Studies of Sebum

8. OBSERVATIONS ON THE SQUALENE AND CHOLESTEROL CONTENT AND THE POSSIBLE FUNCTIONS OF SQUALENE IN HUMAN SEBUM*

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The object of our investigations has been to study the composition of sebum and to elucidate its role in maintaining the health of the skin. In a previous paper on the hydrocarbons of sebum (Boughton, Hodgson-Jones, MacKenna, Wheatley & Wormall, 1955) we observed that the squalene content of sebum showed wide variations, both in samples collected from the same subject on different occasions and between samples collected from different subjects. This variation may be peculiar to squalene or it may indicate a variation in the overall composition of sebum. To investigate this possibility, we have, therefore, studied the comparable variations in another constituent of sebum, cholesterol, and have also extended our observations on squalene in a study of the possible role of this hydrocarbon in the metabolism of the skin. A preliminary report of part of this work has already appeared (MacKenna, Wheatley & Wormall, 1955).

EXPERIMENTAL

Estimation of the squalene and cholesterol contents of sebum. Samples of sebum were collected by the polythene-cup method already described (Boughton *et al.* 1955). The cup was made from the upper part of a polythene filter funnel 6 cm. in diameter, cut across about 2.5 cm. from the top. The cup was pressed against the body and the area of skin enclosed extracted with one or two portions of carbon tetrachloride (glass-distilled). Several adjacent areas of skin were extracted in this manner in order to obtain samples sufficiently large for analysis, and the combined extracts were filtered, evaporated to small bulk under reduced pressure and then diluted to 15 ml. with carbon tetrachloride. Suitable samples of this solution were used for analysis. The sebum content of the solution was estimated by evaporating a sample in a tared microflask and weighing the residue (Hodgson-Jones & Wheatley, 1952), squalene was estimated by the iodometric method (Wheatley, 1953) and cholesterol by the Liebermann-Burchardt method. It was observed, however, that the iodometric titration of squalene was unreliable with amounts of squalene of less than 75 μg .; if therefore the thiosulphate titration required less than 0.5 ml. of 0.005 N thiosulphate the determination was repeated with a larger sample.

Combination with SH groups and inhibition of enzymes. The colorimetric method (Kun & Abood, 1949) was found to be unsuitable for the estimation of succinic dehydrogenase in the presence of squalene, owing to the preferential solubility of the coloured complex in the squalene. Manometric methods were therefore used for the determination of succinic dehydrogenase, cytochrome oxidase, urease, and of whole-yeast fermentation. The SH groups of glutathione solutions were estimated by the ferricyanide method of Mason (1930).

Depilatory studies. The depilatory effect of a number of substances was studied. The test substance (1 ml.) was pipetted on to the skin of a rabbit and lightly rubbed in with the tip of the finger. The site chosen for most experiments was the top of the head between the ears since this site is the least accessible to the animal. It is possible, however, that the hair of this region may be slightly different from that of the rest of the body, and, in order to exclude this possibility, experiments were performed in which the same test substance was applied simultaneously to the head and the centre of the back. After application of the test substance the site of application was examined at daily intervals until hair loss occurred. Preliminary experiments were also performed with the rat, mouse or guinea pig as the test animal, but these animals proved to be much less sensitive to the substances producing depilation than did the rabbit.

Fungicidal and bactericidal activity of squalene. Tests for fungicidal and bactericidal activity were performed for us on squalene from three different sources by Professor L. P. Garrod in the following manner. (1) Glass beads on which *Staphylococcus aureus* or *Candida albicans* had been dried were immersed in squalene and individual beads were removed and cultivated at intervals up to 24 hr. (2) Mixtures of squalene and broth (10–0.07% squalene) with Tween 80 (polyoxyethylene sorbitan mono-oleate) to ensure admixture were incubated for 24 hr. and then plated. (3) Plates of Sabouraud's medium were poured with a thin top layer pre-inoculated with *C. albicans* or *Aspergillus niger*. Before incubation, undiluted squalene was applied to these either in cylinders or simply by allowing a drop to fall on the surface of the medium.

RESULTS

Squalene content of sebum

Normal values. Estimations were made on the sebum from the backs of normal subjects (20 children aged 1–12 years, 20 adult females aged 13–38 and 20 adult males aged 18–74) and the

* Part 7: Wheatley & James (1957).

results are summarized in Table 1. These results show that the squalene content of sebum is significantly lower in children than in adults ($P < 0.001$) but there is no significant difference between the values for adult males and those for adult females ($P > 0.05$). Wide variations in normal subjects were found to occur and this was much more marked in the children than in adults. A dot diagram was plotted of the squalene content and age but no change was found to occur at any age group.

