4α-methyl-5α-cholest-7-en-3β-ol; and lanosterol is 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol.

[2] The term *epidermal mucosa* is used to describe the tissue removed with the keratotome. A single section (0.1–0.2 mm in thickness) is sliced from the surface of the epidermis in a plane parallel to the skin surface. The slices of epidermal mucosa included small amounts of dermis. Most of the sebaceous tissue remains in the dermis (see figures). The term *epidermis* is reserved for true epidermis: epidermal mucosa, sebaceous glands, hair follicles, and other appendages.

including separation of the former as the Diels-Alder adduct or using a reversed phase column chromatographic procedure. Because the latter process is quite laborious and poorly suited for a large number of determinations, the separation of labeled 7-dehydrocholesterol as the adduct was reinvestigated. An adequate separation was obtained.

Brooks, Godefroi, and Simpson (12, 13) demonstrated with differential enzymatic and mechanical separations of mouse skin that the papillary reticulum contains enzymes that catalyze the incorporation of labeled acetate into squalene. Epidermis was required for subsequent conversion of squalene into sterols. The present report describes the biosynthesis of 7-dehydrocholesterol by tissue preparations from differential chemical and mechanical separations of rat skin.

EXPERIMENTAL PROCEDURES

Spectrophotometric Determination of Cutaneous 7-Dehydrocholesterol

Adult male rats of the Holtzman strain, maintained on a commercial ration, were grouped according to age to minimize variations due to hair growth cycles. Dorsal hair was clipped from the skin, the skin was removed by blunt dissection, and the adhering subcutaneous tissue was scraped from the skin with a dull scalpel. Surface sterols were removed with cotton swabs dampened with chloroform.

Disks of skin (3 cm^2) were cut with a punch. Thin slices (0.1–0.2 mm) of epidermal mucosa were cut from the skin surface with a keratotome[3] by the procedure of Blank, Rosenberg, and Sarkany (14). The slices and remaining tissue were examined microscopically. In some experiments thicker slices of skin (0.3–0.5 mm) were prepared with a Stadie-Riggs microtome (15).

Quadruplicate samples of the skin sections were transferred to 50-ml Erlenmeyer flasks fitted with small air condensers. An internal standard of approximately 5 μg of 7-dehydrocholesterol for each cm^2 of skin was added to two of the samples. The samples of skin (3–18 cm^2) were saponified under nitrogen with reflux for 4 hr in a solution of 4 ml of water, 4 ml of ethanol, 2 g of KOH, and 0.75 g of pyrogallic acid. Nitrogen and alkaline pyrogallic acid were used to prevent oxidative destruction of the labile sterols. An equal volume of water was added to the samples, and the sterols and other nonsaponifiable lipids were extracted with a total of 100 ml of petroleum ether (bp 30–60°).

The extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in absolute alcohol, and a sample of the solution was treated with digitonin. The precipitate was centrifuged, washed with acetone and ether, and dried at 110° (16). Glacial acetic acid was added, and the solutions were cooled to room temperature. The absorption at 300, 294, and 282 mμ was measured against a blank containing glacial acetic acid. The amount of 7-dehydrocholesterol in each sample was calculated from the optical densities at the three wavelengths (17). The analytical procedure was verified at frequent intervals by observing ultraviolet spectra with a recording spectrophotometer. Digitonin did not affect the absorbancy. The amount of 7-dehydrocholesterol was calculated from the recovery of 7-dehydrocholesterol, added as an internal standard. The recoveries of the added sterol were between 70 and 90%.

A sample of the same sterol digitonides in glacial acetic acid was treated with Liebermann-Burchard reagent. The concentration of fast- and slow-acting sterols was determined by the method of Moore and Baumann (18). The major slow-acting sterol of skin is cholesterol, and values for the calculated amount of slow-acting sterols are reported as cholesterol. A number of fast-acting sterols (primarily Δ^7-sterols) are present in rat skin (19).

Incubation Procedures

The tissue (thin slices) from 9 cm^2 of skin was incubated in 4 ml of bicarbonate buffer containing 1 μc of acetate-1-C^{14} in 0.2 μmole (8). After incubation for 3 hr at 37°, the buffer was decanted and carrier sterols were added: Δ^7-cholestenol, 2 mg; cholesterol, 2 mg; and 7-dehydrocholesterol, 4 mg. The contents of duplicate flasks were combined and saponified. The nonsaponifiable lipids were fractionated according to the procedure described below. Radioactivity was determined with a liquid scintillation counter. Internal standards of benzoic acid-1-C^{14} were added. The disintegration rate (dpm) is reported for ease of comparison.

In Vivo Studies

A male rat (approximately 250 g) was injected with 25 μc of acetate-1-C^{14}. After 1 hr the rat was decapitated and samples of skin were removed. Four samples totaling 12 cm^2 were treated with ammonium hydroxide to remove the epidermis (plus sebaceous glands and appendages) from the dermis (20). The remaining dorsal skin (18.4 cm^2) was sliced with the keratotome. Carrier sterols were added before saponification.

Preparation of Labeled 7-Dehydrocholesterol and Δ^7-Cholestenol

Cholesterol-4-C^{14} (27 μc)[4] was mixed with 2 g of authentic cholesterol[5] and crystallized. The specific activity was

[3] The keratotome, Castroviejo type, was purchased from the Storz Instrument Company, St. Louis, Mo.

[4] New England Nuclear Corporation, Boston, Mass.

