5. Montagna, W., "The Structure and Function of Skin," 2nd ed., Academic Press, N.Y. 1962.
6. Carruthers, C., "Biochemistry of Skin in Health and Disease," Charles C Thomas, Springfield, Ill., 1962.
7. Montagna, W., R. A. Ellis and A. F. Silver, "The Sebaceous Gland," Vol. 4, Advances in the Biology of Skin, MacMillan, New York, 1962.
8. Montagna, W. and W. C. Lobitz, Jr., "The Epidermis," Academic Press, New York, 1964.
9. Lorincz, A. L., and R. B. Stoughton, Physiol. Rev. *38*, 481–502 (1958).
10. Lorincz, A. L., in "The Human Integument," Ed. S. Rothman, Am. Assoc. for the Advancement of Science, Washington, D.C. 1959, pp. 127–150.
11. Reinertson, R. P., adn V. R. Wheatley, J. Invest. Derm. *32*, 49–59 (1959).
12. VanScott, E. J., J. Invest. Derm. *18*, 377–379 (1952).
13. Nicolaides, N., and S. Rothman, J. Invest. Derm. *24*, 125–129 (1955).
14. Herrmann, F., and P. H. Prose, J. Invest. Derm. *16*, 217–230 (1951).
15. Jones, K. K., M. C. Spencer and S. A. Sanchez, J. Invest. Derm. *17*, 213–226 (1951).
16. Kligman, A. M., and W. B. Shelley, J. Invest. Derm. *30*, 99–126 (1958).
17. Bloom, R. E., S. Woods and N. Nicolaides, J. Invest. Derm. *24*, 97–101 (1955).
18. Nicolaides, N., and R. C. Foster, Jr., JAOCS *33*, 404–409 (1956).
19. Linton, R. G., D. H. Curnow and W. J. Riley, Brit. J. Ophthal. *45*, 718–723 (1961).
20. Moore, P. R., and C. A. Baumann, J. Biol. Chem. *195*, 615–621 (1952).
21. Weitzel, G., A. M. Fretzdorff and J. Wojahn, Z. f. Physiol. Chem. *291*, 46–57 (1952).
22. Murray, K. E., Aust. J. Chem. *15*, 510–520 (1962).
23. Odham, G., Arkiv f. Kemi *21*, 379–393 (1963).
24. Downing, D. T., Aust. J. Chem. *16*, 679–682 (1963).
25. Nikkari, T., and E. Haahti, Acta Chem. Scand. *18*, 671–680 (1964).
26. Haldi, J., G. Gidding and W. Wynn, Am. J. Physiol. *135*, 392–397 (1941).
27. Carruthers, C., and B Davis, Cancer Res. *21*, 82–85 (1961).
28. Haahti, E., Scand. J. Clin. & Lab. Invest. *13*, Suppl. 59, 1–108 (1961).
29. Wheatley, V. R., Am. Perfumer *68*, 37–41 (1956).
30. Nicolaides, N., and G. Wells, J. Invest. Derm. *29*, 423–433 (1957).
31. Nicolaides, N., Chap. XI *in* "The Sebaceous Gland," Vol. 4, Advances in Biology of Skin. Ed. W. Montagna, R. A. Ellis and A. F. Silver, MacMillan, New York 1963, pp. 167–187.
32. Zehender, F., Helv. Chim. Acta *29*, 973–979 (1946).
33. MacKenna, R. M. B., V. R. Wheatley and A. Wormall, J. Invest. Derm. *15*, 33–47 (1950).
34. Rothman, S., *in* "Physiology and Biochemistry of the Skin," The University of Chicago Press, Chicago, Ill., 1954, p. 329.
35. Sobel, H., J. Invest. Derm. *13*, 333–338 (1949).
36. Nicolaides, N., and F. Laves, JAOCS *40*, 400–413 (1963).
37. Haahti, E., and E. C. Horning, Acta Chem. Scand. *15*, 930–931 (1961).
38. Nicolaides, N., J. Invest. Derm. *37*, 507–510 (1961).
39. Haahti, E., T. Nikkari and K. Juva, Acta Chem. Scand. *17*, 538–540 (1963).
40. Haahti, E., and T. Nikkari, Acta Chem. Scand. *17*, 536–538 (1963).
41. Nicolaides, N., JAOCS *42*, 708–712 (1965).
42. Boughton, B., and V. R. Wheatley, Biochem. J. *73*, 144–149 (1959).
43. Dubovii, M. I., Ukr. Biochem. J. *26*, 279–287 (1954).
44. MacKenna, R. M. B., V. R. Wheatley and A. Wormall, Biochem. J. *52*, 161–168 (1952).
45. Festenstein, G. N., and R. A. Morton, Biochem. J. *52*, 168–177 (1952).
46. Kuwata, T., and M. Katuno, J. Soc. Chem. Ind. Japan (Suppl.) *41*, 227–228 (1938).
47. Horn, D. H. S., and F. W. Houghen, J. Chem. Soc. 3533–3538 (1953).
48. Suskind, R. R., J. Invest. Derm. *17*, 37–54 (1951).
49. Kooyman, D. J., Arch. Derm. Syph. *25*, 444–450 (1932).
50. Nicolaides, N., Chap. XXVI *in* "The Epidermis", Ed. W. Montagna and W. C. Lobitz, Jr., Academic Press, New York 1964, pp. 511–538.
51. Glasenapp, I. and G. Leonhardi, Arch. f. Dermat. u. Syph. *196*, 148–154 (1953).
52. Nicolaides, N., R. P. Burns and T. Hansen (in preparation).
53. Wheatley, V., Biochem. J. *58*, 167–172 (1954).
54. Cmelic, S., N. Petrak-Longhino and F. Mihelic, Biochem. Z. *322*, 355–359 (1952).
55. Haahti, E., T. Nikkari, A. M. Salmi and A. L. Laaksonen, Scand. J. Clin. Lab. Invest. *13*, 70–3 (1961).
56. Hou, H. C., Chinese J. Phys. *2*, 345–380 (1928).
57. Kandutsch, A. A., Chap. XXV *in* "The Epidermis", Edit. W. Montagna and W. C. Lobitz, Jr., Academic Press, New York, 1964, pp. 493–510.
58. Wheatley, V. R., and A. T. James, Biochem. J. *65*, 36–42 (1957).
59. Freinkel, R., Chap. VIII *in* "The Sebaceous Gland," Vol. 4, Advances in the Biology of Skin, Edit. W. Montagna, R. A. Ellis and A. F. Silver, MacMillan, New York, 1962, pp. 125–134.
60. Freinkel, R., and R. A. Aadalen, J. Invest. Derm. *42*, 325–331 (1964).
61. Wilson, J., Chap. X *in* "The Sebaceous Gland," Vol. 4, Advances in the Biology of Skin, Ed. W. Montagna, R. A. Ellis and A. F. Silver, MacMillan, New York, 1962, pp. 148–166.
62. Burgess, T., and J. D. Wilson, Proc. Soc. Exp. Biol. Med. *113*, 747–750 (1963).
63. Patterson, J. F., *Ibid.* *105*, 461–463 (1960).
64. Carruthers, C., and A. Heining, Cancer Res. *24*, 1008–1011 (1964).
65. Rouser, G., A. J. Bauman, N. Nicolaides and D. Heller, JAOCS *38*, 565–581 (1961).
66. Tiedt, J., and E. V. Truter, Chem. Ind. 403 (1952).
67. Dawson, R. M. S., Biochem. J. *75*, 45–53 (1960).
68. Bloomstrand, R., S. Fregert and F. Nakayama, Acta Derm.-Vener. *41*, 281–288 (1961).
69. Gerstein, W., J. Invest. Derm. *40*, 105–109 (1963).
70. Carruthers, C., Proc. Soc. Exp. Biol. Med. *115*, 215–218 (1964).
71. Flesch, P., and E. C. J. Esoda, J. Invest. Derm. *39*, 409–415 (1962).
72. Schwarz, H. P., L. Dreisbach, R. Stambough, A. Kleschick and M. Barrinuevo, Arch. Biochem. Biophys. *87*, 171–178 (1960).
73. Carruthers, C., and A. Heining, Cancer Res. *24*, 485–488 (1964).

