ON THE BIOGENESIS OF THE FREE FATTY ACIDS IN HUMAN SKIN SURFACE FAT *

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One of the most conspicuous features of human skin surface fat is the presence of a large amount of free fatty acids (20–35 %). Their biogenesis may be of some dermatological importance.

Past work on the analysis of human skin surface fat (1) as well as on the biosynthesis of the lipids of human skin (2), points to the possibility that the free fatty acids may be the result of a secondary process, namely, the hydrolytic splitting of triglycerides. In this paper chemical and histochemical evidence will be presented which supports and amplifies the view, that in human skin there exists a lipase located at a site where freshly excreted sebum could be lipolyzed. A likely site is the sebaceous gland duct; but the hair follicle or the skin surface itself may also be involved.

COMPOSITION OF SEBUM WHICH IS NOT EMPTIED THROUGH DUCTS AND FOLLICLES ONTO THE SURFACE

Lipid material, which was not exposed to sebaceous gland ducts or skin surface, was obtained from cysts of a young woman who had multiple steatocystoma. Six excisions of cysts containing clear odorless oily material (resembling olive oil) were made.§ Immediately after collection of oil from one cyst histological examination was made and photomicrographs taken. Figure 1 shows the collapsed cyst with its epithelial lining and the intact sebaceous gland opening into the deepest part of the cyst. On serial section no connection with skin surface could be seen. Some of the cysts contained minute free lying hairs.

The composition of the cyst lipids was compared with that of lipids from the skin surface collected at various time intervals. This surface fat was obtained from another subject by a scalp soaking procedure in a manner previously described (1). The method, which involves dipping the scalp in ether 3 times in succession, removes approximately 90 % of the ether extractable lipids on the surface of the skin. Thus fat, which had remained on the skin for a pre-

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§ This patient had older inspissated cysts with cheesy or chalky content, but these were not examined.

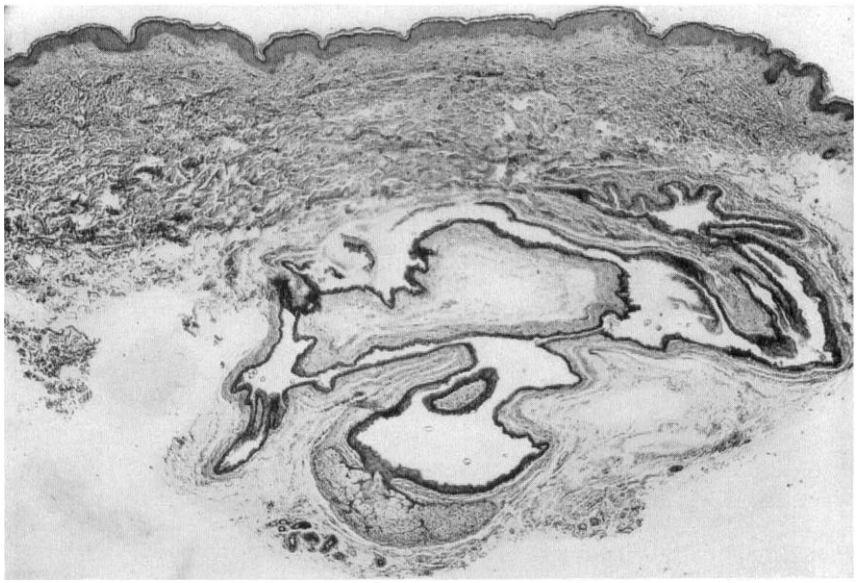

FIG. 1. *Sebocyst.* Paraffin section, stained with hematoxylin and eosin. An intact sebaceous gland opens into the cyst.

determined length of time, could be collected. In this manner two 24-hour samples and an 88-hour sample were taken from the same individual, a 23 year old man having a normal, non-balding scalp. The same subject volunteered to donate a series of half-hour samples. For these samples, after shampooing and thorough rinsing with distilled water, the scalp was soaked 2 times in succession at half-hour intervals for eleven consecutive half-hour periods. The yield of fat for each half-hour fell asymptotically from 45 mg. on the first half-hour to 15 mg. each on the 9th, 10th and 11th half-hour. The last three samples may represent the true rate at which the surface fat was being replenished. They were pooled and analyzed separately. The 3rd through the 7th half-hour soakings were also pooled and analyzed.