TABLE 1 SPECIFIC ACTIVITIES OF SYNTHETIC LABELED 7-DEHYDROCHOLESTEROL AND Δ^7-CHOLESTENOL

	Compound	Treatment	Yield	Specific Activity	% of Original Specific Activity*
			mg	*dpm/mg*	
1.	Cholesterol	Original	2,000	29,500	100
2.	Cholesterol	Recrystallized twice from ethanol	1,870	29,300	99
3.	Cholesteryl benzoate	Product	1,670	27,600	94
4.	Cholesteryl benzoate crystals	Recrystallized twice from ethyl acetate	1,400	27,400	93
5.	Cholesteryl benzoate supernatant		—	26,700	91
6.	7-Dehydrocholesteryl benzoate	Product	1,385†	4,950	100
7.	7-Dehydrocholesteryl benzoate	Recrystallized twice from ethanol	970	2,910	59
8.	7-Dehydrocholesterol	Saponified and chromatographed	590	2,390	48
9*a*	7-Dehydrocholesterol crystals	Recrystallized twice from ethanol	248	2,340	47
b	7-Dehydrocholesterol supernatant		311	2,420	49
10.	Δ^7-Cholestenol	Original	40‡	1,170	100
11.	"	Crude product	37	1,260	100
12.	"	Recrystallized	35	967	83
13.	"	Recrystallized	30	1,010	86

* Based on the specific activities of cholesterol, 7-dehydrocholesterol, and Δ^7-cholestenol. The values were corrected for changes in molecular weight.

† 1.34 g of carrier 7-dehydrocholesteryl benzoate was added. The yields include this amount.

‡ 20 mg of 7-dehydrocholesterol (46,800 dpm) was hydrogenated over Raney nickel. 20 mg of unlabeled Δ^7-cholestenol was added at the end of the hydrogenation.

99% of the original mixture (Table 1). The dry sterol was esterified (benzoyl chloride–pyridine). The product was extracted and separated chromatographically from unchanged sterol. The ester was crystallized from ethyl acetate to a specific activity of 91–94% of the original. Labeled 7-dehydrocholesteryl benzoate was synthesized from the labeled cholesteryl benzoate by the procedure of Wells and Lorah (21). Unlabeled 7-dehydrocholesteryl benzoate (1.34 g) was added as carrier. The yield of radioactivity in the crude product was 18% of the labeled cholesteryl benzoate (Table 1, line 6). The specific activity of the recrystallized product was 59% of the isolated 7-dehydrocholesteryl benzoate (line 7). The ester was saponified in a solution of 50 ml of benzene, 50 ml of methanol, and 4 g of carbonate-free KOH, and the product applied to a column of alumina (Merck) in benzene–hexane 1:3. Benzene–diethyl ether (1:1) eluted the unesterified sterol. Its specific activity remained constant during recrystallization from ethanol (line 9 *a* and *b*). A small sample of labeled 7-dehydrocholesterol (20 mg) was dissolved in absolute ethanol, and the solution was shaken with Raney nickel catalyst under hydrogen for 3 hr. The product was isolated after the addition of 20 mg of authentic Δ^7-cholestenol.[5] The specific activity of the product was 86% of the original specific activity (lines 10–13). Quantitative hydrogenation never obtains; others have reported similar results (22). The reduced product contained less that 1% of the conjugated diene.

TABLE 2 SPECIFIC ACTIVITY OF THE DIELS-ALDER ADDUCT PREPARED FROM LABELED 7-DEHYDROCHOLESTEROL

Sample	Weight Recovered*	Specific Activity*	% of Original Specific Activity
	mg	*dpm/mg*	
Original 7-dehydrocholesterol	100	1170	100
Hydrolyzed adduct†	50.4	1010	86
Regenerated adduct	45.0	998	85
Recrystallized from acetone	27.7	1160	99
Recrystallized from acetone and hexane:			
a. Crystals	4.4	1140	97
b. Supernatant fraction	12.3	1150	98

* Based on the original weight of 7-dehydrocholesterol. The weights were corrected for adduct formation, hydrolysis, and acetylation. The weights do not include the amount of sample that was taken for assay of radioactivity (5–10 mg).

† The ultraviolet spectrum was scanned from 320 to 250 mμ. No absorption characteristic of 7-dehydrocholesterol was observed.

Preparation and Purification of Diels-Alder Adduct of 7-Dehydrocholesterol in the Presence of Δ^7-Cholestenol and Cholesterol

A sample of 100 mg of labeled 7-dehydrocholesterol (1170 dpm/mg) was mixed with 50 mg of Δ^7-cholestenol

[5] Cholesterol was purified as the 5α,6β-dibromo derivative. The product was dehalogenated and crystallized (mp 147–148°, $[\alpha]_D$ −38.5°, $CHCl_3$, c = 0.6). 7-Dehydrocholesterol was obtained commercially as the acetate (mp 129°) from Chemed, Inc., Odenton, Md. The ester was saponified and the 7-dehydrocholesterol was recrystallized from ethanol (final mp 140–142°, $[\alpha]_D$ −110°, $CHCl_3$, c = 0.6). Δ^7-Cholestenol was prepared by catalytic hydrogenation of 7-dehydrocholesterol over Raney nickel catalyst in dioxane solution (mp 122–123°, $[\alpha]_D$ +2.2°, $CHCl_3$, c = 0.6). The product contained less than 1% of unchanged 7-dehydrocholesterol.

and 50 mg of cholesterol. The mixture was heated under reflux for 2 hr with 5 g of freshly sublimed maleic anhydride and 50 ml of xylene (distilled from sodium). The solvent was evaporated under reduced pressure, and excess maleic anhydride was sublimed at 145° under nitrogen at a reduced pressure. The residue was dissolved in 50 ml of ethanol containing 7.5 g of KOH and the solution was heated under reflux for 1 hr. Water was added (200 ml), and the solution was extracted three times with a total of 200 ml of chloroform. The chloroform layer was extracted twice with 75 ml of a solution of 0.1 N NaOH in water; unchanged sterols and neutral products of degradation remained in the chloroform. The aqueous phase and washings were combined, the solution was acidified with sulfuric acid to pH 3 and extracted three times with a total of 225 ml of chloroform. The extract was washed twice with water, twice with a dilute solution of sodium bicarbonate, once more with water, and evaporated to dryness. The crude tan syrup did not crystallize from either acetone or hexane as reported by others (9). A sample of the residue was transferred to a tared counting vial and the hydrolyzed sterol adduct was dried at 145° and a pressure of 23 mm of Hg to a constant weight. The specific activity of the hydrolyzed adduct was 86% of the original specific activity (Table 2).