Skin Lipids. III. Fatty Chains in Skin Lipids. The Use of *Vernix Caseosa* to Differentiate Between Endogenous and Exogenous Components in Human Skin Surface Lipid

N. NICOLAIDES and THOMAS RAY, Department of Biochemistry and the Division of Dermatology, University of Oregon Medical School, Portland, Oregon

Abstract

The literature on the types of fatty chains that occur in skin lipids is reviewed and new data are presented.

To ascertain whether certain unusual fatty acids found in human skin surface lipids are truly products of the human skin or are due to some type of external contamination (possibly bacterial), the fatty acids of *vernix caseosa*, (the lipoidal material covering the human fetus) were analyzed and compared to those found in human skin surface lipid. The same unusual fatty acids were found in *vernix caseosa*. This indicates that these acids are products of human skin.

These acids consist of five classes of saturated branched chain acids and of three classes of monoenes: straight chain, *iso* and *anteiso*. All the monoenes are Δ^6 or derivable from this position by addition of an integral number of 2 carbon units to the carboxyl group. On the polar phase diethylene glycol succinate polyester, the saturated branched chain methyl esters have fractional carbon numbers (by gas chromatography) of 0.15, 0.23, 0.45, 0.63 and 0.75. The series at 0.63 and 0.75 are *iso* and *anteiso*, respectively. The series at 0.15 and 0.23 appear to be two newly identified classes of branched chain fatty acids.

I. Types of Fatty Chains that Occur in Skin Lipids

The unusual nature of the fatty acids present in the skin surface lipids first shown by A. W. Weitkamp in two classic investigations (1,2). In the first (1), he determined the structures of 32 fatty acids that occur in degras, a refined wool grease. He distinguished four homologous series by means of a variety of techniques, especially two novel ones that he de-

veloped, namely, amplified distillation (3) and location of the position of methyl branching on a fatty chain from the melting behavior of binary mixtures (1). The series were 1) normal acids from C_{10} to C_{26} (9 members with an even number of carbon atoms), 2) optically active α-hydroxy fatty acids (2 members: C_{14} and C_{16}), 3) *iso* acids, i.e. acids with the terminal

$$\begin{array}{l} \quad CH_3 \\ \quad | \\ CH_3{-}CH{-}CH_2\text{- - -} \end{array}$$

group, C_{10} to C_{26} (10 members with an even number of carbon atoms) and 4) dextro rotary *anteiso* acids, i.e. acids with the terminal

$$\begin{array}{l} \quad\quad CH_3 \\ \quad\quad | \\ CH_3{-}CH_2{-}CH{-}CH_2\text{- - -} \end{array}$$

group, C_9 to C_{27} and C_{31} (11 members with an odd number of carbon atoms).

