though in practice the two may be indistinguishable over a considerable M_0 range (see Fig. 3).

In Fig. 3 a value of K was assumed but $1/v$ was computed from the S_t (see Table II) obtained using this K value and the actual V_m and K_s of the enzyme used experimentally. It can be seen that the experimental curve (Fig. 2) probably contains only one or two points on the truly linear part of the curve. The apparent linearity over most of the concentration range is due to the very slight concavity of the deviation region (compare with Fig. 3). At very low M_0 the inhibited rate was not sufficiently different from the uninhibited rate to allow a precise determination of the curve form in this region and relatively small errors in v would markedly alter the curve. It is likely that such errors were responsible for the up-curving seen in Fig. 2. Table II gives a comparison of approximate and true values of S for two values of S_0. It can be seen from the table that the error in S_a decreases markedly as S_0 becomes less than K and a corresponding reduction in the amount of deviation in the $1/v$ *vs.* M_0 curves is apparent from Fig. 3.

II. The Dopa-TAT Reaction.—The theory of inhibition discussed in the last section has been based upon the assumption that a dissociable complex was formed between dopa and TAT. It was not possible to obtain detailed information regarding the reaction product, but an estimate of the ratio in which the components occurred was made by means of the method of continuous variation.[22] These experiments (see Fig. 4) showed that the dominant complex species was a 1:1 complex. If hydrolysis was not appreciable and polymerization did not occur to any great extent, it would be reasonable to assume that this complex formation came about through the replacement of a molecule of triethanolamine by one of dopa. Triethanolamine had a negligible effect and Ti^{IV} from the lactate was a weak inhibitor, but an approximately equimolar mixture of these reagents was synergistic, giving considerably greater inhibition than either alone. This strongly suggested that in some manner prior complexing with triethanolamine facilitated formation of a dopa complex (Table III) or contributed to its stability.

(22) For a general discussion see A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 28-34.

TABLE III

Substrate in all cases 5×10^{-3} *M* dopa. Concentrations are final concn. in reaction vessels.

Inhibitor	% Inhibition (total uptake)
2.5×10^{-3} *M* Triethanolamine	2.04
2.0×10^{-3} *M* Titanium lactate	29.20
Triethanolamine 2.5×10^{-3} *M* plus Ti-lactate, 2.0×10^{-3} *M*	53.50

While no proof of the nature of the dopa-TAT product can be offered, the nature of the inhibition kinetics and the experiments above lead to a tentative conclusion that a 1:1 complex was formed.

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MEMPHIS, TENN.

[CONTRIBUTION FROM THE DEPARTMENTS OF MEDICINE (SECTIONS OF DERMATOLOGY AND CARDIOLOGY) AND BIOCHEMISTRY, UNIVERSITY OF CHICAGO]

Studies of the *in vitro* Lipid Metabolism of the Human Skin. I. Biosyntheses in Scalp Skin[1]

BY N. NICOLAIDES, OSCAR K. REISS AND ROBERT G. LANGDON

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The incorporation of isotopic carbon from 1-C^{14} acetate into the lipids by human scalp skin slices was studied. Acetate was incorporated into the fatty acids, squalene, sterols and probably the wax alcohols. Of the total activity, 40% was incorporated into squalene while only 2.6% was found in the sterols. Squalene has been recently implicated as a sterol precursor. The high squalene content of human scalp skin made direct isolation of this hydrocarbon possible. Its specific activity was found to be 10 times higher than that of the sterols, an observation which supports the above implication.

Introduction

The composition of the lipids of human surface fat has been receiving increasing attention recently.[2] Important constituents shown to be present are free fatty acids (25-35%), esterified acids (28-40%), squalene (8-13%), wax alcohols (9-12%) and cholesterol (2-4%).[3] In order to investigate the biosynthesis of these lipids, a study of the incorporation of radioactivity from labeled acetate into the lipids of skin slices was undertaken.