Daily variations. Determinations were made on the sebum collected from the backs of normal subjects, five females and three males. The samples were collected at daily intervals (excluding week-ends) for periods varying from 14 to 28 days. The typical results of a male and a female subject (Fig. 1) indicate the appreciable variation encountered. The range of squalene content from the other six subjects was as follows: the four females, 10.6–15.2, 10.0–12.4, 8.8–11.6 and 6.8–8.8 respectively, and the two males 4.9–10.2 and 6.3–9.5. In the female subjects the changes in squalene content could not be related to the menstrual cycle (see Fig. 1).

Variations over the surface of the body. Samples were collected from a group of normal subjects, four males and four females, from five different areas of the body of each subject, namely, the back, chest, abdomen, arms and legs (Table 2); all the samples from a particular subject were collected on one day and as far as possible during the same part of the day. In view of the wide variations already encountered, detailed analysis of these results has not been attempted, but in practically every case the squalene content of the sebum from the back and chest was consistently higher than that from other areas.

Correlation with sebum level. A dot diagram (Fig. 2) of the squalene content of sebum plotted against the sebum level ($\mu\text{g.}$ of sebum covering 1 cm.^2 of skin) showed that for a sebum level greater than $40 \mu\text{g./cm.}^2$ the squalene content was independent of the sebum level. For sebum levels below this limit the squalene content dropped as the sebum level fell.

Oxidation of squalene in thin films in vitro and in vivo. One explanation of the wide variations in the squalene content of sebum could be that squalene is oxidized after secretion on to the skin surface and that the variations are due to different rates of oxidation caused by variations in local conditions. To test this hypothesis, thin films were prepared by evaporating squalene-containing fatty materials dissolved in carbon tetrachloride in a stream of nitrogen in 100 ml. flasks, kept constantly rotated during the evaporation in order to spread the lipid over the walls of the flasks. These were then set aside and the lipid was allowed to oxidize at room

Table 1. *Squalene content of the sebum from the backs of normal subjects*

Subjects	No. of subjects	Squalene content of sebum (%)	
		Range	Average (and s.d.)
Children	20	1.7–13.8	6.3 ± 3.3
Adults, male	20	6.5–13.4	9.3 ± 2.1
Adults, female	20	7.0–13.0	10.2 ± 1.4

Fig. 1. Daily variation in the squalene content of the sebum from the backs of two normal subjects. Curve A, male subject. Curve B, female subject. ●, Squalene content; the basal-temperature curve (○) has been included to confirm the normal nature of the menstrual cycle.

temperature for fixed periods, at the end of which a squalene estimation was performed. Experiments made with pure squalene, fresh sebum, and sebum to which squalene had been added showed that under these conditions only a negligible amount of squalene was oxidized in 24 hr.

To test the possibility of squalene being oxidized on the skin surface the following experiments were made. A sample of sebum was collected from the back of the subject in the usual manner. The back was then defatted completely by means of a suitable solvent, and after allowing a convenient interval of time to elapse (2–4 hr.) for sufficient sebum for analysis to be secreted, a second sample was collected. The squalene contents of both samples were determined. The second sample had remained on the skin surface for only a limited time, and its squalene content would be expected to be appreci-

Table 2. *Squalene content of the sebum from various areas of the body*

Subject	Sex	Squalene content of sebum (%)				
		Back	Chest	Abdomen	Arm	Leg
J.C.C.	M.	10.3	9.9	5.4	6.8	6.4
J.D.H.	M.	8.6	10.2	5.2	9.1	11.5
R.T.	M.	11.1	11.3	8.3	10.4	5.9
V.R.W.	M.	11.0	11.5	7.5	5.8	6.7
J.H.	F.	9.0	8.0	6.5	6.0	6.1
E.B.	F.	9.9	10.8	7.3	6.0	7.9
B.B.	F.	8.9	12.0	5.2	6.2	3.9
P.H.S.	F.	9.8	9.6	6.4	6.6	9.1
Average		9.8	10.4	6.5	7.1	7.2
Standard deviation		0.9	1.3	1.2	1.7	2.0

Table 3. *Squalene content of sebum samples collected at short intervals after defatting of the skin compared with the squalene content of casually collected samples*

Subject	Solvent used to 'defat' skin	Squalene content (%)			
		Before defatting	After defatting		
			(a)*	(b)*	(c)*
M.E.L.	CCl ₄	7.7	7.5	—	—
B.B.	CCl ₄	8.5	8.3	—	—
E.B.	Acetone	10.4	—	12.9	—
A.R.	Acetone	12.3	—	12.7	—
M.E.L.	Acetone	6.8	—	—	8.3
M.E.L.	Light petroleum	7.2	—	—	9.2
B.B.	Light petroleum	11.5	—	—	13.7

* (a), (b) and (c) were collected after intervals of 2, 3 and 4 hr. respectively.