At a time when it was generally thought that only normal fatty acids with an even number of carbon atoms occurred in biological material, Weitkamp et al. (2) in another classic paper showed that normal fatty acids with an odd number of carbon atoms are present in appreciable quantities in the unesterified (or free) fatty acids of human hair lipid. The position of the double bond in some of the monoenoic acids was also shown to be between the 6th and 7th carbon atoms from the carboxyl end of the molecule, quite contrary to the usual occurrence between the 9th and 10th carbon atoms of the double bond for most biological monoenoic acids.

Subsequent studies have revealed additional fatty chain structures in skin lipid samples, especially in wool wax. Murray and Schoenfeld (4), for example, isolated and characterized 10 branched chain alcohols in the *iso* and *anteiso* series. Alkane-1,2-diols of the normal and *iso* series were also found (5,6) and studied later by gas-liquid chromatography (7). Their occurrence has also been reported in rodent skin surface lipid (8,9) and possibly in human surface lipid (10). ω-Hydroxy fatty acids also occur in wool wax (11, 7). Even naturally occurring hydrocarbons of the normal, *iso* and *anteiso* series have been found (7,12) and the occurrence of pristane (2,6,10,14-tetramethylpentadecane) has been reported (12). Pristane could have been a contaminant from petroleum products (e.g., East Texas crude oil contains ~5% (13)) for their sample was of commercial origin. Unusual branched chain fatty acids occur in the preen gland lipids of the goose (14,15), i.e. 2,4,6,8-tetramethyldecanoic acid and 2,4,6,8-tetramethylundecanoic acid.

Studies of these fatty components in the skin lipids of animals have been made for the guinea pig, rabbit, mouse and rat (8), for the rat (9,16), for the dog (17), and the mouse (18 and the references therein), and sheep (19).

The structures of the fatty components that occur in human skin surface lipids have received the most attention. The fatty alcohols were early shown to constitute a saturated and an unsaturated series of odd and even carbon chain length (20). Hougen (21) later isolated and determined the structures of 12 alcohols. These belonged to three homologous series, normal C_{14} to C_{24}, saturated *iso* C_{20} to C_{24}, and unsaturated *iso* C_{20} to C_{24}. The position of the double bonds of the unsaturated *iso* alcohols was 10 carbon atoms from the methyl end in each case.

Later, by means of gas chromatographic retention data, James and Wheatley (22) confirmed the presence of fatty acids with chain lengths of an odd number of carbon atoms in the surface fat of the human forearm and presented evidence for the existence of two types of branched chain fatty acids. Haahti made an extensive gas chromatographic study of the fatty acids occurring in various esters and also the fatty alcohols of human surface lipid of the back (23). Still later, Haahti and Horning (24) reported gas chromatographic evidence for the presence of four types of saturated branched chain fatty acids as well as mono-unsaturated single branched fatty acids in human surface lipids of the back. Four types of branched chain alcohols were detectable as well as both straight and branched chain mono-unsaturated alcohols in the region C_{10} to C_{24}. In a recent study on the composition of the free fatty acids of human scalp skin surface fat (25), we confirmed the findings of Weitkamp et al. (2) that the main position of the double bonds of the monoenoic acids is between the 6th and 7th carbon atoms. We also showed that the position of the double bond of other monoenoic acids was derivable from the 6–7 position by the addition or subtraction of an integral number of two carbon units to the carboxyl end of the fatty acid and that this applied to straight as well as to branched fatty acid chains.

II. The Use of Vernix Caseosa to Differentiate Between Endogenous and Exogenous Components in Human Skin Surface Lipid

Since these unusual fatty acids occur on the human skin surface, they could be derived from external sources, especially from resident bacteria. They could also be derived from the diet and be excreted through the sebaceous gland. Of course they could also be synthesized, either in part, i.e. by chain extension of some branched unit obtained from the diet, or *in toto* by some type of skin cell, such as those of the sebaceous gland or those of the keratinizing epidermis.

Vernix caseosa, the fatty substance which covers the skin of the human fetus, consists of desquamated epithelial cells and sebaceous excreta. This material would not contain any exogenous lipid components that might be derived from resident bacteria in adult human skin. Thus, if one finds lipids that are common to the human adult skin surface and *vernix caseosa* it is highly unlikely that such components can be due to bacterial or other types of external contamination of the skin surface.

The fatty acids of *vernix caseosa* have been studied in two independent investigations (26,27). The results from these two studies are somewhat at variance: Downing (26), for example, reported the presence of about 10% α-hydroxy acids and no fatty acids suggesting "multi-branching," whereas Haahti et al. (27) did not report the presence of α-hydroxy fatty acids but did report the presence of fatty acids having carbon numbers believed to be due to multi-branching. Neither of these studies reported on the position of the double bonds in the unsaturated fatty acids.

