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THE ROLE OF THE AEROBIC MICROFLORA IN THE GENESIS OF FATTY ACIDS IN HUMAN SURFACE LIPIDS*

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ABSTRACT

The aerobic microflora of the scalp, predominantly cocci, was virtually eliminated by the daily application of 1% aqueous neomycin for one month. This was done to test whether these organisms are an important source of the lipases which liberate fatty acids from the sebaceous triglycerides.

This treatment had no effect on the proportion of free fatty acids in the surface lipids as determined by thin layer chromatography. The follicle-residing anaerobic *C. acnes,* was not attacked by neomycin and the yeast-like fungus *P. ovale* increased somewhat. The percentage of free fatty acids in any individual's sebum before and after treatment was positively correlated with the density of *C. acnes*. The latter organism is probably mainly responsible for lipolysis of triglycerides.

Disparate lines of investigation have established unequivocally that the free fatty acids in surface lipids are secondary products derived by lipolysis of triglycerides. For practical purposes the epidermis makes a negligible contribution to the surface lipid which may be considered as derived from sebaceous glands.

Kellum could find no free fatty acids in microdissected sebaceous glands (1). Neither were these present in closed cysts with sebaceous appendages (steatocystomas) (2). Presumably triglycerides are hydrolyzed by lipases after the lipid filled cells rupture and deliver their sterile contents into the follicular canal. Esterases have been demonstrated histochemically in the follicular canal (2); however, it is practically certain that bacteria are the main source of lipolytic enzymes.

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Two groups of organisms, aerobic coagulase negative cocci and the anaerobic diphtheroid *Corynebacterium acnes* are universal residents of follicles. Free fatty acids are released from various saturated and unsaturated triglycerides (3, 4) and from olive oil (5) in anaerobic cultures of *C. acnes*. Likewise Freinkel observed abundant lipolysis of triglycerides in 48 hour cultures of coagulase negative cocci (6). She found coccal lipase to be even more active than that from *C. acnes*. Theoretically the lipases from either or both of those organisms could account for the free fatty acids in surface lipids. Still another group of organisms, the lipophilic yeasts of the genus *pityrosporium* requires consideration as a source.

P. ovale and *P. orbiculare* densely colonize sebaceous rich areas and may be strongly presumed to attack triglycerides since these usually have to be supplied to obtain growth.

It is clearly impossible to decide from cultural studies which of these lipase producers are in fact mainly responsible for cleaving triglycerides in living skin. The question is more than academic since free fatty acids are thought to play an important role in the pathogenesis of acne (3).

We are following a different strategy in our investigations, namely, to quantify the percentage of free fatty acids after eliminating one at a time each of the lipase producing groups of organisms by selective antibiotics.

In this paper we report on the suppression of aerobic flora of the scalp with topical neomycin and the effect on sebum composition. In later communications we will examine the consequences of suppressing *C. acnes* with oral tetracycline and *Pityrosporium* with topical amphotericin.

MATERIALS AND METHODS

Subjects. Twelve healthy adult male inmates of the Philadelphia County Prison at Holmesburg volunteered for the study.

Treatment. During the four week treatment period 5 ml of 1% aqueous neomycin sulphate was applied to the scalp twice daily and rubbed in by each subject. For a three week period before treatment medicated soaps and creams were prohibited. Throughout the entire study period (8 weeks), the scalp was washed at weekly intervals with a non medicated shampoo.