The free fatty acids for each lipid sample were separated from the neutral lipids by washing a petroleum ether solution of the fat with an alcoholic 0.1 *N* NaOH solution, counter washing the aqueous-alcoholic phase with petroleum ether, then obtaining the free acids in the usual manner. Saponifications of the remaining neutral fat were carried out by refluxing it for two hours under nitrogen using 10% KOH in 90% alcohol. The unsaponifiables were chromatographed on alumina (Brockmann Grade III) eluting with petroleum ether, benzene, ether, and methanol. The ratio of alumina to fat used was 50 to 1 (by weight), and the ratio of column diameter to height was 1 to 10.

The results of these analyses are given in Table 1. It can be seen that the fatty acids of the steatocystoma lipids are all esterified. However, surface fat, collected as early as one half-hour after reaching the surface, showed nearly 24% free acids. Correspondingly, the amount of esterified acids is lower. Further-

TABLE 1

Analytical comparison of steatocystoma lipids with surface fat obtained at various time intervals

	Steatocystoma lipids	Half-hour Hair Surface fat		24-hour Hair Surface fat		88-hour Hair Surface fat
		Middle[a]	Last[b]	I[c]	II[c]	
	%	%	%	%	%	%
Overall analysis:						
Free fatty acids	trace	23.8	23	27.1	28.1	34.0
Esterified acids	61.5	43.6	39	41.8	38.5	29.6
Unsaponifiables	33.0	29.2	33	30.3	30.6	33.8
% recovery	94.5	96.4	95	99.2	97.2	97.4
Unsaponifiable analysis:						
squalene[d]	11.7	11.8	11.0	13.9	13.1	12.0
wax alcohols[d]	15.0	8.5	7.9	11.2	12.2	11.6
sterol[d]	2.7	2.4	2.1	2.5	2.6	3.4
unidentified	1.5	3.0	3.6	.7	1.5	3.6
Loss in unsaponifiable analysis	2.1	3.5	8.4	2.0	1.2	3.2

[a] Pooled 3rd through 7th half-hour soak.

[b] Pooled 8th through 11th half-hour soak, see experimental section.

[c] Duplicate samples.

[d] These materials represent only chromatographic eluates.

more, the proportion of free to esterified acid increases with time in the free flowing sebum, while the total per cent of acid (free and esterified) is roughly the same for all the samples. The remainder of the analysis is also roughly the same for all the samples. Thus sebum which has been exposed to the sebaceous gland duct, the follicle and the skin surface has a significant amount of free fatty acids while sebum which has not been so exposed has none. Furthermore, the proportion of free to esterified fatty acid is dependent on the time of exposure to the surface.

Other data published earlier (2) can be interpreted in a similar way. When freshly excised human scalp skin was sliced and incubated in a suitable medium with 1-C^{14} acetate, 48% of the initial radioactivity was used for synthesis of esterified acids of the lipids while only 4% appeared in the free fatty acid fraction. The low activity in the free acid portion could presumably arise from the fact that here again the enzyme is not available under the experimental conditions of slicing and incubation. Since the slices were thin and made parallel to the skin surface, glandular parenchyma were separated from the ducts.

COMMENT

If hydrolytic splitting of esters in sebum is implicated by either the sebaceous gland duct, the follicle or the skin surface, the question then arises as to which of the three main groups of esters present in human surface fat are split, namely,

triglycerides, waxes or sterol esters. The most likely candidates are the triglycerides because of the following data already published (1):

(a) The more free fatty acids that occur in a given sample of fat the less triglycerides occur there. One pooled sample of fat from scalp soaks contained 30.7 % free fatty acids and 19.5 % triglycerides while another contained 23.2 % free acids and 26.6 % triglycerides.

(b) Both di- and monoglycerides, the intermediate products in lipolysis have also been found to occur in the human hair fat.

(c) If waxes (16 % of the fat) were hydrolyzed, then free wax alcohols would be formed, but these do not occur to any appreciable extent in surface fat.

(d) While both free and esterified sterols do occur in surface fat (1.4 and 2.4 % respectively), from the fact that they occur in such a small amount, it appears highly unlikely that the large quantity of free fatty acids could be derived from them. However, they could contribute to a small extent.