In the present study we have compared the monoenoic and the branched chain fatty acids of human surface lipid with those of *vernix caseosa.* After considerable pains were taken to avoid contamination, 5 types of branched chain fatty acids present in hu-

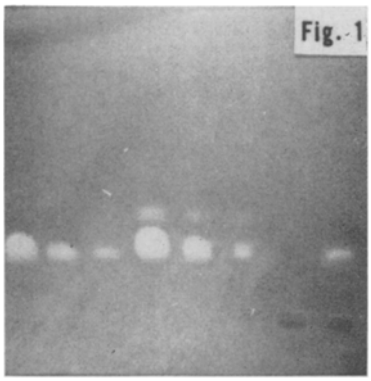

FIG. 1. Thin-layer chromatograms of the saturated non-urea-adducting methyl esters of the free fatty acids from two samples of human surface lipid. Sample 1 was from the pooled lipid from the scalp of an adult male, and sample 2 from hair lipid from pooled cut hair of adult females. In chromatograms a), b) and c), 300, 100 and 30 μg, respectively, of sample 1 was applied at the origin and in chromatograms d), e) and f) the same amounts of sample 2 were applied; g) is 20 μg of 2-hydroxy methyl palmitate and h) is 20 μg methyl palmitate. The thin-layer plates were spread with silica gel plain containing 10% magnesium silicate. The plate was developed with hexane/diethyl ether 95/5, sprayed with Rhodamine 6G and photographed under ultraviolet light. Note that only one spot was obtained for the relatively purer sample 1. For definitive gas chromatography only the material corresponding to the migration with methyl palmitate was scraped off preparative thin layer plates and extracted from the adsorbent. Extraction of the adsorbent from other areas, both above and below the main spots failed to yield any material detectable by gas chromatography under our conditions of operation.

man surface lipid were also found in *vernix caseosa*. For both samples, the double bond positions of 3 classes of monoenoic fatty acids (straight chain, *iso* and *anteiso*) are very similar. The double bond is either Δ^6 or derivable from this position by addition of an integral number of 2 carbon units to the carboxyl group. These results indicate that both groups of fatty acids are products of the human skin and not derived from bacterial or other type of surface contamination. Furthermore, they suggest that these fatty acids are synthesized by the human sebaceous gland.

Procedures

A sample of *vernix caseosa* from a caucasian male was obtained nearly entirely free of blood and stored in a clean stoppered jar until it could be worked up 4 hr later (8.860 g wet weight).[1] It was extracted in 4 batches with 150 ml $CHCl_3/CH_3OH$ 2/1 (v/v) each time and filtered on a medium porosity sintered glass filter. The fourth batch extract contained a negligible amount of lipid. The solvent was removed on a rotary evaporator *in vacuo*, and a yield of 1.0360 g of lipid was obtained. The residue remaining after extraction weighed 0.4412 g.

A 485 mg aliquot of lipid was saponified with 9 ml 10% KOH in ethanol/water 9/1 under reflux in a nitrogen atmosphere for 2 hr. The saponified mixture was diluted with about 15 ml water, the unsaponifiable matter was extracted 4 times with about 15 ml of hexane each time. The pooled hexane washings were washed twice with water. The fatty acid fraction was released from the aqueous alcoholic phase with 6 *N* H_2SO_4 to a pH of 1, then extracted with 4 washes of hexane (15 ml each). The pooled hexane solution of fatty acids was extracted twice with 15 ml 1 *N* KOH in order to free the acids of a small amount (8.8 mg) of unsaponifiable matter which still remained. The fatty acids were released from their potassium salts with 6 *N* H_2SO_4 and taken up again in hexane. The recoveries after solvent removal were 169.4 mg (35%) unsaponifiables and 276 mg (57%) fatty acids; unrecovered 8%. The fatty acids were esterified with BF_3 in MeOH (28).

A 249 mg portion of methyl esters was then chromatographed on a 10 x 2.5 cm column packed with 42 g silicic acid impregnated with $AgNO_3$ in the proportion 3/1 (v/v) (Adsorbosil ADN-1, Applied Science, Inc.), to separate the saturates from the unsaturates. The chromatographic data obtained are as follows:

Tube No. (10 ml each)	Eluent	Wt. mg	
1–7	Hexane	Trace	Discarded (represents the passasge of 2 column volumes of solvent)
8–34	Hexane	113.7	Saturated methyl esters
35–42	Hexane	Trace	
43–59	10% benzene in hexane	Trace	
60–68	10% benzene in hexane	Trace	
69–87	20% benzene in hexane	75.6	Mainly monoenoic methyl esters
88–102	20 %benzene in hexane	9.9	Appreciable amounts of dienoic acids (?)
103–117	20% benzene in hexane	7.6	
118–128	50% benzene in hexane	5.2	
129–151	Benzene	11.5	

Only the saturated (tubes 8–34) and the main monoenoic methyl ester fractions (tubes 69–87) were analyzed by preparative and analytical gas chromatography.

A portion of the saturated methyl ester fraction was hydrogenated to insure that no unsaturates were present. Comparison of the gas chromatograms before and after hydrogenation revealed the disappearance of a few small peaks which indicated that a trace of unsaturated material was present.