Surface lipids. (*a*) *Collection.* After the third weekly scalp washing, collections of lipid were made daily on four consecutive days by dipping the scalp into one liter of redistilled diethyl ether. The ether washings were filtered through a Millipore HA 47 to remove particulate matter. The lipids were recovered by gentle heat and final removal of ether with nitrogen. Neomycin treatment began after the fourth scalp washing and continued for four weeks. The day after treatment ended the scalp was washed and the lipids collected daily for the next four days as before. The samples were frozen until analysis. (*b*) *Lipid analysis.* Portions of the individual samples were redissolved in hexane to a concentration of 1 mg/ml. The major fractions were estimated by quantitative thin-layer chromatography (7). Approximately 10 μg of the samples were applied to 6 mm lanes ruled in a 250 μ layer of Silica Gel G (E. Merck & Co.) on standard 20 × 20 cm glass plates. The chromatograms were developed in three successive solvent systems: hexane (to 19 cm); benzene (to 19 cm); and hexane:ether: acetic acid (70:30:1), to 10 cm, with a 10 min drying period between each development. The resolved lipids were then charred by spraying the chromatograms with 50% H_2SO_4 and heating to 220°. The resulting spots were quantitated by scanning each lane with a photodensitometer (Photovolt Corp., Model 530) and calculating the relative concentrations of constituents in each mixture from the peak areas on the recorder chart. This procedure has a precision of ±5% for the major constituents (8).

Bacteriological methods. (*a*) *Quantitative sampling.* The detergent scrub technique of Williamson and Kligman was utilized (9). Two areas near the vertex of the scalp were clipped closely with scissors and a glass cylinder 3.8 sq cm in area held to the skin. At each site two one minute scrubs in one ml of 0.1% Triton X-100 in phosphate buffer were made and the two suspensions pooled.

The first pretreatment sample was taken prior to the first ether dipping. The second pretreatment sample was secured one week later just before the fourth scalp washing and start of treatment. This permitted appraisal of the effect of four consecutive days of ether dipping on the bacterial flora. The scalp was sampled after two weeks of treatment and finally at the end. The sample was always taken immediately before shampooing. (*b*) *Laboratory methods.* The general procedures have been previously described (10).

Four tenfold serial dilutions of the sample were made in half strength wash fluid and 0.25 ml quantities incorporated into the following media.

(1) Trypticase Soy Agar (TSA) (BBL—Division of Bio Quest) for aerobes.
(2) TSA with 0.5% 'Tween 80' (Atlas Chemical, Wilmington, Del.) as a growth supplement for lipophilic diphtheroids.
(3) Thioglycollate agar (DIFCO) without dextrose or indicator supplemented to 1% with glucose for *Corynebacterium acnes.*

Two plates of Marshall and Kelsey's medium (11) and a TSA plate were streaked from the undiluted sample to facilitate identification of bacterial groups.

The aerobic plates were incubated for 3 days at 37°C and counted with the Quebec colony counter. Bacterial groups were identified after a further 4 days incubation at room temperature.

We emphasize the necessity of maintaining strict anaerobic conditions for quantifying *C. acnes.* Without this, some strains grow very poorly and some cells in all strains fail to form colonies on primary isolation. The anaerobic plates were incubated at 37°C for 7 days in an atmosphere of 10% CO_2 in nitrogen. To remove traces of oxygen, steel wool moistened with saturated copper sulphate was placed in each jar. These measures combined with the reducing medium used allowed reproducible quantitation of *C. acnes,* colonies of which were readily recognized by the domed shape, pinkish tinge and smooth texture. Confirmation by microscopic morphology of the cells was seldom necessary and no difficulty was experienced in separating *C. acnes* colonies from facultatively anaerobic coccal colonies.

Yeast counts. We have found no cultural methods for quantifying the lipophilic *Pityrosporum ovale* and *P. orbiculare* which are normal scalp residents. We therefore had recourse to the less satisfactory method of visually counting the cells collected on a Millipore filter.

To the remainder of the first tube dilution was added 3 drops of 1:1 mixture of Huckers crystal violet and basic fuchsin. We have employed this stain to visualize horny cells as well as fungi (12). A volume of 0.5 ml of the stained suspension was added to 100 ml of particle free water and the cells collected on a membrane filter (Millipore HAWG 047). The dye was removed from the filter by treatment with 70% alcohol. For counting, part of the filter was mounted in immersion oil and all the yeast cells in 100 oil immersion fields were counted.

Yeast counts were made once before treatment and on the final treatment samples.

RESULTS

Twelve subjects entered the study. However, only 8 were available at the end of the four week treatment period due to administrative decisions unconnected with the study.