DIRECT TEST FOR LIPOLYTIC ACTIVITY BY HUMAN SKIN SURFACE

Perhaps the most straightforward piece of evidence for the case that skin surface has lipolytic activity was the demonstration of the ability of the skin to split a labeled triglyceride. This experiment was performed by applying to the skin surface pure tripalmitin which was labeled in the carboxyl ester carbon atoms with C^{14}. After a suitable period of time, the lipid mixture consisting of surface fat already present plus split products derived from the tripalmitin plus the unreacted tripalmitin was wiped off and the free fatty acid fraction was separated and examined for radioactivity.

Technics

Tripalmitin (Volk Mfg. Co.) was purified by chromatographing it on neutral alumina (Woelm) which had an adsorption activity of V on the Brockmann scale. After eluting with petroleum ether the tripalmitin could be cleanly separated from any mono- or diglyceride or free fatty acid present. The purified material melted at 65.0–65.5° C. A portion of this (~0.5 mg.) having a total activity of approximately 0.8 μc and a specific activity of 4.2 mc/mM was dissolved in about 2 ml. petroleum ether then applied to the back of a male test subject, age 27, who had normal skin. The drops of petroleum ether quickly evaporated leaving the radioactive tripalmitin spread out over an area of about 300 sq. cm. After remaining on the back at room temperature (27° C) for 2.5 hours, the mixture of radioactive lipids plus the lipids which were already on the back were wiped off with fat-free cotton pledgets soaked in ether. (After the subject had bathed himself, no traces of radioactivity could be found on his back when sensitive monitoring equipment was used.) The fat was removed from the cotton pledgets by extracting them with petroleum ether until negligible radioactivity was observed in the fat residue extracted and the extracts were then pooled. A total of 33.9 mg. was obtained and an aliquot of 4.4 mg. was removed for duplicate combustions.

A technic was developed for separating quantitatively the neutral material from the free fatty acid fraction, during which procedure, hydrolysis of triglycerides was minimized to the extent of only 0.3% of the amount used. Chemical separation with dilute base was found to give variable and significantly more hydrolysis (2 to 3%). Chromatography on alumina of activity stronger than V on the Brockmann scale was also found to be unsuitable because appreciable hydrolysis occurred on the column. Furthermore, the free acids could

not be quantitatively recovered. The method here used was to chromatograph the whole fat on neutral alumina (Woelm—activity V) using a column 9 cm. long and 0.8 cm. diameter. The fat mixture was put on the column with benzene and then eluted further with benzene, then with ether, then with methanol (taking a 50 and a 25 ml. fraction with each of these solvents) and finally with a mixture consisting of 20% HCl 12*N* and 80% absolute methanol. Using this sequence of eluents the neutral tripalmitin was eluted with benzene along with most of the fat, and the free fatty acids were eluted in the methanolic-HCl fraction. The free fatty acids were recovered from the latter fraction by first adding an equivalent amount of water than extracting twice with petroleum ether. To test the procedure a blank experiment was performed in which comparable amounts of purified labeled tripalmitin and human surface fat were mixed and put through the same chromatographic procedure. The intermediate fractions (i.e. ether and methanol eluates) were designed to elute mono- and diglycerides which though not yielding significant radioactivity in the blank experiment could conceivably show appreciable radioactivity in the lipids which were exposed to the skin. This was not found to be the case, however.

Samples of fatty acids and total fat were combusted, the CO_2 trapped as $BaCO_3$ and the latter counted as an infinitely thick sample as previously described (2).

Results

Results of this experiment are given in Table 2. Here are tabulated weights and radioactivity data for the analysis of fat obtained from the back of an individual after labeled tripalmitin was spread out over an area of about 300 sq. cm. for a period of 2.5 hours. Also presented is the analysis of a control sample of fat to which labeled tripalmitin was added. It can be seen that after the chromatographic separation, the free fatty acids of the control sample showed only 0.3% of the total radioactivity employed, whereas the fatty acids of the fat containing radioactive tripalmitin which was exposed to the skin showed 6.17% of the radioactivity employed. This 20-fold increase shows definitely that hydrolysis of the tripalmitin occurred on the skin.