The anhydride of the adduct was regenerated by heating the residue with 25 ml of redistilled acetic anhydride under reflux for 3 hr. The solution was concentrated to approximately 5 ml. Tan crystals (45 mg) with a specific activity of 998 dpm/mg (Table 2) were collected. The ultraviolet spectrum in acetic acid showed no characteristic absorption of 7-dehydrocholesteryl acetate. The infrared spectrum[6] of the crystals indicated three maxima that may be ascribed to carbonyl residues: 5.30, 5.55, and 5.70 μ; and a pronounced shoulder at 5.80 μ (Fig. 1, curve *A*). 7-Dehydrocholesteryl acetate shows absorption maxima at 5.70 and 8.00 μ (curve *B*, Fig. 1). Broad and less specific absorption was obtained at the higher characteristic wavelengths of the adduct (7.9–8.0 μ, curve *A*). The crude product was recrystallized from acetone, and the crystals were recrystallized from a mixture of hexane and acetone. The specific activity of the product remained constant (Table 2). The infrared spectrum of the product had the absorption maxima at 5.30, 5.55, and 7.90 μ characteristic for cyclic anhydrides (Fig. 1, curve *C*) in addition to those corresponding to 7-dehydrocholesteryl acetate (5.70 and 8.00 μ). The shoulder at 5.80 μ observed in curve *A* that

[6] Qualitative infrared spectra were determined with an Infracord model 137-b (Perkin-Elmer Co.). Samples of approximately 3 mg were mixed with 300 mg KBr, and the mixture was pressed into a disk (1 cm diameter). The absorption was measured against an air blank.

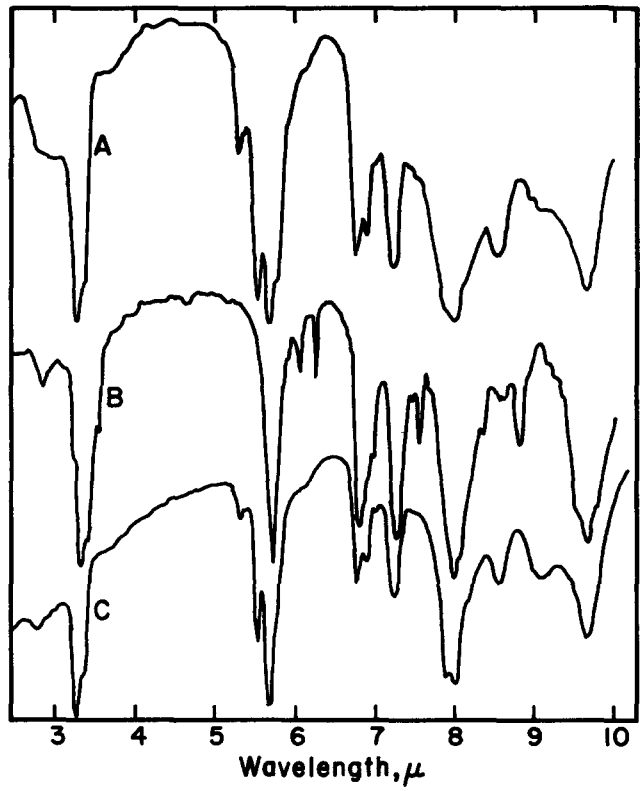

FIG. 1. Infrared spectra of crude 5α, 8α Diels-Alder adduct of 7-dehydrocholesteryl acetate (*A*); 7-dehydrocholesteryl acetate (*B*); and the purified adduct (*C*).

indicated contaminating acyclic anhydride (presumably acetic anhydride) was absent from the final product. The final product (second crop, approximately 7 mg) was collected by centrifugation: mp 171–173°; reported 178° (9). The retention of the original specific activity indicated that the adduct has been obtained essentially free from Δ^7-cholestenol, cholesterol, and products of degradation.

Separation of Labeled 7-Dehydrocholesterol, Δ^7-Cholestenol and Cholesterol

Labeled and unlabeled 7-dehydrocholesterol (8 mg), Δ^7-cholestenol (4 mg), and cholesterol (4 mg) were combined (Table 3). Each mixture was heated under reflux with nitrogen and 0.2 g of maleic anhydride in 2 ml of xylene. The solvent and excess of maleic anhydride were removed, and the residue was saponified for 2 hr in 4 ml of alcohol containing 14.5 g of carbonate-free KOH per 100 ml. Water (4 ml) was added and the neutral sterols were extracted with redistilled chloroform (25 ml total). The hydrolyzed adduct was extracted with 25 ml of chloroform after acidification. The neutral sterols were esterified with *p*-phenylazobenzoyl chloride, and the esters were chromatographed on silicic acid (23). The *p*-phenylazobenzoates were eluted, assayed spectro-

TABLE 3 SEPARATION OF LABELED 7-DEHYDROCHOLESTEROL, Δ^7-CHOLESTENOL, AND CHOLESTEROL

Mixtures of sterols were prepared as follows: 7-dehydrocholesterol, 8 mg; Δ^7-cholestenol, 4 mg; and cholesterol, 4 mg. Duplicate samples contained either: labeled 7-dehydrocholesterol (Table 1), 2340 dpm/mg; labeled Δ^7-cholestenol, 1010 dpm/mg; or labeled cholesterol, 13,700 dpm/mg. The other two sterols in each mixture were unlabeled.

