Studies of Sebum

2. SOME CONSTITUENTS OF THE UNSAPONIFIABLE MATTER OF HUMAN SEBUM

By R. M. B. MACKENNA, V. R. WHEATLEY and A. WORMALL
Departments of Biochemistry and Dermatology, Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 6 *November* 1951)

Our earlier studies (MacKenna, Wheatley & Wormall, 1950) have shown that human sebum (surface skin fat) contains on an average about 29 % free fatty acids, 36 % combined fatty acids (present in glycerides, waxes and other esters) and 32 % unsaponifiable matter. The unsaponifiable matter contains hydrocarbons, cholesterol and some unidentified compounds, and squalene was identified as one of the major constituents, accounting for about 16 % of the unsaponifiable matter and about 35 % of the hydrocarbon fraction. Some preliminary chemical tests were made for the fat-soluble vitamins, and the presence of a small amount of vitamin E in human sebum was reported.

A more complete study of the unsaponifiable matter has now been made, and this material has been more thoroughly examined for vitamins, especially for vitamin E. A preliminary account of part of this work has already been given (MacKenna, Wheatley & Wormall, 1951).

EXPERIMENTAL

Collection of sebum. Sebum was collected by the method of Ricketts, Squire & Topley (1948, 1951), modified as already described (MacKenna *et al.* 1950). Single specimens were obtained by immersing the forearm of a normal male (age within the range 18–30) in 2 l. of twice-redistilled acetone contained in a tall glass cylinder. At the end of 3 min. the arm was removed from the cylinder, and the other arm extracted for a similar period. The acetone was then distilled off, and the residue of lipid, cellular debris and dirt was extracted with $CHCl_3$; the $CHCl_3$ solution was then filtered and evaporated to dryness and the residue weighed. About 0·1 g. sebum was obtained from each subject.

Bulked collections were obtained from a number of male medical students. In the earlier experiments described here the method used for the collection of single specimens was adopted, the same 2 l. of acetone being used for several extractions up to a maximum of ten. The acetone was then distilled off and the distillate used for a further batch of ten subjects; all the residues from the acetone extracts were pooled and put through the $CHCl_3$ stage together. In later experiments a large glass tank (24 × 12 × 12 in.; 60 × 30 × 30 cm.) containing 15 l. acetone was used, and with this apparatus two subjects were each able to immerse both their forearms simultaneously.

With some batches of sebum precautions were taken to reduce, as far as possible, the oxidation of readily oxidizable constituents. In these cases the acetone was distilled off under reduced pressure (28–32 cm. Hg) in a stream of N_2 and the residue was extracted with ether instead of $CHCl_3$; the ether was then removed from this extract under reduced pressure at room temperature.

Saponification. This was carried out as recommended by the Society of Public Analysts (Cocks, 1933) with appropriate alterations in the volumes of reagents according to the amount of lipid saponified. With one batch of sebum (*T*) saponification was carried out in the presence of pyrogallol, using the method of Tošić & Moore (1945).

Chromatography. Alumina (British Drug Houses Ltd. 'For chromatography') which had been slightly weakened by storage was used in all experiments except one in which full-strength alumina (Savory & Moore) was used. The solvents used were light petroleum (b.p. 40–60°), 5 % (v/v) and 10 % (v/v) $CHCl_3$ in light petroleum (referred to below as 5 % $CHCl_3$ and 10 % $CHCl_3$, respectively), $CHCl_3$, ether and methanol.

An approximately 1 % solution of the unsaponifiable matter in warm light petroleum was prepared (when this solution was allowed to stand, a fraction tended to separate out and in some experiments this insoluble fraction was examined separately; see below, and also Table 1). This solution was filtered through a column of alumina (for a 1 g. sample a column approximately 1·4 × 10 cm. was prepared from 20 g. alumina) and the column was then eluted with light petroleum, 5 % $CHCl_3$, 10 % $CHCl_3$, $CHCl_3$ (or ether) and methanol in succession. During the development of the chromatogram a series of bands was obtained which fluoresced in ultraviolet light. In earlier experiments the elution of these bands was followed and various fractions were collected and examined. In later work the eluates were collected in 100 or 150 ml. portions and the weight of dissolved matter in each portion was determined. By plotting these weights graphically the development of the chromatogram could be assessed.

Chemical and physical constants. Iodine numbers were determined in samples of about 3 mg. by the method of Yasuda (1931), and acetyl numbers by the method of West, Hoagland & Curtis (1934) modified so that samples of 30–100 mg. could be analysed. Molecular weights were determined by Rast's camphor method; all other microanalyses were made by Weiler and Strauss of Oxford. All melting points recorded are uncorrected.