If it is assumed that only triglycerides are hydrolyzed, then the question may be raised as to why such a small amount (6%) was hydrolyzed in a 2.5

TABLE 2

	Control expt. Surface fat diluted with 1-C^{14} tripalmitin	Expt. where skin is exposed to 1-C^{14} tripalmitin
Total Fat		
weight (%)	36.52 mg. (100%)	29.49 (100%)
Specific activity	7,384 counts/min./mg.	8,715 counts/min./mg.
Total activity[a] (%)	207,400 counts (100%)	197,800 counts (100%)
Neutral Fat		
Benzene eluate (wt.-%)	26.62 mg. (73%)	21.20 mg. (72%)
Other eluates	traces	traces
Free Fatty Acids		
Weight (%)	9.96 mg. (27%)	7.69 mg. (26%)
Specific activity	83 counts/min./mg.	2,112 counts/min./mg.
Total activity[a] (%)	622 counts (0.30%)	12,200 counts (6.17%)

[a] Total activity = specific activity × weight in mg. × fraction of material due to carbon. The total fat was estimated at 76.9% C and the fatty acids at 75.2% C.

hour period when sebum secreted after only half an hour is about 30%* hydrolyzed. This could be due to a number of causes. In the first place, the enzyme may not have been very accessible under the conditions of the experiment. For example, if the lipase is located in the sebaceous gland duct, as histochemical data seem to indicate, the labeled tripalmitin may not have had an opportunity to penetrate that deeply to any appreciable extent. Furthermore, if it did penetrate somewhat, it may have been quickly washed away by newly formed sebum. Also, the free fatty acids already present may serve as inhibitors to the enzyme.

HISTOCHEMICAL EVIDENCE FOR A NON-SPECIFIC ESTERASE IN THE SEBACEOUS GLAND DUCT

Technics

Freshly excised normal skin was fixed in 10% formol saline at 4° for 24 hours. Frozen sections were mounted on slides, dried at room temperature for a few hours and stored at 4°. These slides were subsequently incubated in the appropriate substrate mixtures with suitable controls. Biopsies of acne vulgaris and of sebocystoma multiplex were treated in the same way. Three histochemical methods for non-specific esterases were employed.

(a) *alpha-Naphthyl acetate.* This substrate was used as outlined by Gomori (3) according to details given by Burstone (4) and Gomori's trishydroxymethylaminomethane buffer was employed.

(b) *Naphthol AS acetate.* This substrate, the acetate of 2-hydroxy-3-naphthoic acid anilide was synthesized according to Pearse (5). The method of Gomori (3) was used routinely and Gomori's buffer was employed. A recent modification of Holt (6) gave the best results with clearest detail of esterase location.

(c) *Indoxyl acetates.* The indoxyl acetate method of Holt and Whithers (7) using potassium ferricyanide as oxidizing catalyst was employed according to details given by Pearse (5). The substrate used was 0-acetyl-5-bromo-indoxyl.

Results

Hydrolysis of alpha-naphthyl acetate was rapid, and false localization of azo-dye easily occurred, so that this substrate was considered unreliable for location of simple esterases. Results with naphthol AS acetate and with 0-acetyl-5-bromo-indoxyl were comparable and the methods seemed reliable. Location of simple esterase has therefore been judged by these latter two methods. With these substrates, normal skin showed no esterase activity in the surface epithelium except for a variable amount in the surface film. In the dermis scattered elongated cells (probably histiocytes or fibroblasts) showed slight esterase activity. Some acinar cells of sweat glands were strongly esterase positive and activity could be seen in some sweat ducts.

In pilosebaceous follicles intense esterase activity was seen at the point where the sebum enters the hair canal (Fig. 2) and the esterase could sometimes be traced up the canal to the pilosebaceous opening. The section has to be made through the right part of the follicle to show this effect, and this type of esterase

* The total glyceride percentage is here assumed to be the sum of the free fatty acids plus mono- and diglycerides plus triglycrides, i.e. seome 60% of the fat.