Pretreatment microflora. In all instances coagulase negative cocci completely dominated the aerobic flora usually exceeding 95% of the population. Small numbers of lipophilic diphtheroids, usually less than 1000/sq cm were found in most samples. Occasional colonies of *Micrococcus luteus* and miscellaneous diphtheroids were recovered in about a third. A large number of *C. acnes* was always present.

Four daily treatments with ether had no significant effect on the aerobic and anaerobic flora (Table I).

Effect of treatment on the microflora. The geometric mean densities at each sample time are displayed on a logarithmic scale with 95% confidence limits about the mean in Figure 1. The elimination of the aerobic flora by neomycin was virtually complete, a reduction exceeding 99% by two weeks of treatment and remaining at this level.

There was no significant change in density of *C. acnes* in either the mid-treatment or final samples. The mean yeast count doubled in the post treatment samples. Although this difference was statistically significant ($p < 0.05$), we do not have high confidence in the accuracy of the visualization method.

In summary the effect of neomycin treatment was to virtually eliminate coagulase negative cocci without affecting *C. acnes* or *Pityrosporum*.

Lipids. The composition of the surface lipid is given for each subject in Table II. In the final tabulation, the results of the first lipid sample of each set of four consecutive samples was discarded and the remaining three results averaged.

There was no evidence of a reduction in the proportion of free fatty acids. Indeed in 5 of the 8 subjects the value tended to increase. Treatment did not significantly alter any other component of the surface lipid. Nor was there any significant change in the total lipid recovered from the scalp before or after treatment.

TABLE I

Geometric mean count per sq cm

Organism	Pretreatment		Treatment	
	1	2	2 weeks	4 weeks
Aerobes	572,000	960,000	1,670	2,960
C. acnes	317,500	603,000	240,700	640,000
Yeasts	535,800	—	—	1,127,000

Correlation of bacterial densities with free fatty acids. A plot of the percentage of free fatty acids against aerobic density before and after treatment is shown in Figure 2. This resulted in a random distribution, but again shows the marked reduction in aerobic density produced by treatment. The percentage of free fatty acids remained similar where coagulase negative cocci were both abundant and scarce.

By contrast when *C. acnes* is plotted against the percentage of free fatty acids, a straight line relationship is observed ($r = 0.78$) (Fig. 3). This is highly significant at the 1% level. The equation of the orthogonal regression line derived from all 19 available pairs of observations is: $-\%\ \text{f.f.a} = 12.7\ (\log\ C.\ acnes/\text{sq cm}) - 37.6$.

Before treatment the density of aerobes and

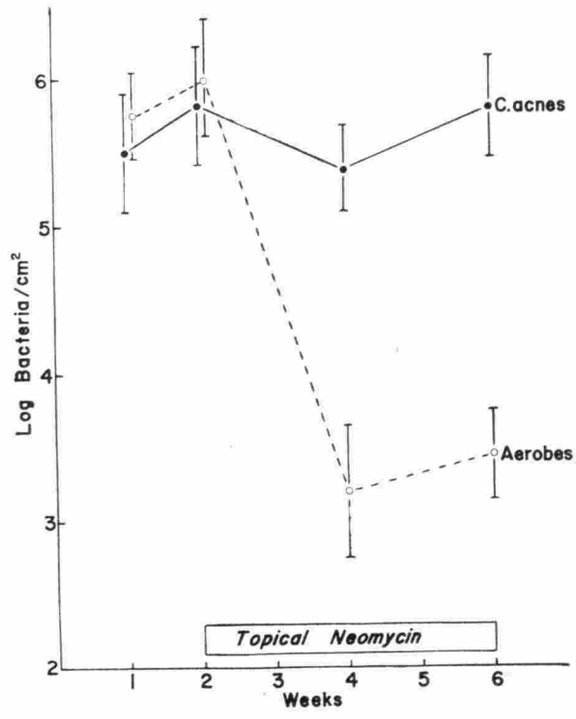

FIG. 1. Neomycin rapidly suppressed the aerobic coccal flora without influencing the anaerobic population. Bars indicate 95% confidence limits of the mean.