An enrichment of the branched chain material present in the saturated fatty acids was accomplished by urea adduct formation as follows: First a saturated solution of recrystallized urea (Baker's Analyzed Reagent) in methanol was prepared. An aliquot of 82.7 mg of the saturated, hydrogenated methyl esters was dissolved in 1 ml petroleum ether (bp 30–60C) in a glass stoppered tube. Then 2 ml of the urea-saturated methanol solution was added and adduct crystals formed immediately. The tube was stoppered and vigorously shaken. The petroleum ether layer (containing the branched chain material) was carefully pipetted off and the adduct crystals and methanol solution washed two more times with 1 ml portions of petroleum ether. The solvent was blown off with nitrogen and 23 mg (28%) of non-urea-adducting methyl ester was recovered. The urea-adducted compounds yielded 52.2 mg (63%) of methyl esters after decomposition of the adduct with water. Both the urea-adducted and non-urea-adducted methyl esters yielded straight and branched chain methyl esters, but most of the branched chain compounds were concentrated in the nonadducted fraction. The non-urea-adducted methyl ester fraction was further purified by preparative thin-layer chromatography (TLC) and analyzed by gas chromatography. By means of column chromatography on Adsorbosil and the TLC purification any methoxy

[1] In the work up of this material scrupulous care was taken to avoid the possible inclusion of minor amounts of extraneous material in the fatty acid fraction. Thus, all solvents were redistilled, adsorbents washed until negligible residues were obtained, quantitative separations were employed, etc.

derivative which might conceivably arise from the methyl ester preparation (29) should have been eliminated.

A sample of human scalp surface lipid (6.12 g) from a young man was obtained from the pooled yield of scalp soaking every 48 hr (30). The free acids were removed with a 0.06 *N* KOH wash (30) whereupon 2.001 g free fatty acids (33.2%) and 3.920 g neutral fat (65.0%) were recovered. A 970 mg aliquot of the free fatty acids was esterified with methanol (as above), and an aliquot of 772 mg of the methyl esters was made up to 25 ml with petroleum ether, then treated 3 times in succession with 10 ml of a saturated methanolic urea solution (as above) to yield 3 batches of urea-adducting material (224, 197 and 49 mg respectively) and a batch on non-urea-adducting material (245 mg). By this successive extraction of the straight chain material, it was hoped that monomethyl branched material might be separated from the more highly branched material but this procedure did not prove to be practicable. A 95 mg aliquot of the non-urea-adducting material was chromatographed on silicic acid/silver nitrate 3/1 as described above and 29 mg saturated methyl esters was obtained and analyzed by gas chromatography.

A third sample of saturated, non-urea-adducting methyl esters was obtained from the nonesterified fatty acid fraction of a large sample of adult female hair fat obtained from an earlier study (31). The fatty acids were stored in a tightly stoppered flask in a refrigerator. A 7.23 g sample was treated with 170 ml of a urea saturated methanol solution, boiled, then cooled first to room temperature then to 4C where it remained for 1 hr. The crystals were filtered, washed thoroughly 3 times with petroleum ether, dried in air then washed again with petroleum ether. The methanol phase was evaporated to dryness in a stream of nitrogen and the resulting crystals were washed 3 times with petroleum ether. The pooled petroleum ether washings were dried in a stream of nitrogen, and yielded 2.51 g (34.7%) of a non-urea-adducting fraction of fatty acid. This material was then esterified with methanol (as above) and a 238 mg aliquot purified by chromatography on silicic acid (Mallinckrodt, 100 mesh silicic acid, Analytical Reagent Catalog No. 2847). The silicic acid had previously been washed with 6 *N* HCl, then distilled water until neutral then dried in a 3-necked flask at 120C under vacuum and a fine stream of nitrogen. Column dimensions were 10 cm x 2.5 cm. The methyl esters (148 mg) were eluted with 300 ml 50% benzene in hexane, after 80 ml hexane, 120 ml 10% benzene in hexane and 240 ml 25% benzene in hexane were first eluted. These esters were then separated into a saturated (42.4 mg) fraction and an unsaturated (92.4 mg) fraction (as above). The saturated fatty acid fraction was analyzed after preparative gas chromatography.

To check the purity of the saturated methyl ester samples, they were chromatographed by preparative TLC under conditions described in the legends of Figs. 1 and 2. Only the material with an Rf of the standard, methyl palmitate, was collected and gas chromatographed.

Preparative gas chromatography and reductive ozonolysis were performed as already described (25,33). Isothermal gas chromatography was performed on a 20-foot stainless steel column 1/8 in. O.D. packed with 18% diethylene glycol succinate (DEGS) on silanized

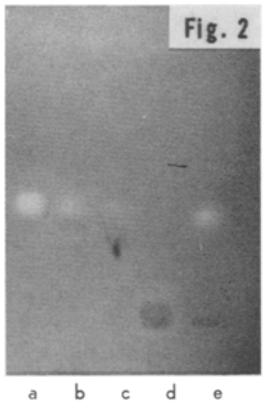

FIG. 2. In a), b) and c) are 300, 100 and 30 μg, respectively, of the saturated non-urea-adducting methyl esters of *vernix caseosa*, and d) and e) the same amounts of the same standards as in Fig. 1. The preparation of the plate and development of the chromatogram are also as in Fig. 1. The dark spot in the lower middle part of the plate is an artifact.

Chromosorb-W 30–60 mesh with helium as the carrier gas (flow rate ~30 ml/min). The gas chromatograph was a Perkin-Elmer Model 800, dual column instrument equipped with a hydrogen flame ionization detector.