Fig. 2. Relationship between squalene content of sebum and the sebum level of the area of skin from which the sample was collected of adult male (□) and female (●) subjects, and of children (○).

ably higher than that of the casually collected sample if the squalene in sebum on the skin surface is extensively oxidized. The results (Table 3) showed that the squalene content of the second sample was usually slightly higher than that of the first sample, but the difference was often negligible. The most reasonable interpretation is that while some oxidation of squalene may take place on the skin surface it is not sufficient to explain the wide variations in the squalene content of sebum.

Inhibition of enzymes by squalene. Flesch (1951) observed an inhibition of succinic dehydrogenase by squalene, which he attributed to a combination with the SH groups of the enzyme. Dr E. D. Wills kindly undertook to investigate this inhibition for us. The effect of squalene on succinic dehydrogenase, urease, cytochrome oxidase (a non-SH enzyme) and whole-yeast respiration were studied manometrically. The results (Table 4) show that there is no inhibition of succinic dehydrogenase and only slight inhibition of urease by relatively large quantities of squalene. Slight inhibition of whole-yeast respiration occurred but this may well have been due to a surface effect.

Combination with SH groups. The possible combination of SH groups with squalene was studied for us by Miss P. Handoll. Estimations were performed on solutions of glutathione in the presence and absence of squalene with sodium dodecyl sulphate as an emulsifying agent. No evidence of combination with the SH of glutathione was observed.

Depilatory action of squalene and other lipids. The depilatory action of a variety of lipids and other substances was tested on the rabbit with the results shown in Table 5. Many of these fatty materials had a depilatory effect similar to that of squalene and this activity was not confined to the unsaturated compounds only. The time required for the hair loss to be noticeable after the application of the test substance varied appreciably in these experiments,

Table 4. *Possible inhibition of certain enzymes by squalene*

Enzyme system	Squalene (ml.)*	Oxidized squalene† (ml.)*	Inhibition (%)
Succinic dehydrogenase from pig heart	0.5	—	0
	0.25	—	0
	0.1	—	0
	—	0.5	0
	—	0.2	0
	—	0.1	0
Succinic dehydrogenase from rat liver	0.5	—	0
	0.25	—	0
	0.1	—	0
	—	0.5	0
	—	—	—
Urease (cryst.)	1.0	—	15
	0.75	—	15
	0.5	—	15
	—	1.0	15
	—	—	—
Cytochrome oxidase	1.0	—	0
	0.5	—	0
	0.25	—	0
	0.1	—	0
	—	—	—
Whole-yeast fermentation	1.0	—	23
	0.5	—	8

* Amount of squalene or oxidized squalene added to each manometer, in which the total aqueous phase was 3 ml.

† Squalene which had been allowed to autoxidize for several months.

Table 5. *Depilatory action on the rabbit of squalene and some other lipids*

Substance	No. of animals		Depilatory effect*
	Tested	Showing hair loss	
Squalene	8	7	++
Liquid paraffin B.P.	5	1	±
Hair dressing†	4	3	+
Castor oil	2	1‡	+
Oleic acid	5	5	+++
Methyl oleate	3	3	+++
Methyl stearate	3	3	++
Olive oil	4	2	+
Shark-liver oil	3	3	++
Benzene	3	3	++

* Assessed from the appearance of the treated area after hair loss.

† A proprietary brand containing liquid paraffin.

‡ One doubtful.

Table 6. *Cholesterol content of the sebum from the backs of normal subjects*

Cholesterol content of sebum (%)

Subjects	No. of subjects	Average (and S.D.)	
		Range	
Children	20	5.8–36.3	16.0 ± 5.3
Adults, male	20	2.1–5.4	3.1 ± 0.7
Adults, female	20	2.3–5.2	3.1 ± 0.6

but it was usually about 7 days with most of the substances; with olive oil, the hair dressing, benzene and occasionally with squalene, the period was

longer (10–15 days). In the experiments in which the test substance was applied simultaneously to the top of the head and the centre of the back no difference in the depilatory effect on the two areas was observed.

Bactericidal and fungicidal action. No inhibition of the growth of the bacterium *Staph. aureus* or of the fungi *C. albicans* or *A. niger* by squalene was observed under the conditions studied.