TABLE II

Comparison of lipids before and after neomycin treatment

Subject	Treatment	Diglycerides	Cholesterol	Fatty acids	Triglycerides	Wax esters	Cholesterol esters	Squalene
1	Pre.	3.3	0.8	22.13	36.57	26.17	2.47	8.57
	Post.	4.07	0.6	22.37	32.9	26.13	3.17	10.8
2	Pre.	4.1	1.3	22.63	40.97	19.0	2.43	9.6
	Post.	2.83	0.57	27.53	30.17	22.33	3.43	13.13
3	Pre.	5.13	1.0	38.0	23.4	22.7	2.53	7.27
	Post.	3.37	0.87	40.93	19.93	23.17	2.9	8.8
4	Pre.	5.17	0.67	34.0	23.93	27.13	3.43	5.7
	Post.	5.3	1.07	45.87	13.6	24.83	3.5	5.83
5	Pre.	5.03	0.93	34.27	26.4	20.37	2.7	10.37
	Post.	5.53	1.43	45.0	18.27	18.37	2.27	9.17
6	Pre.	4.83	0.83	42.97	13.6	23.83	2.0	11.9
	Post.	4.27	1.03	42.03	14.1	22.4	2.57	13.5
7	Pre.	3.4	1.05	42.85	15.9	23.55	3.55	9.7
	Post.	2.97	0.73	30.77	26.0	24.03	3.33	12.1
8	Pre.	3.67	1.33	26.17	39.87	14.57	1.73	12.62
	Post.	4.77	1.47	31.2	31.7	16.4	2.27	12.27

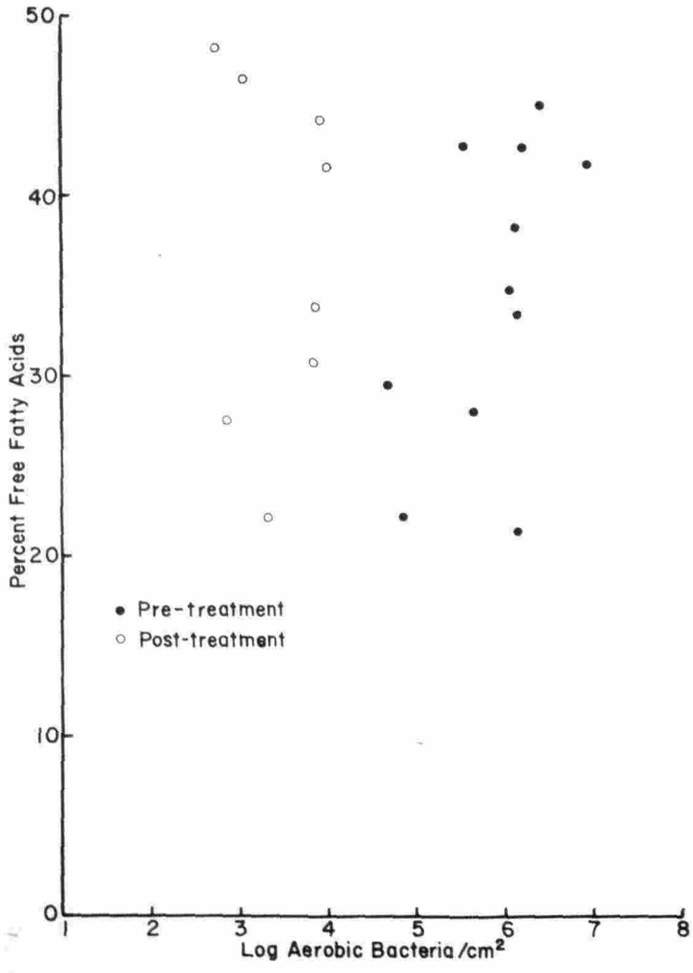

FIG. 2. Lack of relationship between percentage of free fatty acids and aerobic flora.