In rat skin, the *in vitro* synthesis of cholesterol from acetate has been demonstrated.[4] The experiments to be reported here show that human skin can also synthesize lipids from 1-C^{14} acetate. These findings are in accord with the established fact that acetate is a precursor of fatty acids, cholesterol and squalene.[5,6] The amount of isotope incorporated into the squalene of skin lipids, however, is considerably greater than that found for other tissues.

While this work was in progress two publications have appeared in which was reported the specific

(1) This investigation was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-411.

(2) S. Rothman, "Physiology and Biochemistry of the Skin," University of Chicago Press, Chicago, 1954, Chapter 13.

(3) R. E. Bloom, S. Woods and N. Nicolaides, *J. Invest. Dermatol.*, in press.

(4) P. A. Srere, J. L. Chaikoff, S. S. Treitman and L. S. Burstein, *J. Biol. Chem.*, **182**, 629 (1950).

(5) R. G. Langdon and K. Bloch, *ibid.*, **200**, 129 (1953).

(6) R. G. Langdon and K. Bloch, *ibid.*, **200**, 135 (1953).

activity of squalene formed in systems synthesizing cholesterol from 1-C^{14} acetate. Popják,[7] on one hand, concluded from a study of the incubation of rat liver slices that squalene *per se* can be excluded as an intermediate in the conversion of acetate to cholesterol. Schwenk, *et al.*,[8] on the other hand, isolated squalene from pig liver perfused with 1-C^{14} acetate, and found a much higher specific activity in the squalene than in the cholesterol. In the work reported here, the specific activity of the squalene isolated from human skin was more than 10 times that of the sterols isolated from the same incubate, thus indicating that squalene could be an intermediate on the direct pathway of cholesterol synthesis.

Experimental

Preparation of Sample and Incubation.—Normal skin was obtained from the scalp of a woman, age 53, who had a benign tumor removed. For this experiment the uninvolved skin surrounding the tumor was used. After the subcutaneous fat was carefully removed, the skin was cut into pieces approximately 1 cm.^{2} and sliced by hand into slices approximately 0.5 mm. thick, parallel to the skin surface. The total duration of time from the removal of the skin from the scalp to the beginning of incubation was 40 minutes at room temperature, and one hour in ice-cold normal saline.

The incubation mixture consisted of 3.0 g. of wet skin slices (the dry weight of the lipid free sample was later found to be 0.3245 g.), 20 mg. of glucose, and 50 micromoles of 1-C^{14} acetate, counting 7×10^5 counts per minute as an infinitely thick sample of $BaCO_3$, in 20 ml. of Krebs-Ringer phosphate buffer, *p*H 7.4. The gas phase was 100% oxygen. The incubation mixture was shaken for 6 hours at 36.8°, and 40 ml. of 95% ethanol was added at the conclusion of the incubation.

Isolation of Lipids from Skin Slices.—The skin tissue lipids were obtained by boiling the skin successively 5 times with 30-ml. portions of absolute alcohol followed by the same treatment with absolute ether. The extracts were pooled, the solvents removed under reduced pressure and the residue was taken to dryness *in vacuo*. The fat was re-extracted from this residue with ether, the remaining solids were acidified with dilute H_2SO_4 and again extracted with ether. All the ether extracts were combined, then washed 8 times with water. The ether was distilled and the lipids taken up in petroleum ether and again washed 5 times with water to ensure the complete removal of un-utilized labeled acetate. There remained 110.2 mg. of an oil which counted about 2000 counts/min./mg., as an infinitely thin sample.

This oil was then divided into 2 parts. For a more accurate determination of the total activity of the major components, 20.6 mg. of the skin tissue lipids was diluted with 1.017 g. of scalp surface fat (expt. I). The pooled scalp surface fat from 3 female adults was obtained by soaking the scalp in ether in a manner to be described elsewhere.[3] For an accurate specific activity determination, the components of the remaining 89.6 mg. of skin tissue lipids were separated without dilution and their isotopic content determined (expt. II).