	Experiment I		Experiment II	
	Specific Activity	% of Activity Added	Specific Activity	% of Activity Added
	dpm/mg		*dpm/mg*	
Radioactivity in adduct*				
1. Labeled 7-dehydrocholesterol	1,430	61	2,200	94
2. Labeled Δ^7-cholestenol	73	14	114	22
3. Labeled cholesterol	116	1.7	201	2.9
Radioactivity in Δ^5-zone*				
4. Labeled 7-dehydrocholesterol	684	15	257	5.5
5. Labeled Δ^7-cholestenol	995	98	965	95
6. Labeled cholesterol	135	1.0	935	6.8
Radioactivity in Δ^5-zone*				
7. Labeled 7-dehydrocholesterol	55.5	1.2	57.1	1.2
8. Labeled Δ^7-cholestenol	38.6	3.9	74.9	7.4
9. Labeled cholesterol	9,310	68	12,900	94

* Values for both Experiments I and II are averages of duplicate samples. Each was corrected for changes in molecular weight.

photometrically, and counted as described previously (15). The Δ^7-zone contained primarily esterified Δ^7-cholestenol and traces of esterified 7-dehydrocholesterol. The Δ^5-zone contained the ester of cholesterol. The hydrolyzed adducts from the chloroform extract of the acidified solutions were filtered into tared counting vials, the solutions were evaporated, and the vials heated to constant weight (±0.3 mg). The specific activities, corrected for the addition of the maleic acid moiety or esterification, are reported in Table 3. Subsequent calculations are based upon similar measurements of specific activity.

Of the added labeled 7-dehydrocholesterol, 61% was recovered as the adduct (Table 3, line 1, Experiment I). When the length of time for adduct formation was increased to 4 hr, and that for hydrolysis to 3 hr, 94% of the added activity was isolated with the adduct (Experiment II). The percentage of total counts from 7-dehydrocholesterol in Δ^7-cholestenol decreased from 15 to 5.5 (line 4). Cross-contamination with cholesterol was not significant (lines 3, 6, 7, 8). Substantially more than 100% of added Δ^7-cholestenol-C^{14} was recovered in both experiments (lines 2, 5, 8). Significant amounts of C^{14} activity were isolated with the adduct (line 2). If the adduct were contaminated by Δ^7-cholestenol or a degradation product of the same specific activity, marked dilution of the specific activity of the adduct of labeled 7-dehydrocholesterol would have been observed here (line 1, Experiment II) and previously (Table 2). Possibly, the synthetic labeled Δ^7-cholestenol contained a high-counting contaminant that was carried through the recrystallization.

The weight of the hydrolyzed adduct was between 3.8 and 6.8 mg. The yield of esters from Δ^7-cholestenol and cholesterol (approximately 50 to 65%) was somewhat lower than usual (15, 23).

The amount of label associated with each sterol in a given area of skin was calculated from the specific activity of the isolated product, the amount of carrier sterol added, and the original area of skin. In the case of 7-dehydrocholesterol, the amount of carrier (8000 μg) was substantially greater than the amount of endogenous sterol (approximately 97 μg/18 cm^2 of whole skin—see Table 4 below). The ratio of carrier to endogenous sterol was not as high for Δ^7-cholestenol and cholesterol. However, in most experiments, only thin sections of skin were used, and the contributions of the endogenous Δ^7-cholestenol and cholesterol were not appreciably significant.

Separation of Labeled Sterols Obtained from Skin

The crude nonsaponifiable lipid extract was dissolved in hexane, and the solution transferred to a column of 7.5 g of Merck alumina (24). Squalene was eluted with hexane (100 ml). The sterols were eluted with diethyl ether (100 ml) and transferred to a column of 7.5 g of acid-washed alumina prepared according to the method of Schneider, Clayton, and Bloch (24). The following fractions were obtained: I, 20 ml of hexane (residual squalene); II, 80 ml of benzene–hexane 15:85 (lanosterol, C_{28}-, C_{29}-sterols, and companions); III, 20 ml of benzene–hexane 15:85 (overlapping zone); and IV, 80 ml of benzene–hexane 30:70 (cholesterol, Δ^7-cholestenol, 7-dehydrocholesterol, and C_{27}-sterol companions). The amount of lanosterol (or dihydrolanosterol) and fast-acting sterols of Fraction II were determined by means of the Liebermann-Burchard reaction (15, 18, 25). A sample of the solution was counted. The cholesterol and companions were treated as described above for Experiment II of Table 3. The amount of label in each sterol was calculated for a constant area of skin.

Relatively few of the known skin sterols would be expected to contaminate the isolated Δ^7-cholestenol, cholesterol, and 7-dehydrocholesterol. Fraction IV may contain small amounts of desmosterol and traces of Δ^8-cholestenol and $\Delta^{7,24}$-cholestadienol (19). Small amounts of highly labeled companions may contaminate the products, but the retention of trace contaminants is minimal in the absence of carriers. The activity recovered in Δ^7-cholestenol, cholesterol, and 7-dehydrocholesterol

TABLE 4 CONCENTRATIONS OF FAST-ACTING STEROLS, CHOLESTEROL, AND 7-DEHYDROCHOLESTEROL IN VARIOUS PREPARATIONS OF RAT SKIN

The epidermal mucosa was obtained with the keratotome after the skin was shaved. The dermis plus sebaceous glands remained. The epidermal mucosa plus sebaceous glands was obtained by blunt dissection of the skin after treatment with ammonium hydroxide. The sterols of the dead keratin layer were determined by the difference between samples of whole skin that were shaved before analysis and samples of untreated skin.