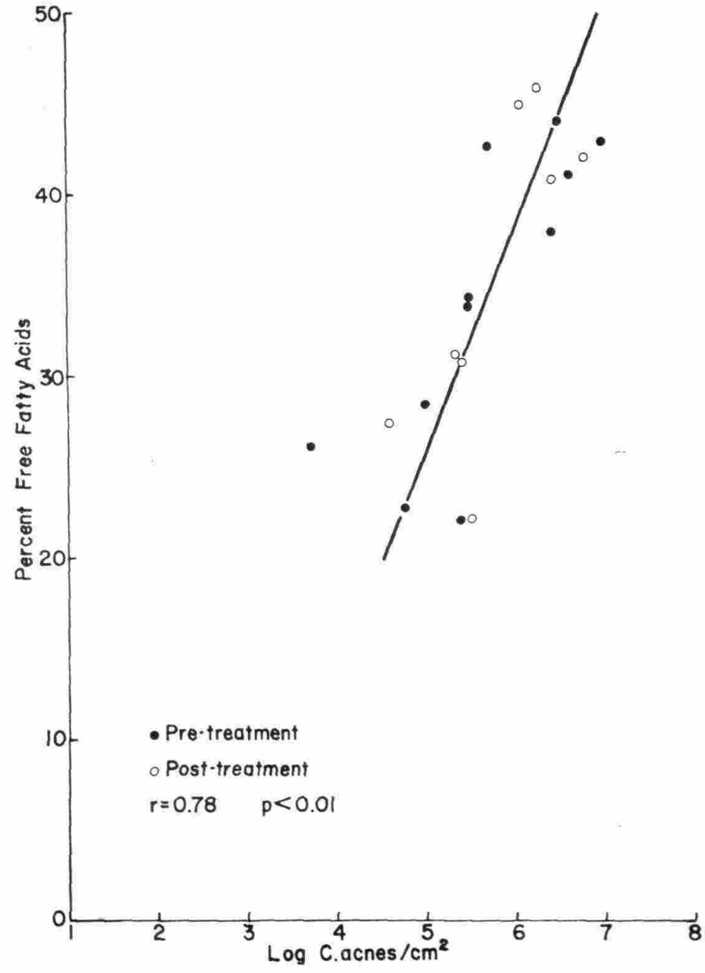

FIG. 3. The percentage of free fatty acids is strongly correlated with the density of the anaerobic flora. This was evident before and after treatment.

of *C. acnes* were positively correlated; this, of course, was abolished by treatment.

DISCUSSION

The scalp is a particularly favorable territory in which to identify the organism chiefly responsible for triglyceride lipolysis. It is both sebaceous-rich and bacteria rich. It turns out that the very bacteria we wished to compare, the anaerobic *C. acnes* and the aerobic cocci, comprise essentially all of the bacterial population and are present in about equal numbers, amounting to millions per sq. cm. The customary designation of the aerobic cocci as *S. albus* and *S. epidermidis* is now obsolete. Using the Baird-Parker classification, we have established that the normal scalp cocci are predominantly *Micrococcus* Type 3, while elsewhere *Staphylococcus* Type 2 predominates. We call attention to these fine points mainly to indicate that the species utilized by Freinkel *et al.* in their *in vitro* work (5, 6) may be different from the scalp cocci. It is, however, likely that most of the various skin cocci are lipase producers.

The principal finding in this study is that virtual elimination of the aerobic cocci had no effect on the proportion of free fatty acids. The aerobes were held to very low levels for four weeks. The *C. acnes* population meanwhile remained unchanged, possibly because the organism lives within follicles and so is inaccessible to topical aqueous antibiotics. Here it is also in a position to attack the sebum before it exits to the surface where the cocci chiefly congregate. This partial separation may be very important in the light of Freinkel's recent demonstration that the lipases produced by these two organisms are strongly inhibited by low pH (5). The latter may be the controlling mechanism which stops the lipolysis at a point far short of completion. The triglycerides are usually more abundant than free fatty acids in surface lipids.

Thus assuming that the free fatty acids are mainly products of lipases from *C. acnes* these may have accumulated to an inhibitory pH level by the time sebum surfaces to the region where it can be acted upon by coccal lipases. It seems more reasonable to suppose that the lipases produced by cocci are inhibited rather than not produced at all *in vivo*.