Separation of Lipid Constituents.—The free fatty acids were removed from the lipid mixture by shaking its ether solution (34 ml.) with 1% aqueous KOH in 6 successive 20-ml. extractions, counter washing each phase with ether and the free fatty acids recovered in the usual manner.[9] The neutral fraction was refluxed under nitrogen for 2 hours with 14 ml. of 10% KOH in 95% ethanol. The esterified acids and the unsaponifiable matter were recovered by standard procedures. Resaponification of the unsaponifiable matter yielded a negligible amount of acid.

Chromatography of the Unsaponifiable Matter.—The unsaponifiable matter from expts. I and II was chromatographed separately on alumina (Merck, reagent grade, "Suitable for Chromatographic Adsorption"), which was found to be Grade III on the Brockman Scale.[10] The ratio of alumina to unsaponifiable matter was 40:1. Eluates of petroleum ether (b.p. 30–60°), benzene, anhydrous ether and methanol were collected, until further elution with each of the solvents yielded negligible quantities and/or radioactivities. The benzene eluate contained chiefly wax alcohols, the ether eluate chiefly sterols and the methanol eluate unidentified materials.

The petroleum ether eluates from the alumina chromatographs, which contained a considerable amount of isotope, were rechromatographed on a dry packed column of silica gel (Davison, 100–200 mesh). The ratio of silica gel to lipid was about 100:1. The silica gel column was eluted successively with petroleum ether, anhydrous ether and methanol. An appreciable weight of residue (saturated hydrocarbon) was recovered in the petroleum ether eluates from the silica gel chromatographs from both experiments, but these fractions were not radioactive. All of the radioactivity was concentrated in the ether eluate which normally removes squalene from the column. This squalene fraction was rechromatographed on alumina to remove a tiny amount of brown coloring matter that forms after silica gel chromatography. A clear water white oil was thus recovered.

Squalene.—The squalene fraction obtained by the above procedure for the lipids of expt. I was compared to a sample of squalene derived from shark liver oil. This shark liver oil squalene was purified by chromatographing technical squalene (Distillation Products) on alumina, and distilling the petroleum ether eluate *in vacuo*. After discarding a liberal forerun and residue, the middle third of the distillation was redistilled, and the middle third of this second distillation (b.p. 183–185° (0.2 mm.)) was chromatographed through silica gel, then alumina. This final material was used for comparison with skin squalene.

The iodine value[11] of skin squalene (an average of 3 values) was 473 ± 5, while that of shark liver squalene (an average of 4 values) was 474 ± 3.[12] Both samples gave identical infrared spectra and the same index of refraction, $n^{25}D$ 1.4941. They also gave identical optical densities when examined by the modified Liebermann–Burchard reaction of Sobel.[13] And finally, the molecules from both sources had the same length when this length was measured in the thiourea adduct crystal lattice,[14] indicating that both samples had the same *cis–trans* configuration.[15]

After the purification processes, the total squalene content of the incubate was estimated to be a minimum of 1.60 mg. For counting purposes an aliquot of the skin tissue squalene (expt. II) was diluted 96.8-fold with shark liver squalene (obtained as described above), which was further purified by recrystallizing 3 times from acetone at −55°. Squalene can be crystallized as a flocculent precipitate at temperatures below −49°, which is its approximate melting point.

From an aliquot of the squalene samples obtained from both experiments, the hexahydrochloride[16] was prepared, washed with cold acetone, recrystallized from acetone to yield a product melting at 128°. The specific activities of the squalene and its hexahydrochloride were determined as described below. No attempt was made to separate the higher and lower melting isomers because these have been found[5,8] to have the same specific activity.

Sterols.—65% of the ether eluate from the alumina chromatograph of expt. II was precipitable with digitonin. This precipitate, when subjected to the Liebermann–Burchard

(7) G. Popják, *Arch. Biochem. Biophys.*, **48**, 102 (1954).

(8) E. Schwenk, D. Todd and C. A. Fish, *Arch. Biochem. Biophys.*, **49**, 187 (1954).

(9) By employing the same procedure on pure tripalmitin it was found that approximately 1-2% was hydrolyzed, thus the free fatty acid fraction could contain a small amount of acids which were originally esterified.

(10) L. Zechmeister, "Progress in Chromatography," Chapman and Hall, London, 1950, p. 27.