Carbon numbers of unknown and standard compounds were obtained by the method of Woodford and van Ghent (34). Standards used have already been described (25). The accuracy of the carbon number determination depended somewhat on the amount of time it took for a peak to emerge from the column. Maximum accuracy was achieved from 5 to 15 minutes, consequently, column temperature was controlled so that the peaks were eluted in this time interval. In many cases internal standards were added to the samples especially to those collected from preparative gas chromatography. Internal standards could be selected to emerge both before and after the unknown peak so as to increase substantially the accuracy of the carbon number value determination. These values, reported in Table I, are generally the average of at least three determinations. A conservative estimate of the accuracy is ±0.05 of a carbon number unit.

Results and Discussion

The fractional carbon numbers for the saturated chain lengths of the non-urea-adducting material, purified as described above, are grouped in Table I. The samples were derived from: a) the free fatty acids of scalp surface lipid from a man; b) the free fatty acids from a pooled sample of hair lipid obtained from women; and c) the total fatty acids from *vernix caseosa*. The values for the two surface lipid samples show excellent agreement with those of *vernix caseosa*. These esters had the same migration on TLC (see Figs. 1 and 2) as did methyl palmitate so it is unlikely that there are additional oxygenated functional groups on the fatty chain. Therefore, there must be 5 series of branched chain fatty acids present in human surface lipid and in lipids of *vernix caseosa*. The average fractional carbon numbers of the methyl esters of these groups of branched chain fatty acids on liquid phase diethylene glycol succinate (DEGS), are, respectively, 0.15, 0.23, 0.45, 0.63 and 0.75.

If branched chain components are not concentrated by urea adduct formation, then large peaks of straight

chain material could mask not only peaks of fractional carbon numbers of 0.15 but even those at 0.23.

Three of these series show members that differ from each other by 2 carbon atoms, i.e. the series at fractional carbon numbers of 0.23, 0.63 and 0.75. This suggests that these series have a common branched chain stem on which successive members are built up by the addition of two carbon units.

Although it had long been suspected from gas chromatographic retention data that fatty acids having the *iso* and *anteiso* structures were present, no proof of structure for these substances has as yet been reported. In unpublished work (35) we have found by mass spectrometry that the esters with carbon numbers of 0.63 and 0.75 on DEGS have the *iso* and *anteiso* structures, respectively. It is noteworthy that in the human the *iso* series has an *even* total number of carbon atoms whereas the *anteiso* has an *odd* number. This agrees with the finding by Weitkamp for sheep (1), although Pelick and Shigley found both even and odd members for both the *iso* and the *anteiso* series (19). Downing also reported (26) that the *vernix caseosa* branched chain fatty acids show slightly lower retention times for the even members than for the odd ones. From this he suggested that the even members are *iso* and the odd ones *anteiso*.

The structures of the esters with the other carbon numbers have not yet been identified. Some carbon number values have been reported by Haahti et al. (27) for *vernix caseosa* fatty acid methyl esters determined on Apiezon L. They report a series (which they believe to be highly branched) 13.16, 14.18, 15.21, 16.23 and 17.28, another about which they do not comment, 12.47, 14.46 and 16.46, a third which they believed to be *iso*, 11.59, 13.59, 14.59, 15.60, 19.60, and a fourth which they believe to be *anteiso*, 12.69, 14.69 and 16.70. In a later study of the lipids of human surface done on 200-foot capillary columns coated with Dow Corning F-60 silicone fluid, Haahti and Horning (24) did not report the presence of material with fractional carbon numbers between 0.1 and 0.2. They did report that the series between 0.4 and 0.5 consists of 2 series of "double branched" material, one of fractional carbon number of 0.45 and another at 0.55.

It is noteworthy that some of the values for carbon numbers reported by Haahti et al. (27) correspond to those found by us even though they used the nonpolar phase Apiezon L while we used the polar phase DEGS. The meaning of this is not clear. It is difficult to conclude much about these minor peaks in their study in view of their own statement that ". . . It might be possible that some of these peaks are caused by higher free alcohols. . . ."

From gas chromatographic retention data alone one cannot tell whether there are one or two methyl branches on a fatty acid chain or indeed if these substances are due to methylated branching at all. They could, for example, be due to the presence of other functional groups on the fatty chain such as keto, epoxy, cyclopropyl, etc., or to other nonmethyl ester contaminants. Even a single methyl branch located in positions on a fatty chain not usually encountered can cause striking changes in carbon numbers. For example, on DEGS the methyl esters of 8-methyloctadecanoic acid, 3-methyloctadecanoic acid and 2-methyloctadecanoic acid (gifts of Dr. James Cason) had carbon numbers of 18.38, 18.24 and 17.97, respectively. The latter could easily be mistaken for methyl stearate whose carbon number is 18.00. These same esters when gas-chromatographed on the nonpolar phase SE-30 had carbon numbers of 18.41, 18.41 and 18.38, respectively.

The branched chain fatty acids make up 15% of the free fatty acid fraction of human surface lipid and an estimated 12% of the entire lipid (see Table I in Ref. 36). About half of the branched chain acids have the *iso* structure and one fourth the *anteiso* structure. The group with fractional carbon numbers of 0.45 is next greatest in amount and the 0.23 and the 0.15 groups make up the remainder.

The main position of unsaturation of the monoenoic fatty acid methyl esters found in *vernix caseosa* are listed in Table II. (No attempt was made to carry the analysis for the *vernix caseosa* lipids above C_{19}). These results correlate well with the positions of unsaturation of the free fatty acid fraction of human surface lipid previously reported (25).