Our results are in conflict with those presented by Scheimann *et al.* (13). They claimed that there was inhibition of hydrolysis of C^{14} tripalmitin when the surface (back) was first "sterilized" by a single spraying with tetracycline. We greatly doubt that such treatment would make it impossible to recover aerobic organisms from the skin and hair in 8 of 10 subjects. It is a certainty that *C. acnes* would not be affected. They found about 10% hydrolysis in 24 hours on unsterile skin and about half this much after sterilization. An alternate explanation for their findings is contained in the demonstration by Shalita and Wheatley (14) that tetracycline in comparatively high concentrations can inhibit the enzymic action of lipase.

It is not only by exclusion that we focus upon *C. acnes* as the responsible organism. Additional evidence stems from the observation that the fatty acids fall after oral tetracycline (15). This is associated with a decrease in the number of *C. acnes*, and no major change in the density of aerobic organisms, owing to these having required resistance to the antibiotic (10). Thus the evidence to date points away from any correlation between the aerobic flora and the percentage of free fatty acids.

Finally, our data do not give support to an important role for Pityrosporum species as a lipase source. These organisms increase after treatment probably by reduction of aerobic competitors. Assuming that these yeast-like fungi can produce lipases, this should have increased the free fatty acids. The more conclusive study will be the one which measures free fatty acids after elimination of the yeast-like fungi, perhaps after prior reduction in the density of *C. acnes*.

REFERENCES

1. Kellum, R. E.: Human sebaceous gland lipids. Analysis by thin layer chromatography. Arch. Derm., *95:* 218, 1967.
2. Nicolaides, N. and Wells, G. C.: On the biogenesis of the free fatty acids in human skin surface fat. J. Invest. Derm., *29:* 423, 1957.
3. Kellum, R. E. and Strangfield, K.: Triglyceride hydrolysis by *Corynebacterium acnes in vitro*. J. Invest. Derm., *52:* 255, 1969.
4. Reisner, R. M., Silver, D. Z., Puhvel, M. and Steinberg, T. H.: Lipolytic activity of

Corynebacterium acnes. J. Invest. Derm., *51:* 190, 1968.
5. Freinkel, R. K. and Shen, Y.: The origin of free fatty acids in sebum II. Assay of the lipases of the cutaneous bacteria and effects of pH. J. Invest. Derm., *53:* 422, 1969.
6. Freinkel, R. K.: The origin of free fatty acids in sebum. I. Role of coagulase negative staphylococci. J. Invest. Derm., *50:* 186, 1968.
7. Downing, D. T.: Photodensitometry in thin layer chromatographic analysis of neutral lipids. J. Chromatog., *38:* 91, 1968.
8. Downing, D. T., Strauss, J. S. and Pochi, P. E.: Variability in the chemical composition of human skin surface lipids. J. Invest. Derm. *53:* 322, 1969.
9. Williamson, P. and Kligman, A. M.: A new method for the quantitative investigation of cutaneous bacteria. J. Invest. Derm., *45:* 498, 1965.
10. Marples, R. R. and Williamson, P.: Effects of systemic demethylchlortetracycline on human cutaneous microflora. Appl. Micro., *18:* 228, 1969.
11. Marshall, J. H. and Kelsey, J. C.: A standard culture medium for general bacteriology. J. Hyg. (Camb.), *58:* 367, 1960.
12. McGinley, K. J., Marples, R. R. and Plewig, G.: A method for visualizing and quantitating the desquamating portion of the human stratum corneum. J. Invest. Derm., *53:* 107, 1969.
13. Scheimann, L. G., Knox, A., Sher, D. and Rothman, S.: The role of bacteria in the formation of free fatty acids on the human skin surface. J. Invest. Derm., *34:* 171, 1960.
14. Shalita, A. R. and Wheatley, V.: Inhibition of pancreatic lipase by tetracyclines. J. Invest. Derm., *54:* 413, 1970.
15. Strauss, J. S. and Pochi, R. E.: Effect of orally administered antibacterial agents on titratable acidity of human sebum. J. Invest. Derm., *47:* 577, 1966.