(11) G. H. Benham and L. Klee, *J. Am. Oil Chemists' Soc.*, **27**, 127 (1950).

(12) It should be noted that the iodine value for squalene is abnormally high (the theoretical value being only 370.8), indicating that appreciable substitution was occurring. Bromination with Br_2 in CCl_4 also evolved HBr fumes. The high iodine value does not, of course, alter the argument that the two samples of squalene are similar.

(13) H. Sobel, *J. Invest. Dermatol.*, **13**, 333 (1949).

(14) N. Nicolaides and F. Laves, This Journal, **76**, 2596 (1954).

(15) The similar chemical and physical properties of the two samples of squalene derived from different sources does not constitute conclusive proof of their purity.

(16) I. M. Heilbron, E. D. Kamm and W. M. Owens, *J. Chem. Soc.*, 1630 (1926).

color reaction,[17] gave no color at 90 seconds. At 33 minutes the color intensity indicated a maximum cholesterol content of 66%.[18] In expt. I, where the tissue lipids were diluted with surface fat, 4.0% of the digitonin precipitable sterols gave a color at 90 seconds, while the color intensity at 33 minutes indicated a maximum cholesterol content of 96%.

Measurement of Radioactivity.—All samples were converted to CO_2 in a micro-combustion furnace and the gas trapped as $BaCO_3$. The $BaCO_3$ was collected on aluminum planchets of 3.47 cm.^{2}, and counted in a gas flow counter for a sufficiently long time to reduce the statistical error of counting to less than 3%. All specific activities were corrected to infinite thickness by the correction curve of Reid, *et al.*[19]

Results and Discussion

The major component of the lipids from expt. I (scalp skin tissue lipids diluted with scalp surface lipids) and their isotope concentrations are given in Table I. While it is true that the diluting lipids are not of the same percentage composition as that of the original skin tissue lipids, the same components were present in both samples and one could thus determine the total activity incorporated into the main constituents. The fact that the lipids are radioactive shows, of course, that acetate is utilized in their syntheses. Of the total isotope available from the acetate, 12.2% is incorporated into the total skin lipids, which is roughly 3 to 6 times that reported for rat liver slices.[20]

TABLE I

BIOSYNTHESIS OF LIPIDS FROM SLICED HUMAN SCALP SKIN[a] (EXPERIMENT I)

Incubation medium: 0.05 m*M* of $CH_3C^{14}OOK$, specific activity 7×10^5 c. p. m.

	Weight, mg.	Specific activity[b]	Total activity[c]	Isotope recovery, %
Total lipids	1003.0	23	1600[d]	100.0
Free fatty acids	256.7	4	62[e]	3.9
Esterified acids	429.2	28	765[e]	47.8
Sterols	29.6	20[f]	41	2.6
Squalene	107.7	80	634	39.6
Squalene hexahydrochloride	....	87	...	...
Recovery, %	82.1	..	...	93.9

[a] 18.7% of the total lipids isolated was diluted 50.4-fold with scalp surface fat. [b] Counts per minute of an infinitely thick $BaCO_3$ sample. [c] Total activity = millimoles $C \times$ specific activity of $BaCO_3$. [d] Millimoles *C* estimated from amount conversion of total lipids to $BaCO_3$. [e] Millimoles *C* computed from determined neutralization equivalent of 277. [f] Isolated as sterol digitonide and corrected for non-radioactive carbon dilution.

In the lipid components, half of the activity resides in the total acids and approximately half in the unsaponifiable matter. It should be noted that the distribution of radioactivity between the free and esterified acids of expt. I does not parallel the weight distribution. While the free fatty acids comprise 38% of the total weight of free and combined acids, they contain less than 8% of the total isotope found in these acids. In expt. II where the undiluted scalp tissue lipids were worked up, only about 1% by weight of fatty acids were isolated and even these could have originated from the hydrolysis of esters (see experimental section footnote 9).

Sterols precipitated with digitonin account for 2.6% of the total isotope incorporation into the skin lipids whereas squalene accounts for 40%. While the total amount of radioactivity incorporated into the lipids of human skin is larger than that of rat liver, the latter incorporates 10 to 20 times as much isotope into the sterols as does the human skin.