Sterol	Keratin Layer I	Epidermal Mucosa II	Epidermal Mucosa + Sebaceous Glands III	Dermis + Sebaceous Glands IV	Dermis V	Total
	$\mu g/cm^2$	$\mu g/cm^2$	$\mu g/cm^2$	$\mu g/cm^2$	$\mu g/cm^2$	$\mu g/cm^2$
Cholesterol	51.3	7.07 ± 2.94*	21.9 ± 6.43	65.8 ± 25.6	51.6 ± 14.6	124.5†
Fast-acting	19.5	4.64 ± 1.41	22.2 ± 6.88	19.3 ± 4.05	6.5 ± 2.19	45.8
7-Dehydrocholesterol	2.61	0.40 ± 0.14	1.85 ± 0.78	1.76 ± 0.50	0.93 ± 0.34	5.08‡

* Standard deviations of the means. Each value is the average of 16 determinations. No attempt was made to assess the error term for the amount of sterol that was determined by difference (keratin layer) or by summation (total).

† The amount of sterol in the total was obtained by summation of appropriate sections. For example, the amount of cholesterol was similar for each procedure of separation: (51.3 + 7.1 + 65.8 = 124.2 and 51.3 + 21.9 + 51.6 = 124.8); accordingly, a mean of 124.5 $\mu g/cm^2$ is reported in the table.

‡ The amount of 7-dehydrocholesterol in the whole sample was calculated from the amount of 7-dehydrocholesterol in the keratin (I) and the epidermis and dermis with and without the sebaceous glands: (2.61 + 0.40 + 1.76 = 4.77 $\mu g/cm^2$; 2.61 + 1.85 + 0.93 = 5.39 $\mu g/cm^2$; mean = 5.08 $\mu g/cm^2$). Because the dermis (V) from the ammonium hydroxide separation contained some sebaceous gland material that was not removed, the former calculation from the keratotome separation (I, II, IV) probably represents a more valid estimation. A more detailed discussion is in the text and the heading to Table 5.

accounted for 86–100% of the Fraction IV activity isolated from skin (see below).

RESULTS

Histological Examination of Tissue Preparations

Samples of skin from normal rats had clearly defined areas of dead keratin, epidermal mucosa, and sebaceous glands, Fig. 2A. The keratin layer was removed completely by shaving, together with some of the epidermal mucosa (Fig. 2B). By slicing the skin with the keratotome, the keratin and epidermal mucosa were completely removed (Fig. 2C). The sebaceous glands and hair follicles were not damaged. Although treatment of skin with ammonium hydroxide permits a facile separation of epidermis (with sebaceous glands and appendages) from dermis (20), residues of atrophic sebaceous glands and entrapped sebum were apparent in all samples (Fig. 2D). Hence, one may obtain complete separations of the epidermal mucosa from the remaining tissue (Fig. 2C vs. 2A), but the separation of sebaceous gland tissue from the dermis was poorer (Fig. 2C vs. 2D). Accordingly, the amount of 7-dehydrocholesterol in the different layers of skin was calculated from the concentration of the sterol in the clearly defined epidermal components, namely epidermal mucosa obtained by the keratotome separation, and whole epidermis from the ammonium hydroxide procedure.

Localization of Sterols within Rat Skin

The layer of dead keratin contained approximately 40–50% of the cutaneous cholesterol, fast-acting sterols, and 7-dehydrocholesterol (Table 4). Most of the cholesterol in the live portion of skin (epidermal mucosa, sebaceous glands, and dermis) was concentrated in the dermis (51.6 $\mu g/cm^2$). Small amounts of cholesterol were isolated with the epidermal mucosa and sebaceous glands. Most of the fast-acting sterols of skin were isolated

TABLE 5 DISTRIBUTION OF 7-DEHYDROCHOLESTEROL WITHIN RAT SKIN

The concentration of 7-dehydrocholesterol in sebaceous glands was determined as the difference between the samples of epidermal mucosa with and without sebaceous glands (Table 4, column III minus column II). Some of the 7-dehydrocholesterol that remains in the dermis following the separation with ammonium hydroxide represents a portion of the sebaceous tissue that remains (Fig. 2D; see footnote to Table 4). Accordingly, the amount of dermal 7-dehydrocholesterol was calculated from the more clearly defined slices that were prepared with the keratotome. Hence, the amount of 7-dehydrocholesterol in the dermis was estimated from the total amount of the sterol in the epidermal mucosa plus the dermis (with sebaceous glands) (Table 4, columns II + IV) minus the amount of the epidermal mucosa plus sebaceous glands (Table 4, III).

Tissue	Concentration of 7-Dehydrocholesterol	% of 7-Dehydrocholesterol in Live Tissue
	$\mu g/cm^2$	
Epidermal mucosa	0.40	18
Sebaceous glands	1.45	67
Dermis	0.31	14
Total in live tissue:	2.16*	—
Total in dead keratin:	2.61	—

* Calculated total.