Chemical tests for vitamins

Vitamin E. The total $FeCl_3$-reducing substances were determined by the method of Emmerie & Engel (1938, 1939). Attempts were then made to remove non-tocopherols by saponification (Emmerie, 1940), by treatment with 85 % (v/v) H_2SO_4 (Parker & McFarlane, 1940) and by chromatography (Tošić & Moore, 1945). Finally, the effect of

acetylation (Emmerie & Engel, 1943) was tried both on the total unsaponifiable matter and on the fractions obtained in the chromatographic separation (method of Tošić & Moore, 1945). In all these experiments control tests were made with sebum to which had been added a small known amount of α-tocopherol.

Other vitamins. The colorimetric Carr & Price (1926) test was used for vitamin A, but since this invariably gave turbid solutions when applied to sebum, tests were also made with activated dichlorohydrin (Sobel & Snow, 1947) which gave no turbidity. Rosenheim's (1929) test with 90% trichloroacetic acid was used for ergosterol and similar provitamins D; according to Schoenheimer & Evans (1936) 7-dehydrocholesterol and 22-dihydroergosterol give positive reactions in this test.

The colorimetric test of Irreverre & Sullivan (1941) was used to test for vitamin K. It is claimed that this test is specific for 2:3-dialkyl-substituted α-naphthaquinones and that it is not given by the synthetic substitute, 2-methyl-1:4-naphthaquinone. It was not possible for us to carry out control tests with sebum to which had been added the natural vitamin, for our efforts to obtain a pure sample of this vitamin were unsuccessful.

Tests were also made for the presence of two water-soluble vitamins, in case these vitamins were secreted in the sweat and carried over into the sebum. The fluorimetric thiochrome method was used for thiamine, and a photometric 2:6-dichlorophenolindophenol method for ascorbic acid. In these two cases the tests were made on acid extracts of sebum, using 0·1 N-HCl and 10% (v/v) acetic acid, respectively.

Wherever possible control tests were also made with sebum containing added vitamin, in order to assess the sensitivity of the tests and the possibility of interference by other constituents of the sebum.

RESULTS

Properties of fractions obtained by chromatography

Eight batches of sebum (*D*, *E*, *F*, *H*, *K*, *S*, *T* and *U*, obtained, respectively, from groups of 25, 82, 22, 23, 55, 60, 15 and 43 normal medical students) were fractionated by the chromatographic method. Five principal fractions were obtained from each batch, and details of the relative amounts of the fractions obtained from each of the six principal batches are given in Table 1. The fractions from each batch were studied separately and the composite results of these studies are as follows.

Fraction I. This fraction was a white or pale-yellow semi-solid wax composed of hydrocarbons, with an average iodine number of 130–170. Squalene accounted for 30–40% of this fraction. Recrystallization of the crude fraction from ethanol gave a solid hydrocarbon, m.p. 57·6 and 56·2°; mol.wt. (Rast) 428. In order to decide whether this solid hydrocarbon was a normal or a branched-chain paraffin the infrared absorption of a sample was examined (curve *A*; Fig. 1). This showed that this substance is a normal-chain paraffin, and it is suggested that it is probably pentacosane (m.p. 55·5–56·0°; Heilbron, 1936), or a mixture of closely related paraffins.

Table 1. *Chromatographic fractionation of the unsaponifiable matter of six batches of mixed human sebum, using alumina*

	Batch					
Fraction*	*E* (%)	*F* (%)	*H* (%)	*K* (%)	*S* (%)	*T* (%)
I	45·6	37·5	37·3	39·7	30·0	40·3
II	17·6	21·6	23·1	23·7	25·1	18·6
III	13·8	16·3	14·8	13·7	18·8	16·1
IV	4·0	7·1	10·8	6·2	11·8	6·4
V†	2·2 (6·1)‡	6·3	6·4	9·1	6·2 (12·6)‡	13·2

* For details of the separation of these fractions see the text.
† This fraction contained a small amount (about 5% of its weight) of an aluminium compound.
‡ Including the material insoluble in light petroleum.

The crude hydrocarbon fraction gave an immediate green coloration which slowly changed to brown in the Liebermann-Burchard test; this suggested the presence of substances other than paraffin and squalene (e.g. *tetracyclo*squalene; cf. Sobel, 1949). In an attempt to obtain further information about this fraction the infrared spectrum of the material which remained after removal of the solid hydrocarbon was examined. The result (curve *B*; Fig. 1) was, however, not conclusive.