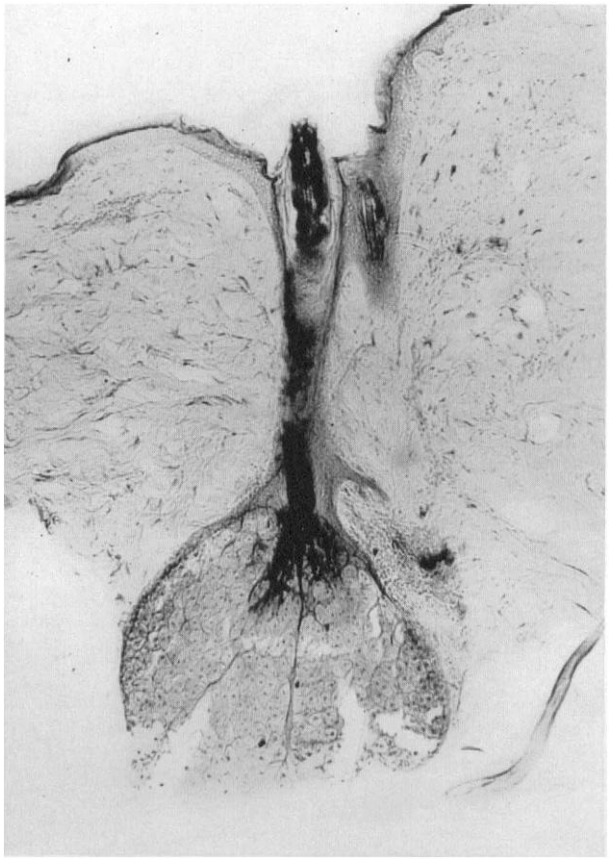

FIG. 2. *Normal skin.* Holt's modification of the naphthol AS method. The darker colour shows the site of esterase activity in the sebaceous gland duct and along the hair shaft canal. Scattered esterase-positive dermal cells may be seen.

was not present in the periphery of the human sebaceous gland. In a single biopsy from a case of sebocystoma multiplex, the fatty content of a sebocyst was demonstrable in frozen sections (Fig. 3 stained with Sudan IV and hematoxylin), but it contained no esterase (Fig. 4 Indoxyl acetate method). By contrast the comedone of acne vulgaris showed well marked esterase activity which seemed to track up through the distorted hyperkeratotic pilosebaceous outlet (Fig. 5 Indoxyl acetate method).

The following chemicals had no inhibitory effect on the follicular esterase: sodium fluoride, 10^{-3} M; lysergic acid, 3×10^{-6} M; hydrocinnamic acid, 10^{-3} M; hydroxylamine, 10^{-3} M. Strong irreversible inhibition was produced by silver nitrate 2×10^{-3} M and by di-isopropyl fluorophosphate (DFP) 10^{-6} M. Sodium taurocholate, 10^{-3} M, and sodium dodecyl sulphate, 10^{-3} M were unsatisfactory as activators or inhibitors as they interfered with the deposition of dye.

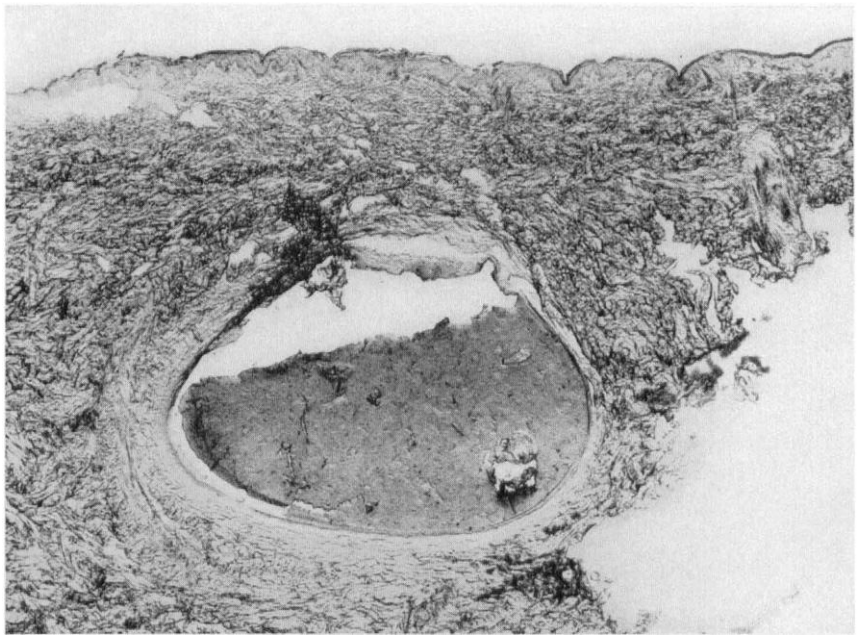

FIG. 3. *Sebocyst.* Frozen section stained with Sudan IV and Hematoxylin, the cyst is seen to be full of fat.