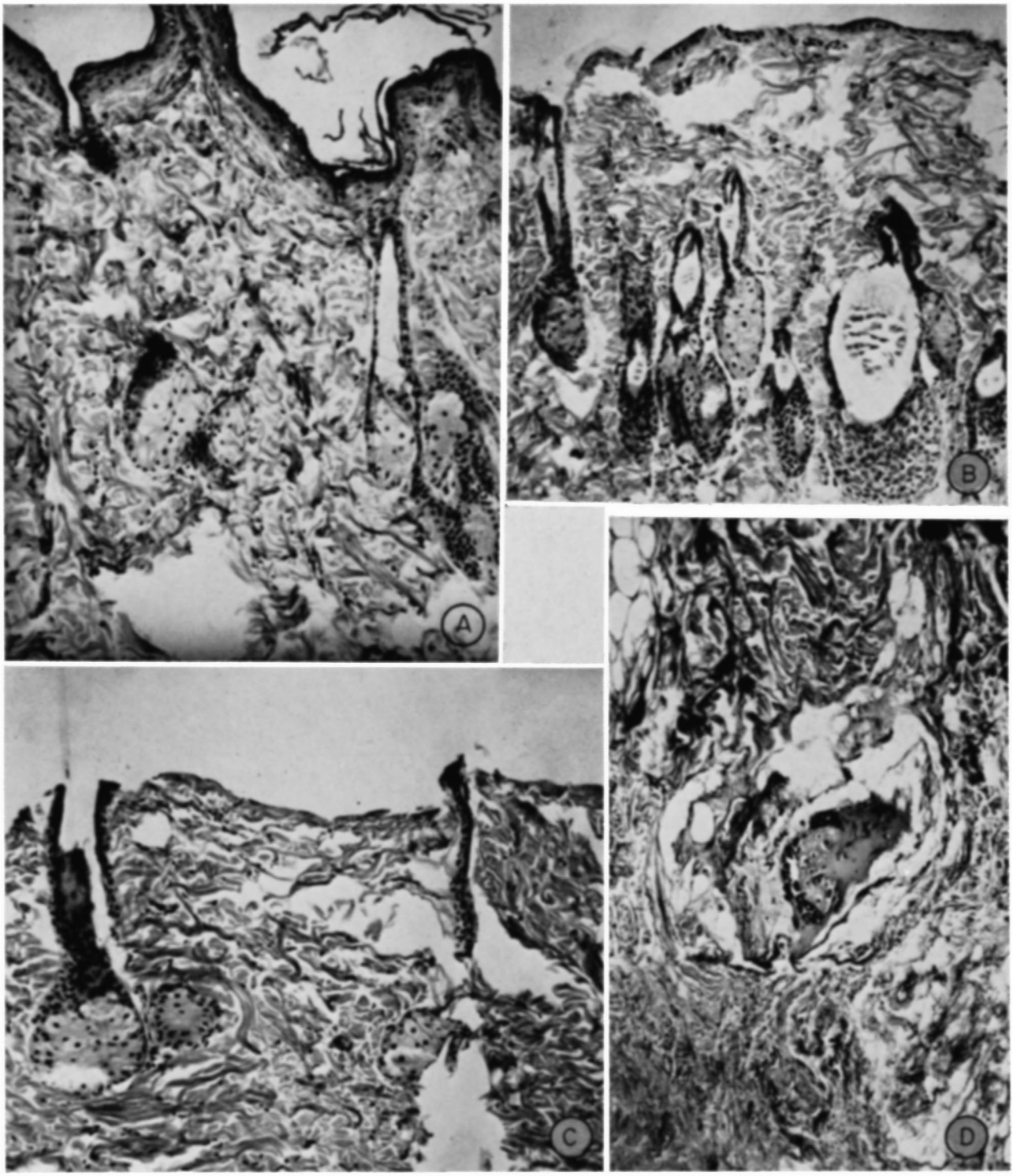

FIG. 2. Preparations of normal rat skin were fixed in formalin, embedded in paraffin, and sectioned (approximately 6μ). The tissue was stained with hematoxylin and eosin. A: untreated normal rat skin, × 130. The sebaceous glands, epidermal mucosa, hair follicles, and dermis are clearly visible. B: normal rat skin, washed with a neutral detergent and shaved, ×130. Staining of the keratin layer (not clearly visible in black and white prints) further indicated that most of the keratin and adhering materials were removed by shaving. C: dermis from whole skin after sectioning with the keratotome, × 130. The epidermal mucosa was removed completely, and the sebaceous tissue remained unaltered in the dermis. Note the hair that remained protruding into the follicle on the left. D: atrophic sebaceous gland tissue that remained in the dermis after epidermis had been removed with ammonium hydroxide and blunt dissection.

from slices that contained sebaceous glands. These results agree with the histological observations of Brooks, Lalich, and Baumann (7). The percentage (3.2) of 7-dehydrocholesterol in the total skin sterols agrees with the value reported by Miller and Baumann (2).

Most of the 7-dehydrocholesterol in the live tissue of skin was in the sebaceous glands (67%) (Table 5). Small amounts were isolated with the dermis (14%) and the epidermal mucosa (18%). More 7-dehydrocholesterol was extracted from the layer of dead keratin than from the combined live cellular fractions (2.61 vs. 2.16 $\mu g/cm^2$).

Formation of Labeled 7-Dehydrocholesterol In Vitro

Approximately 77% of the radioactivity incorporated into the labeled sterols was isolated from the slice of skin that contained dermis, sebaceous glands, and other appendages of the epidermis (Table 6). Approximately 78% of the labeled 7-dehydrocholesterol was associated with the same preparation of tissue. The 7-dehydrocholesterol radioactivity was 17 to 18% of the combined activity in the cholesterol and companions of both tissue preparations.

Formation of Labeled 7-Dehydrocholesterol In Vivo

Most of the activity of the crude Fraction IV sterols of skin was obtained from cellular layers that contained sebaceous tissue (Table 7). An average of 16.5 dpm/cm^2 of C^{14} radioactivity was recovered. Hence, the mean recovery of 12.2 dpm/cm^2 of radioactivity in the sebaceous glands represents approximately 74% of the total radioactivity. The amount of label in sebaceous gland 7-dehydrocholesterol was 26% of the combined activity of the crude cholesterol and companions.

Relative Rates of Formation of Δ^7-Cholestenol, Cholesterol, and 7-Dehydrocholesterol In Vitro

Others suggested that Δ^7-cholestenol may be converted sequentially into 7-dehydrocholesterol and cholesterol by enzymes of liver (10, 26, 27) and intestine (28). Labeled acetate was biosynthetically incorporated into squalene by incubation of slices of epidermis (0.3–0.5 mm in thickness) under nitrogen in a fortified medium (29). (Small amounts of activity were isolated in the sterol fraction, but this amount of activity accounted for no more than 5% of the activity that was in the combined fractions of sterols and squalene.) Following the anaerobic incubation, the slices of epidermis contained an average of 3090 ± 889 dpm/cm^2 of squalene (15). The slices that contained the labeled squalene in situ were washed with cold buffer, transferred to 6 ml of oxygenated buffer, and incubated with 4 μmoles of unlabeled acetate for 2–60 min under oxygen. Labeled squalene was converted to sterols, and Δ^7-cholestenol, cholesterol, and 7-dehydrocholesterol were isolated with the carrier technique.