TABLE I

Comparison of the Carbon Numbers of Methyl Esters of Fatty Acids from Skin Surface Lipid with *Vernix Caseosa*[a, b, c]

Straight chain	Branched chain series				
	1	2	3	4	5
A. Saturated non-urea-adducting fraction of the free fatty acids of human scalp skin surface lipid (male)					
				9.66	
			10.50		
11.00			11.50	11.63	
12.00			12.48		12.74
13.00	13.14	13.24	13.48	13.62	
14.00	14.1		14.50		14.76
15.00	15.14		15.49	15.67	
16.00	16.1		16.48		16.72
17.00	17.1	17.24	17.48		
B. Saturated non-urea-adducting "branched chain" fraction of the free fatty acids of human hair lipid (female)					
7.00					
8.00	8.18				
9.00	9.15			9.63	
10.00	10.1		10.49		10.75
11.00	11.15	11.26	11.49	11.61	
12.00	12.13		12.50		12.74
13.00	13.14	13.20	13.47	13.62	
14.00	14.15		14.46		14.74
15.00	15.15	15.20	15.47	15.63	
16.00	16.15		16.50		16.78
17.00	17.1	17.23	17.48	17.63	
18.00	18.15		18.40 and 18.5		18.78
19.00	19.15		19.4	19.64	
20.00	20.15		20.42		20.73
C. Saturated non-urea-adducting "branched chain" fraction of the total fatty acids of *vernix caseosa*					
9.00				9.60	
10.00			10.42		10.73
11.00			11.48	11.72	
12.00	12.13		12.47		12.74
13.00	?	13.25	13.49	13.62	
14.00	14.14		14.50		14.75
15.00	15.15		15.47	15.64	
16.00	16.11		16.47		16.73
17.00	17.1	17.23	17.47	17.67	
18.00			18.46		18.73
19.00				19.68	
20.00					20.76

[a] Carbon numbers computed as in Ref. 34 for methyl esters run on diethylene glycol succinate (DEGS).

[b] Only homologues up to C_{20} were examined. There were good indications that for *vernix caseosa* higher homologues were present as was also found by Downing (26).

[c] Where only one figure is given after the decimal the results are somewhat less accurate, otherwise the values are estimated at ±0.05 carbon number unit.

Further Comments

Since *vernix caseosa* and human surface lipid both contain the same types of unusual fatty acids of Tables I and II, we conclude that these acids are products of human skin and not of bacteria or other external contamination.

The next question is from what part of the skin do these acids originate? The sebaceous gland is a likely source for the monoenoic acids. This is in-

TABLE II

Position Isomers of Largest Amounts at Each Chain Length of the Monoenoic Fatty Acid Methyl Esters for Skin Surface Lipid Compared with Those of *Vernix Caseosa*

Total chain length	Double bond position from the carboxyl group	
	For free acids of human surface lipids[a]	For total acids of *Vernix caseosa*
A. Straight		
C_{13}	4=5; 6=7	6=7
C_{14}	6=7	6=7
C_{15}	6=7	6=7
C_{16}	6=7	6=7
C_{17}	6=7; 8=9	6=7; 8=9
C_{18}	6=7; 8=9; 9=10	6=7; 8=9; 9=10
C_{19}	8=9; 10=11	8=9; 9=10 10=11
C_{20}	8=9; 10=11	
C_{21}	10=11	
C_{22}	12=13	
C_{24}	14=15	
C_{26}	14=15	
B. Branched		
C_{14} iso[a]	4=5	
C_{15} iso	6=7	
C_{16} iso	6=7	6=7
C_{17} iso	6=7	
C_{17} anteiso	6=7	6=7
C_{18} iso	6=7; 8=9;	6=7; 8=9
C_{19} iso	6=7; 8=9;	6=7; 8=9
C_{19} anteiso	6=7; 8=9;	6=7; 8=9
C_{20} iso	8=9;	
C_{22} iso	10=11	
C_{24} iso	12=13; 14=15	
C_{26} iso	14=15	

[a] Taken from reference (25.).

ferred from the fact that they comprise a large fraction of scalp surface lipid, 40% of the free acids and triglycerides, and these substances in turn constitute about 70% of the surface lipid. This lipid sample is well over 90% sebum (32, and Table I in 36). Similar reasoning argues for a sebaceous gland origin for the *iso* and *anteiso* acids.

If these acids originate in the sebaceous gland are they derived from the diet as such and excreted or are they synthesized there *de novo*? Downing has considered these questions for sheep (37) and for man (26). He compared the branched chain fatty acid content of wool lipid and depot fat of newly born lambs with those of the wool lipid and depot fat of the adult sheep and found that for wool lipid it was nearly the same for the lamb as it was for the adult sheep, whereas for lamb depot fat it was 0.2%, but for adult sheep depot fat it was 3%. He also noted the relatively high content of branched fatty acids in *vernix caseosa* (60%) compared to that of surface lipid of the human forearm (12%) as analyzed by James and Wheatley (22) and ". . . in view of the probability of low placental permeability to lipids . . . ," he suggests that these compounds are not likely to be derived directly from the maternal blood but are synthesized in the human sebaceous gland (26).