Human skin incorporates 4.8% of the medium acetate into squalene. Some basis for comparison of squalene synthesis in human skin slices and rat liver slices may be obtained by examining the rate of squalene synthesis in both tissues. It has been observed that during short term experiments the acetate to squalene conversion proceeds in rat liver slices at a rate of 0.04–0.06 micromoles/g. tissue/hour during the first hour and decreases rapidly after that.[21] For skin slices however, it can be calculated that the rate of incorporation of acetate into squalene proceeds at an average rate of 0.13 micromoles/g. tissue/hour, assuming linear rate of synthesis during the six-hour incubation period. It is obvious that if skin slices did not carry on their synthetic function at a uniform rate over the entire incubation period their maximum rate of squalene synthesis would be correspondingly higher.

Because of the minute quantities of squalene normally present in rat liver, it has not been possible to isolate squalene directly from that tissue in order to compare its specific activity with that of cholesterol. However, the large squalene content of skin slices together with the high incorporation of acetate into squalene made it feasible to isolate undiluted squalene for the determination of its specific activity. This was done in expt. II. The specific activity of squalene along with the other major components as obtained in expt. II is shown in Table II. Available evidence indicated that squalene is an intermediate in the biosynthesis of cholesterol[5,6] from acetate. However, the specific activity of the squalene synthesized during the conversion of acetate to cholesterol had not previously been directly determined. In this experiment the specific activity of squalene was determined and found to be 10 times higher than that of the sterols. A higher specific activity for squalene is consonant with the requirement for its serving as an intermediate in cholesterol synthesis.

TABLE II

BIOSYNTHESIS OF LIPIDS OF SLICES OF HUMAN SCALP SKIN (EXPERIMENT II)

	Specific activity[a]		Specific activity[a]
Total lipids	1170[b]	Squalene	12000
Esterified acids	700	Squalene hexahydrochloride	12900
Sterols	1070[c]		

[a] Counted as an infinitely thick sample of $BaCO_3$. [b] Computed from the diluted sample in expt. I. [c] Isolated as the digitonide and corrected for non-radioactive carbon dilution.

(17) C. A. Baumann and P. R. Moore, *J. Biol. Chem.*, **195**, 615 (1952).

(18) Because of the small amount of digitonide only 1 set of determinations could be made.

(19) Reid, *et al.*, "Preparation and Measurement of Isotopic Tracers," J. W. Edwards, Ann Arbor, Mich., 1947, pp. 91–93.

(20) The comparison of lipids of sliced human skin with those of rat livers is made because these two organs were studied under identical experimental conditions.

(21) R. G. Langdon, unpublished observations.

When the purity of squalene is examined by comparison of its specific activity with that of its hexahydrochloride, it was found in earlier work that the hexahydrochloride had a higher specific activity which varied considerably from that of the crude squalene from which it was derived.[5] The additional purification employed in these experiments reduces this difference to approximately 8%.

The fact that no color was observed in 90 seconds in the Liebermann–Burchard reaction on the sterol digitonides of human scalp skin tissue indicates that this material contains less than 10% of sterols, that are "fast-acting." Baumann and Moore[17] have found 22 to 36% of fast acting sterols in rat skin tissue lipids. The color developed at 33 minutes for human skin tissue indicates a cholesterol content of 66%. The amount of digitonin precipitable material available was insufficient for a purification of cholesterol *via* its dibromide derivative. However, in order to controvert the argument for the precursor relationship of squalene to cholesterol, less than 10% of the digitonin precipitable matter would have to be cholesterol and the remainder non-radioactive material, assumptions which do not appear likely.

Of the minor components of the human skin tissue lipid unsaponifiable matter, the fraction of the chromatograph corresponding to the wax alcohols contained radioactivity. This suggests that the alcohols are at least in part derived from acetate. This fraction is being investigated further.