TABLE 6 INCORPORATION OF LABELED ACETATE INTO 7-DEHYDROCHOLESTEROL IN VITRO

Strips of rat skin were separated into epidermal mucosa and dermis plus sebaceous glands with the keratotome. Tissue from 9 cm^2 of skin was incubated as described previously (8). The labeled sterols were separated into Fractions II and IV. The labeled 7-dehydrocholesterol was removed from Fraction IV with maleic anhydride. Each value is the average of eight samples from four separate incubations.

Tissue	Fraction II A	Fraction IV B	7-Dehydrocholesterol C	C/B × 100
	dpm/cm²	*dpm/cm²*	*dpm/cm²*	%
Epidermal mucosa	153 ± 18*	326 ± 41	54 ± 16	17
Dermis + sebaceous glands	182 ± 19	1050 ± 165	188 ± 46	18

* Standard error of the mean.

C^{14}-Activity accumulated in each of the sterols during the course of the incubation (Table 8). The initial counting rates of Δ^7-cholestenol and 7-dehydrocholesterol were essentially the same. With additional incubation, C^{14} activity accumulated to a greater extent in Δ^7-cholestenol. Because C^{14} activity accumulates with further incubation, a non-integrated expression of incorporation of radioactivity into each of the three sterols may illustrate the relationship more clearly:

$$dpm/cm^2/min = \frac{(dpm/cm^2)_t - (dpm/cm^2)_0}{t \text{ in min}}$$

The rate of incorporation of activity into Δ^7-cholestenol exceeded the rate of incorporation of activity into 7-dehydrocholesterol. The rate of incorporation of activ-

TABLE 7 INCORPORATION OF LABELED ACETATE INTO 7-DEHYDROCHOLESTEROL IN VIVO

One rat was injected with 25 μc of acetate-1-C^{14} and killed after 1 hr.

Tissue Slice	Sebaceous Tissue Present	Activity in Fraction IV: Activity	Activity in Fraction IV: Δ Sebaceous Glands	Activity in 7-Dehydrocholesterol: Activity	Activity in 7-Dehydrocholesterol: Δ Sebaceous Glands	%
		dpm/cm²		*dpm/cm²*		
Epidermis plus sebaceous glands	+	14.0	—	3.9	—	—
Epidermal mucosa	−	1.2	12.8	0.53	3.4	26
Dermis plus sebaceous glands	+	14.7	—	4.1	—	—
Dermis	−	3.2	11.5	0	4.1	28

TABLE 8 RELATIVE RATES OF FORMATION OF Δ^7-CHOLESTENOL, 7-DEHYDROCHOLESTEROL, AND CHOLESTEROL

Time	C^{14}-activity*			Rate of Change†		
	Δ^7	$\Delta^{5,7}$	Δ^5	Δ^7	$\Delta^{5,7}$	Δ^5
	dpm/cm²			*dpm/cm²/min*		
0	52	41	7	—	—	—
2	130	112	13	39	35	3
5	178	134	37	25	19	6
10	295	169	70	24	13	6
30	727	223	101	22	6	3
60	1160	335	167	19	5	3

* Δ^7 = Δ^7-cholesterol. $\Delta^{5,7}$ = 7-dehydrocholesterol. Δ^5 = cholesterol. Each number represents the average of values from eight separate incubations. The statistical variation was similar to that reported earlier (15).

† The rate of change of C^{14} activity was calculated with the formula given in the text.

ity into cholesterol was maximal between 5 and 10 min of incubation.

Recoveries of 86–100% of the activity in the crude Fraction IV were obtained in the 3 sterols. The final amount of radioactivity in 7-dehydrocholesterol was 18% of the activity of Fraction IV. A similar percentage of activity was obtained in experiments involving a single incubation (Table 6). This similarity suggests that the formation of 7-dehydrocholesterol was not affected by the conditions of the anaerobic incubation that preceded the aerobic time-course study.

Human Skin

Duplicate samples of a surgical specimen of human skin were sliced with the keratotome. Epidermal mucosa plus keratin layer contained an average of 0.40 μg/cm² of 7-dehydrocholesterol. Dermis plus sebaceous glands contained an average of 0.57 μg/cm². Little fast-acting sterol other than 7-dehydrocholesterol was detected. The average concentration of cholesterol was 34.3 and 52.7 μg/cm² in the epidermal mucosa and dermis plus sebaceous glands, respectively. The concentration of 7-dehydrocholesterol (1%) agreed with values reported by others (2). The distribution is not in agreement with the data of Wheatley and Reinertson (5), who reported 7-dehydrocholesterol to be present primarily in the Malpighian layer of human skin (epidermal mucosa in the present study). Further work is needed.

DISCUSSION

The results of this study indicate that most of the 7-dehydrocholesterol of rat skin is localized in the sebaceous glands and layer of dead keratin. More than 75% of labeled 7-dehydrocholesterol of skin was associated with slices of skin that contained sebaceous glands and other appendages of the epidermis. The recent report of Brooks et al. (12, 13) and the present results suggest that squalene is formed by cells of the papillary reticulum and 7-dehydrocholesterol may be formed from the squalene by cells of the sebaceous glands. Presumably, the sterol is stored as sebum and translocated onto the skin surface for irradiation (30).