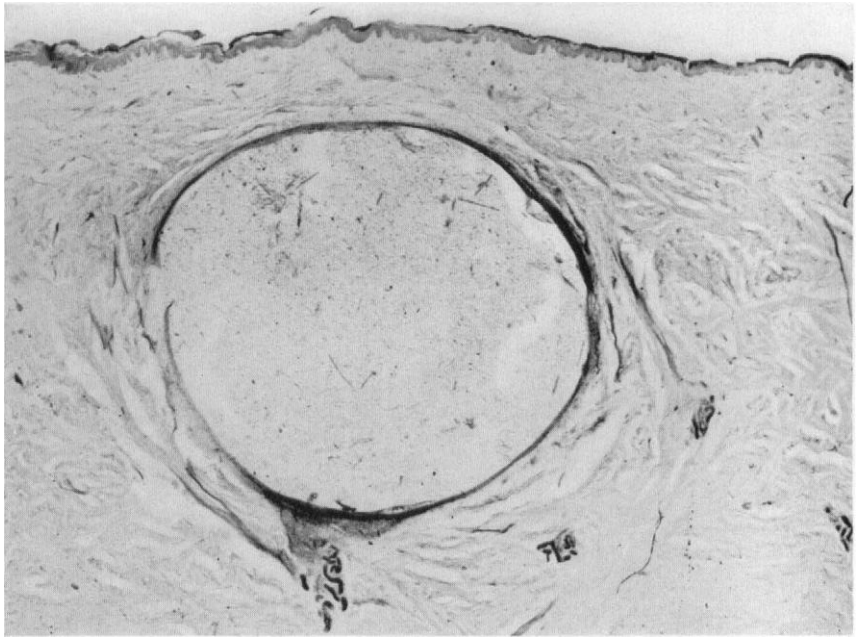

FIG. 4. *Sebocyst.* O-acetyl-5-bromo-indoxyl method, with eosin and fuchsin counterstain. There is no esterase activity inside the cyst. A sweat duct just below the cyst contains esterase.

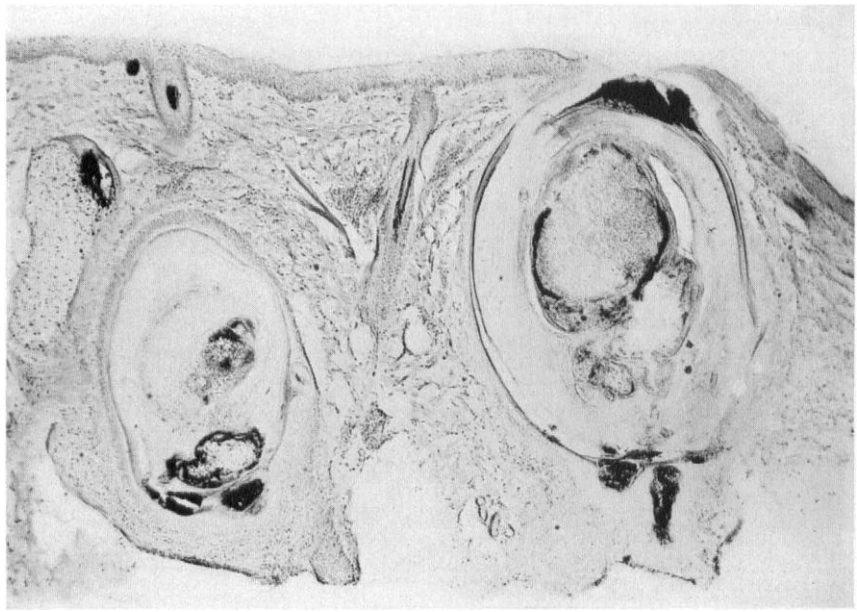

FIG. 5. *Comedones of acne vulgaris*. O-acetyl-5-bromo-indoxyl method with eosin and fuchsin counterstain. Esterase activity can be traced up through the hyperkeratotic masses.