Cholesterol content of sebum

Normal values. Estimations made on sebum from the backs of normal subjects (20 children aged 1–12 years, 20 female adults aged 19–48 and 20 male adults aged 19–72) showed (Table 6) that the cholesterol content of children's sebum is markedly higher than that of adults. The results for adult males and females were almost identical and there was no general correlation between cholesterol content and the age of the subject. One subject, aged 75, who had obvious senile changes of the skin (seborrhoeic warts, etc.) gave a very high value (32.7%) which was not included in the present series.

Daily variations. Daily determinations were made on three male and three female subjects. With one exception all showed little daily variation and a typical result is shown in curve A (Fig. 3). The subject exhibiting wide daily variation (curve B, Fig. 3) had a consistently low sebum level on the back (11–27 µg./cm.²).

Fig. 3. Daily variations in cholesterol content of the sebum from the backs of two normal adults. Curve A, results with a male; B, those with a female subject. Two other males and two females gave curves similar to A.

Fig. 4. Relationship between cholesterol content of sebum and the sebum level of the area of skin from which the sample was collected. Adult male subjects (□), adult females (●) and children (○).

Variations over the surface of the body. The cholesterol content of samples of sebum collected from the back, chest, abdomen, arms and legs of eight subjects, four male and four female, are given in Table 7. Here again the subject (B.B.) who exhibited wide daily variations in cholesterol content also gave consistently high values for all areas, whereas in the other subjects the values for the back and chest were generally lower than for other areas.

Correlation with sebum level. A dot diagram of the cholesterol content of sebum plotted against the sebum level (Fig. 4) showed a striking correlation. For high sebum levels the cholesterol concentration in the sebum was very constant and was independent of the sebum level, but with low sebum levels the cholesterol content increased rapidly as

Fig. 5. Relationship between the squalene and cholesterol contents of sebum samples collected from adult male (□) and female (●) subjects, and children (○).

Table 7. Cholesterol content of the sebum from various areas of the body

Subject	Sex	Cholesterol content of sebum (%)				
		Back	Chest	Abdomen	Arm	Leg
J.C.C.	M.	3.3	3.8	8.8	8.8	16.3
J.D.H.	M.	2.8	2.4	8.8	5.8	16.9
R.T.	M.	3.5	3.3	3.5	6.1	10.4
V.R.W.	M.	3.3	4.2	7.3	7.1	8.3
J.H.	F.	3.1	3.3	3.9	3.7	10.0
E.B.	F.	2.7	2.5	7.1	13.0	5.7
B.B.	F.	10.3	16.8	12.0	12.6	20.4
P.H.S.	F.	2.4	2.6	6.5	7.6	11.1
Average		3.9	4.9	7.2	8.0	12.4
Standard deviation		2.6	4.9	2.7	3.2	5.0

the sebum level fell. The limiting value dividing the two types of results was about $50 \mu\text{g./cm.}^2$. The sebaceous activity of the subject, of which the sebum level gives a rough indication, thus markedly influences the cholesterol content of the film of sebum on the skin surface.

Squalene-cholesterol correlation. A dot diagram (Fig. 5) showed that although there was a general tendency for the cholesterol content to increase as the squalene content fell there was no convincing relationship between these two components of sebum.

DISCUSSION

In a previous paper (Boughton *et al.* 1955) we summarized the known and suggested biochemical actions and functions of squalene. These are (a) its role as an intermediary in sterol metabolism (Langdon & Bloch, 1953; Cornforth & Popják, 1954), (b) its ability to inactivate free SH groups and to inhibit SH-containing enzymes (Flesch, 1951), (c) its action as a reversible depilatory when applied to certain animals (Flesch, 1951) and (d) its fungistatic action against certain dermatophytes (Sobel, Marmorston & Arzangoolian, 1954). In the present study an attempt has been made to investigate certain of these reported actions of squalene. We have been unable to confirm the claims that squalene is able to inactivate SH groups and inhibit SH-containing enzymes, and that it has a significant bactericidal or fungicidal action. When applied to the skin of rabbits it caused reversible hair loss, but similar hair loss was also caused by a variety of saturated and unsaturated lipids and similar substances. We were unable to demonstrate any convincing hair loss when squalene was applied to guinea pig, mouse or rat skin. These results are in agreement with those of Kandutsch & Baumann (1955) who failed to cause depilation of the mouse by repeated application of undiluted squalene, though they did observe some hyperplasia of the epidermis. In previous work (Boughton *et al.* 1955) we did not observe any action on pigmentation or hair growth when squalene was applied to the skin of normal human subjects, nor did it show any therapeutic action when used to treat, by topical application, certain diseases of the skin. We have, therefore, been unable to assign a direct action on the skin which is peculiar to squalene. It has, however, been shown recently by El Ridi, Azouz & Hay (1955) that squalene is essential for promoting lactation in the rat and, since the mammary gland can be regarded as a modified sebaceous gland, an indirect action of squalene on sebaceous activity is still possible.