In both expts. I and II a small but definite quantity of lipid was found in the petroleum ether eluate of the silica gel chromatograph. In the analysis of human hair fat[22] this material was found to consist of saturated hydrocarbons.[23] Since no radioactivity could be detected in this material, it must be assumed that under the conditions of this experiment, acetate is not utilized as such for the synthesis of their carbon skeletons. Further investigation is needed to decide whether these materials are truly endogenous.

(22) N. Nicolaides and S. Rothman, *J. Invest. Dermatol.*, **21**, 9 (1953).

(23) Schwenk, *et al.*, (ref. 8) also separated non-radioactive hydrocarbons from squalene in their pig liver perfusion experiments.

Chicago, Illinois

[Contribution from the Lilly Research Laboratories]

1,2,4-Triazole-3-alanine

By R. G. Jones and C. Ainsworth

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1,2,4-Triazole-3-alanine has been synthesized and found to be an antagonist of histidine when tested against certain strains of *E. coli*. 1,2,4-Triazole and formalin gave 3-hydroxymethyl-1,2,4-triazole in moderate yield.

A number of α-aminocarboxylic acids related to histidine have been synthesized in this Laboratory and tested for chemotherapeutic activity against bacteria, viruses and cancers. One objective has been to find antagonists of histidine. Of some twenty compounds in which the imidazole nucleus of histidine was replaced by variously 1-substituted imidazoles,[1] thiazoles,[2] pyrazoles[3] and other hetero cycles, only one compound, 2-thiazolealanine,[2] has shown any appreciable activity as a growth inhibitor for *E. coli*. The inhibition was reversed by addition of histidine to the culture medium. This communication reports the synthesis of another amino acid, 1,2,4-triazole-3-alanine (I), which appears to be a specific histidine antagonist when tested against certain strains of *E. coli*.

Compound I was obtained in 70% yield by the reaction of 3-chloromethyl-1,2,4-triazole (II) with the sodium derivative of N-formylaminomalonic ester according to previously published directions for this general method.[4] The chief problem encountered in the synthesis of I was the preparation of the intermediate 3-hydroxymethyl-1,2,4-triazole (III) from which II was obtained by reaction with thionyl chloride.

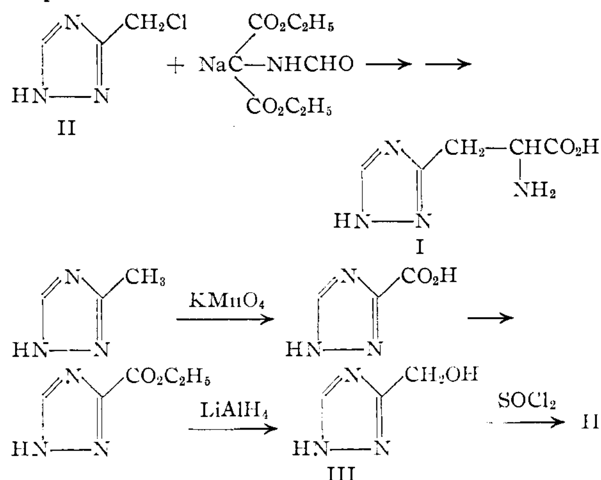
(1) R. G. Jones and K. C. McLaughlin, This Journal, **71**, 2444 (1949).

(2) R. G. Jones, E. C. Kornfeld and K. C. McLaughlin, *ibid.*, **72**, 4526 (1950).

(3) R. G. Jones, *ibid.*, **71**, 3994 (1949).

(4) H. R. Snyder, J. F. Shekleton and C. D. Lewis, *ibid.*, **67**, 310 (1945).

The first method used in obtaining III was a straightforward series of reactions starting from 3-methyl-1,2,4-triazole. The latter was oxidized with permanganate to 1,2,4-triazole-3-carboxylic acid. This was esterified, and the ester was reduced with lithium aluminum hydride. Although reasonably good yields were obtained in each step, this sequence was cumbersome because of the number of steps involved.



A second and more direct synthesis of III was from the readily available 1,2,4-triazole (IV)[5] by

(5) C. Ainsworth and R. G. Jones, *ibid.*, **77**, 621 (1955).