Discussion

The finding of simple esterase in the pilosebaceous canal is in broad agreement with the observations of Montagna (8) and Findlay (9) who relied mainly on the alpha-naphthol esters. With the substituted indoxyl acetate and with naphthol AS acetate this esterase was more clear-cut, and with these methods there was no esterase shown in the periphery of the human sebaceous gland. The arrangement of this esterase in the lining of the sebaceous canal, and following up alongside the hair shaft to the skin surface suggests that it might have something to do with hydrolysis of lipids. The inhibitor studies rule out the presence of a cholinesterase or pseudocholinesterase, and also rule out certain peptidases. While these studies do not characterise the esterase as a lipase, they do not rule it out as a possibility. Furthermore, the absence of this esterase from the cyst of sebocystoma multiplex while requiring further confirmation, is consistent with the biochemical finding of no free fatty acids in these cysts.

It is not uncommon for lipolytic activity to be found in gland ducts. B. F. Martin reports the widespread occurrence of a lipase (using Gomori's Tween technic) in duct epithelia of different glands of many species (10).

SUMMARY

1. In lipids from steatocystoma, which do not contain ducts, no free fatty acids were found.

2. Tripalmitin is lipolyzed to free fatty acids to the extent of 6% when it is allowed to remain on the skin for 2.5 hours.

3. Histochemical data clearly demonstrate the presence of a non-specific esterase in sebaceous ducts.

4. The above data and earlier published data indicate that there is a lipase which is probably located in the sebaceous gland duct. This lipase is very likely responsible for the large free fatty acid fraction found in human surface fat.

ACKNOWLEDGMENTS

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DISCUSSION

Dr. Franz Herrmann (New York, N. Y.): I should like to ask two of the questions which crossed my mind during the presentation of this fascinating study:

1. Dr. Nicolaides consistently referred to "a" lipase when discussing the tripalmitate lipolysis. Does he really think that only one lipase exists at this level of the skin?

2. Does he assume that the lipolytic activity (at the mouth of the sebaceous ducts) is caused by the metabolism of microbes or by enzymes physiologically preformed in the tissues?

Histochemical findings well compatible with those presented here were demonstrated by Montagna, as well as by Steigleder. These investigators pictured a concentration of esterase activity in the duct of the sebaceous gland, hardly any activity in the center of the lobes—and mild activity in the periphery of the latter (apparently not due to the presence of microbes).

Dr. Nicholas Nicolaides (in closing): To answer Dr. Hermann's first question, I must state that I do not know whether more than one lipase is present.

What we mean here when we say "a lipase is present" is that there is lipolytic activity. Whether this activity is due to one or more enzymes cannot be said at the present time.

To answer his second question as to whether this lipolytic activity is due to bacteria, we have already done some work to try to pin this point down, but since we got conflicting results I did not include this in my talk.

The questions we posed were these: If we cut down the bacterial population on the skin of an individual (1) would the sebum excreted then show the same free fatty acid content as that of "non-sterile sebum"? And (2) would the "sterile" skin of the back show the same ability to split radioactive triplamitin as the non-sterile skin reported in the paper?

Bacterial cultures were taken from the back of the same individual used in the experiment on the hydrolytic splitting of radioactive tripalmitin, the sebum was wiped off with cotton pledgets and discarded, then neomycin and bacitracin antibiotics were applied to the back frequently over a period of 2 days. After the first day the sebum was again wiped off and discarded. The back was also kept well covered with sterile gauze during the entire 2 day period. Bacterial cultures were taken at the end of the 2 day period and the sebum wiped off again. This "sterile sebum" was analyzed for free fatty acids. After removing the "sterile sebum", radioactive tripalmitin was applied as before over a 300 sq. cm. area and allowed to remain for 2.5 hours. The sebum was wiped off again and analyzed for radioactivity in the free fatty acid fraction as described in the paper. Bacterial cultures were also taken just prior to wiping off the radioactive sebum.

The results were as follows: 1) Antibiotics were very effective in cutting down bacterial population both before and after the application of the radioactive tripalmitin. 2) Chemical analysis of the "sterile sebum" for free fatty acids prior to the application of the radioactive tripalmitin showed a free fatty acid content identical to "non-sterile sebum", showing that the antibiotic *did not* affect production of free fatty acids. 3) But the per cent of the applied radioactive tripalmitin which was split into free fatty acids was only 2% when lipolysis was tested on sterile skin as compared to 6% when tested on non-sterile skin. This indicates that bacteria *may* have something to do with the formation of the free fatty acids.

Further work is now in progress to attempt to resolve these conflicting results.