After the hydrocarbon fraction had been eluted from the column a band of yellow pigment followed. At first it was suspected that this compound was a carotene, but further tests showed that it was not of this type. Its nature has not yet been established.

Fraction II. The crude fraction was a pale-yellow wax, m.p. 48–52° and iodine number 40. It gave an unusual yellow coloration with a green fluorescence in the Liebermann-Burchard test. Refractionation on full-strength alumina yielded two fractions; a white, saturated wax, m.p. 49–50°, and a pale-yellow, viscous oil. The white wax on recrystallization from acetone gave a substance, m.p. 62·5°, acetyl number 167, which yielded an

acetyl derivative with m.p. 48·3°. The same substance (m.p. 64 and 65°; acetyl derivative, m.p. 48·8°) was obtained by crystallizing the crude fraction from acetone without preliminary rechromatography. (Found: C, 79·2; H, 13·5. Calc. for $C_{20}H_{42}O$ (eicosanol): C, 80·5; H, 14·1%.) It is thought that this substance is eicosanol (m.p. 65·5–66·5° (Levene & Taylor, 1924), 70° (Ameseder, 1907); acetyl number, 168; acetyl derivative, m.p. 44° (Ameseder, 1907)), but it has not yet been characterized to our satisfaction.

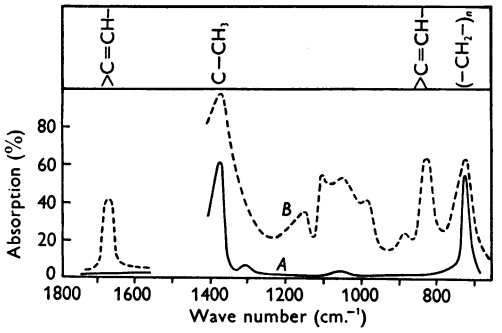

Fig. 1. Infrared absorption spectra of fractions *A* (——, in CS_2 solution) and *B* (- - - -, liquid film 50 μ. thick) from the hydrocarbons (fraction I, Table 1) of the unsaponifiable matter of human sebum. *A* contains the solid saturated hydrocarbons and *B* the residue of fraction I after removal of *A*. (Measurements made by Dr H. P. Koch and Miss J. M. Fabian, using a Grubb-Parsons single-beam instrument.)

The yellow viscous oil obtained on rechromatographing the crude fraction gave the above-mentioned unusual coloration in the Liebermann-Burchard test. This material on analysis gave C, 75·1; H, 11·9; mol.wt. (Rast) 497 and 477 (calc. for $C_{30}H_{50}O_4$: C, 75·9; H, 10·6%; mol.wt. 474) and it appeared to be identical with the primary thermal oxidation product of squalene described by Bolland & Hughes (1949).

Fraction III. This fraction consisted of practically pure cholesterol with traces of pigments. Recrystallization from ethanol gave typical cholesterol crystals, m.p. 148°, and an acetyl derivative, m.p. 114°, was also prepared. Both the crude fraction and the recrystallized material gave a negative Rosenheim (1929) test.

Fraction IV. The crude fraction was a brown gum which gave a positive Liebermann-Burchard reaction, and an immediate brown coloration which slowly changed to green or blue-green in the Rosenheim test. Recrystallization from acetone yielded a small amount of a dirty-white solid, m.p. 76–77° (another sample gave m.p. 95°). Treatment of the crude fraction with a solution of digitonin in methanol yielded a copious precipitate apparently of unchanged digitonin. The material not precipitated by digitonin was a brown gum and gave positive Liebermann-Burchard and Rosenheim tests. The colour obtained in the latter test was qualitatively different from that produced by ergosterol, an observation which was confirmed by the spectrophotometric investigations of Festenstein & Morton (1952). Mr G. N. Festenstein in Prof. Morton's laboratory kindly examined the ultraviolet absorption spectrum of this material and found no evidence of provitamins D_2 and D_3 (see Festenstein & Morton, 1952). If sebum contained such provitamins it is probable that the bulk of them would be in this fraction. This fraction thus contains an unidentified sterol or sterol-like substance which is not precipitated by digitonin and is difficult to crystallize.

Fraction V. This fraction was a dirty-white wax-like substance which gave a brown coloration in the Liebermann-Burchard test. Recrystallization from methanol gave a white amorphous substance, m.p. 79–81° and 78–82°; mol.wt. (Rast) 717.