The wide variations in the squalene content of sebum, both from day to day and between different

individuals, have been confirmed. There is, however, no evidence for wide overall variations in sebum composition, since the cholesterol content of sebum appears to be remarkably constant. No explanation for this variation in the squalene content of sebum has been found; from the evidence available it appears probable that the variations are not due solely to variation in the rates of oxidation of squalene once it reaches the surface of the skin. The simple excretion through the skin of an excess of squalene over that required in sterol synthesis cannot be excluded.

The cholesterol in the film of lipid on the skin surface arises from two sources, the sebaceous glands and the cells of the keratinizing epidermis. In other work (Hodgson-Jones, MacKenna & Wheatley, 1952) little evidence was obtained that the amount of lipid arising from the horny-layer cells of the epidermis was significant in comparison with that coming from the sebaceous glands, and we have therefore referred to this lipid film as sebum. We have now shown that for low sebum levels the amount of cholesterol arising from the epidermis can appreciably alter the composition of the surface film. We can, therefore, no longer regard the surface skin lipid collected from areas of skin with low sebum levels, e.g. the arms and legs, as being pure sebum. For experimental work in which the composition of the sebum of different subjects is being compared, sites of high sebaceous activity, e.g. the back, should be used. Even then measurement of the 'sebum level' of the area should also be made and due consideration given to the possible contamination with epidermal lipid should the sebum level fall below the critical value of $50 \mu\text{g./cm.}^2$. This finding also explains why the cholesterol content of the 'sebum' of children is higher than that of the adult, since the 'sebum' of children, who generally have 'sebum levels' below the critical limit, is appreciably diluted with epidermal cholesterol. A similar explanation has recently been put forward by Nicolaides & Rothman (1955).

Study of the variations of only two components of sebum has proved to be of limited value. Histochemical and other evidence indicates that appreciable lipid synthesis takes place in the sebaceous gland; in order to be able to detect changes in this metabolic activity due to disease and other factors a much more detailed study of the overall variations in sebum composition is required. Until recently such a study has not been possible owing to a lack of suitable small-scale methods of lipid analysis; recent advances (James & Martin, 1956; James & Wheatley, 1956), however, have made such analysis possible, and a much more detailed investigation of the variations in sebum composition is now being undertaken.

SUMMARY

1. The squalene and cholesterol contents of the sebum from the backs of normal children and adults have been studied. It has been confirmed that the squalene content of sebum is lower and the cholesterol content higher in children than in adults.

2. A relationship between the sebaceous activity of a particular area of skin and the squalene and cholesterol content of the sebum derived therefrom has been demonstrated. It is considered that dilution of the surface-film lipids by lipids from the horny-layer cells significantly alters the composition of the 'sebum' in areas of skin with low sebaceous activity, e.g. most skin areas of the child and the arms and legs of adults. This apparent dilution could account for the observed differences between the squalene content of the 'sebum' from children and that of adults. To avoid this dilution effect, it is suggested that for studies of the composition of sebum the sample should be collected from the back.

3. The wide variations in the squalene content of sebum, both from day to day and between different individuals, has been confirmed. This is not due to epidermal contamination of the sample nor solely to variable rates of oxidation of squalene in different samples of sebum.

4. Claims that squalene can inactivate SH groups and inhibit certain enzymes have not been confirmed, and squalene does not appear to possess significant fungicidal or bactericidal properties. It can cause reversible depilation in the rabbit but not in the guinea pig, mouse or rat, and this property is not specific for squalene but is shared by a number of saturated and unsaturated lipids.

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Metabolism of Polycyclic Compounds

11. THE CONVERSION OF NAPHTHALENE INTO 2-HYDROXY-1-NAPHTHYL SULPHATE IN THE RABBIT*

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By the use of paper-chromatographic analysis, Corner & Young (1954, 1955), Sato, Yamada, Suzuki, Fukuyama & Yoshikawa (1956) and Solomon (1955) detected the presence of a metabolite of naphthalene in the urine of rats dosed with naphthalene, which gave the colour reactions of

1:2-dihydroxynaphthalene after the chromatograms had been treated with mineral acid. By injecting ³⁵S-labelled sodium sulphate into the dosed animals, the first two groups of workers were able to show by the use of radiochromatograms that the metabolite was probably a sulphuric ester of 1:2-dihydroxynaphthalene. Furthermore, Corner & Young (1955) detected 1:2-dihydroxynaphthalene

* Part 10: Boyland & Solomon (1956).