Circumstantial evidence supports this hypothesis. Immature mammals, which have less sebaceous gland secretion than adults, require larger amounts of exogenous vitamin D (4). Indeed, the requirement for vitamin D by normal adults is variable and difficult to establish. Kandutsch and co-workers demonstrated that testosterone stimulates the formation and accumulation of skin sterols in adult, immature, and castrated mice (31). Lorincz (32) compared the relative activity of sebaceous gland secretions and the simultaneous biosynthesis of sterols and squalene by sebaceous glands in children and adults. The importance of the coincidental stimulation of cutaneous 7-dehydrocholesterol formation and the onset of rapid growth at puberty is apparent.

As pointed out previously (15), data from time-course studies only suggest precursor–product relationships. Endogenous sterols are stored in the sebaceous glands and the labeled intermediates probably do not equilibrate with these stored sterols (8). Changes in specific activity may be misleading. The hypothesis that Δ^7-cholestenol may be a precursor of cutaneous cholesterol was supported by recent studies with arsenite inhibition of mevalonate incorporation into Δ^7-cholestenol and cholesterol by slices of epidermis (33). Cholesterol synthesis was inhibited by concentrations of arsenite (0.17 mM) that did not affect the accumulation of radioactivity into Δ^7-cholestenol. The biosynthesis of the latter was not depressed until the concentration of arsenite was increased to 1.7 mM. These data suggest that perhaps one of the two steps in this conversion proposed by Dempsey et al. (27) may be sensitive to arsenite. Kandutsch observed that the microsomal 7-dehydrocholesterol Δ^7-reductase of mouse liver is inhibited by chloromercuribenzoate and the inhibition was reversed by reduced glutathione (34).

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REFERENCES

1. Windaus, A., and F. Bock. *Z. Physiol. Chem.* **245:** 168, 1937.
2. Miller, W. L., Jr., and C. A. Baumann. *Proc. Soc. Exptl. Biol. Med.* **85:** 561, 1954.

3. Bills, C. E. *J. Am. Med. Assoc.* **110:** 2150, 1938.
4. C. E. Bills in *The Vitamins*, edited by W. H. Sebrell, Jr., and R. S. Harris. Academic Press, Inc., New York, 1954, Vol. 2, p. 162.
5. Wheatley, V. R., and R. P. Reinertson. *J. Invest. Dermatol.* **31:** 51, 1958.
6. Wells, W. W., and C. A. Baumann. *Arch. Biochem. Biophys.* **53:** 471, 1954.
7. Brooks, S. C., Jr., J. J. Lalich, and C. A. Baumann. *Am. J. Pathol.* **32:** 1205, 1956.
8. Brooks, S. C., and C. A. Baumann. *J. Biol. Chem.* **229:** 329, 1957.
9. Schenck, F., K. Buchholz, and O. Wiese. *Chem. Ber.* **69:** 2696, 1936.
10. Kandutsch, A. A., and A. E. Russell. *J. Biol. Chem.* **235:** 2256, 1960.
11. Gaylor, J. L., and C. A. Baumann. *Federation Proc.* **18:** 233, 1959.
12. Brooks, S. C., V. C. Godefroi, and W. L. Simpson. *J. Invest. Dermatol.* **40:** 305, 1963.
13. Brooks, S. C., V. C. Godefroi, and W. L. Simpson. *Federation Proc.* **22:** 589, 1963.
14. Blank, H., E. W. Rosenberg, and I. Sarkany. *J. Invest. Dermatol.* **36:** 303, 1961.
15. Gaylor, J. L. *J. Biol. Chem.* **238:** 1643, 1963.
16. Gaylor, J. L., R. W. F. Hardy, and C. A. Baumann. *J. Nutr.* **70:** 293, 1960.
17. Morton, R. A., and A. L. Stubbs. *Analyst* **71:** 348, 1946.
18. Moore, P. R., and C. A. Baumann. *J. Biol. Chem.* **195:** 615, 1952.
19. Clayton, R. B., A. N. Nelson, and I. D. Frantz, Jr. *J. Lipid Res.* **4:** 166, 1963.
20. Baumberger, J. P., V. Suntzeff, and E. V. Cowdry. *J. Nat. Cancer Inst.* **2:** 413, 1941–42.
21. Wells, W. W., and C. L. Lorah. *J. Biol. Chem.* **235:** 978, 1960.
22. Frantz, I. D., Jr., A. T. Sanghvi, and R. B. Clayton. *J. Biol. Chem.* **237:** 3381, 1962.
23. Idler, D. R., and C. A. Baumann. *J. Biol. Chem.* **195:** 623, 1952.
24. Schneider, P. B., R. B. Clayton, and K. Bloch. *J. Biol. Chem.* **224:** 175, 1957.
25. Gaylor, J. L. *J. Biol. Chem.* **238:** 1649, 1963.
26. Schropfer, G. J., Jr., and I. D. Frantz, Jr. *J. Biol. Chem.* **236:** 3137, 1961.
27. Dempsey, M. E., J. D. Seaton, and R. W. Trockman. *Federation Proc.* **22:** 529, 1963.
28. Mercer, E. I., and J. Glover. *Biochem. J.* **80:** 552, 1961.
29. Gaylor, J. L. *Proc. Soc. Exptl. Biol. Med.* **106:** 576, 1961.
30. Gaylor, J. L. *N. Y. State J. Med.* **62:** 251, 1962.
31. Kandutsch, A. A., E. D. Murphy, and M. E. Dreisbach. *Arch. Biochem. Biophys.* **61:** 450, 1956.
32. Lorincz, A. L. In *Conference on the Biology of Cutaneous Cancer*, F. Urbach, National Cancer Institute Monograph No. 10. U. S. Public Health Service, Bethesda, Md., 1963, p. 45.
33. Gaylor, J. L. *Arch. Biochem. Biophys.* **101:** 409, 1963.
34. Kandutsch, A. A. *J. Biol. Chem.* **237:** 358, 1962.