Although the conclusion may be correct, there are a number of difficulties with the argument. There may be considerable differences in organ specificity. For example, skin may be much more predisposed to select the branched chain fatty acids from the maternal blood than adipose tissue. Furthermore, the whole matter of placental permeability to lipids seems to be poorly understood. In the sheep and the rabbit there is, however, some evidence that labeled palmitic acid gets across the placenta (38).

Wheatley et al. (39) have provided more suggestive evidence that the sebaceous glands synthesize branched chain fatty acids, at least of the *anteiso* type. From perfusion studies of dog skin with uniformly labeled isoleucine, they found considerable activity present in what appears to be an *anteiso* C_{15} acid (36).

ACKNOWLEDGMENT

This work was supported in part by Research Grant No. AM-05120-04 and Training Grant No. 5-TI-AM-5300-04 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service; and U.S. Army Medical Research and Development Command, Department of the Army, Research Contract No. DA-49-139-MD-2184.

Technical assistance given by Miss Kathe Strangfed.

REFERENCES

1. Weitkamp, A. W., J. Am. Chem. Soc. *67*, 447–454 (1945).
2. Weitkamp, A. W., A. M. Smiljanic and S. Rothman, J. Am. Chem. Soc. *69*, 1937–1939 (1947).
3. Weitkamp, A. W., JAOCS *24*, 236–238 (1947).
4. Murray, K. E., and R. Schoenfeld, JAOCS *29*, 416–420 (1952).
5. Horn, D. H. S., and F. W. Hougen, Chem. & Ind. 670 (1951).
6. Horn, D. H. S., and F. W. Hougen, J. Chem. Soc. 3533–3538 (1953).
7. Downing, D. T., Z. H. Krantz and K. E. Murray, Aust. J. Chem. *13*, 80–94 (1960).
8. Wheatley, V. R., and A. T. James, Biochem. J. *65*, 36–42 (1957).
9. Nikkari, T., and E. Haahti, Acta Chem. Scand. *18*, 671–680 (1964).
10. Boughton, B., and V. R. Wheatley, Biochem. J. *73*, 144–149 (1959).
11. Horn, D. H. S., and D. Ilse, S. Afr. Ind. Chem. 309 (1956).
12. Mold, J. D., R. E. Means, R. K. Stevens and J. M. Ruth, Biochem. J. *3*, 1293 (1964).
13. Bendoraitis, J. G., B. L. Brown and L. S. Heppner, Anal. Chem. *34*, 49–53 (1962).
14. Murray, K. E., Aust. J. Chem. *15*, 510–520 (1962).
15. Odham, G., Arkiv f. Kemi *21*, 379–393 (1963).
16. Kishimoto, Y., and N. S. Radin, J. Lipid Res. *4*, 139–143 (1963).
17. Wheatley, V. R., and D. W. Sher, J. Invest. Derm. *36*, 169–170 (1961).
18. Carruthers, C., and A. Heining, Cancer Res. *24*, 1008–1011 (1964).
19. Pelick, N., and J. W. Shigley, A rapid method for identifying the fatty acids of Degras. Presented in the symposium on Composition and Analytical Methodology of the Meeting of the American Oil Chemists' Society, held in New Orleans, La., April 22, 1964.
20. Brown, R. A., W. S. Yound and N. Nicolaides, Anal. Chem. *26*, 1653–1654 (1954).
21. Hougen, F. W., Biochem. J. *59*, 302–309 (1955).
22. James, A. T., and V. R. Wheatley, Biochem. J. *63*, 269–273 (1956).
23. Haahti, E., Scand. J. Clin. Lab. Invest. *13*, Suppl. 59, 1–108 (1961).
24. Haahti, E., and E. C. Horning, Scand. J. Clin. Lab. Invest. *15*, 73–78 (1963).
25. Nicolaides, N., R. E. Kellum and P. V. Woolley III, Arch. Biochem. Biophys. *105*, 634–639 (1964).
26. Downing, D. T., Austr. J. Chem. *16*, 679–682 (1963).
27. Haahti, E., T. Nikkari, A. M. Salmi and A. L. Laaksonen, Scand. J. Clin. Lab. Invest. *13*, 70–73 (1961).
28. Metcalf, L. D., and A. A. Schmitz, Anal. Chem. *33*, 363–364 (1961).
29. Lough, A. K., Biochem. J. *90*, 4C (1964).
30. Nicolaides, N., and R. C. Foster, Jr., JAOCS *33*, 404–409 (1956).
31. Nicolaides, N., and S. Rothman, J. Invest. Derm. *21*, 9–14 (1953).
32. Nicolaides, N., Skin Lipids. II, JAOCS *42*, 697–702 (1965).
33. Stein, R. A., and N. Nicolaides, J. Lipid Res. *3*, 476–478 (1962).
34. Woodford, F. P., and C. M. van Ghent, J. Lipid Res. *1*, 188–190 (1960).
35. Unpublished work in collaboration with E. A. Day and L. M. Libby.
36. Nicolaides, N., Skin Lipids, IV, JAOCS *42*, 708–712 (1965).
37. Downing, D. T., J. Lipid Res. *5*, 210–215 (1964).
38. Van Dyne, C., and R. Havel, Clin. Res. *8*, 111 (1960).
39. Wheatley, V. R., D. C. Chow and F. D. Keenan, Jr., J. Invest. Derm. *36*, 237–239 (1961).