Fraction insoluble in light petroleum. This fraction on recrystallization from methanol gave a white wax, m.p. 78–79°, which on analysis gave C, 73·1; H, 12·1%; mol.wt. (Rast) 746. The nature of this fraction, which appears to be identical with fraction V, has not been established, but it is believed to contain oxidation products of squalene.

Chromatography of oxidized squalene. Some of the above-mentioned fractions almost certainly contained oxidation products of squalene, and in order to obtain information about the behaviour of these compounds on alumina columns a sample of squalene that had undergone autoxidation was fractionated by the chromatographic method.

Unchanged squalene (28% of the total material) was eluted first (i.e. corresponding to fraction I). Then came a colourless oil (7%) corresponding to fraction II; this gave the yellow-brown coloration with green fluorescence in the Liebermann-Burchard reaction. A yellow viscous oil (35·4%) was eluted with $CHCl_3$ (corresponding to fraction IV of sebum) and this gave a dark-brown coloration in the Liebermann-Burchard test. The remainder of the material was eluted slowly with methanol (corresponding to fraction V of sebum), and consisted of a dirty-white substance which gave a dark brown Liebermann-Burchard reaction. It would appear, therefore, that fractions II, IV and V of sebum might be expected to contain any squalene oxidation products present in the unsaponifiable matter of sebum.

In order to decide whether these oxidation products were present in sebum prior to collection or were produced during the subsequent manipulations, one batch of sebum (*T*) was collected under conditions designed to reduce oxidation to a minimum. The acetone was removed by distillation in N_2 and under reduced pressure, on the day of collection, and the sebum was then saponified in the presence of pyrogallol using the method of Tošić & Moore (1945). Oxidation products of squalene were still apparently present, and fraction II still gave the peculiar yellow-brown Liebermann-Burchard coloration.

From the results of these experiments it is concluded that some oxidation of squalene takes place during the normal removal of acetone and subsequent saponification, but that significant amounts of squalene oxidation products are present in the sebum prior to its removal from the skin.

practically all the $FeCl_3$-reducing substances from sebum, but added α-tocopherol was also destroyed; this modified method is, therefore, useless for tocopherol determinations on sebum. With another batch of sebum (*O*) a comparison was made of the amounts of non-saponifiable, acidic and neutral

Table 2. *Tocopherol determinations on human sebum*

Method	Sebum (mg.)	Tocopherol added (mg.)	Tocopherol found (mg.)	Recovery of added tocopherol (%)	Apparent tocopherol content of sebum (mg./g.)
Emmerie (1940) (saponification)	—	0·1	0·048	48	
	200	—	0·085	—	0·42
	200	0·05	0·105	40	
Tošić & Moore (1945) (saponification in the presence of pyrogallol)	—	0·05	0·038	76	
	200	—	0·084	—	0·42
	200	0·05	0·116	64	
Parker & McFarlane (1940) (removal of 'impurities' with 85% (v/v) H_2SO_4)	—	0·06	0·057	95	
	200	—	0·009	—	0·05
	200	0·06	0·020	18	

Vitamin E and other $FeCl_3$*-reducing substances*

One of the most widely used chemical methods for determining vitamin E is that in which the colour given with ferric chloride and 2:2′-dipyridyl is measured colorimetrically (Emmerie & Engel, 1938, 1939). Determination of the total $FeCl_3$-reducing substances in sebum by this method showed that appreciable amounts (0·8–3·3 mg./g. sebum, calculated as α-tocopherol) were present, while recovery of added α-tocopherol was found to

Table 3. $FeCl_3$*-reducing substances in sebum*

	(Calc. as mg. α-tocopherol/g. of sebum)
Total	1·78
Acidic	1·35
Neutral*	0·59

* A similar value (0·60) was obtained with the unsaponifiable fraction of this sebum. This would suggest that the loss of reducing substances during the saponification was balanced by reducing substances liberated by hydrolysis of esters.

be fairly satisfactory (82%). Further study was made of these $FeCl_3$-reducing substances to decide whether, in fact, tocopherols were present. The effect of saponification, and of treatment with 85% (v/v) sulphuric acid, was examined first. The results (Table 2) showed that significant amounts of $FeCl_3$-reducing substances were present in the unsaponifiable fraction, though the control tests showed that appreciable destruction of added α-tocopherol occurred even when the saponification was carried out in the presence of pyrogallol as advocated by Tošić & Moore (1945). Treatment with 85% (v/v) sulphuric acid (Parker & McFarlane, 1940) removed $FeCl_3$-reducing substances present, and it was found (Table 3) that the largest amount was in the acidic fraction, the neutral or unsaponifiable fractions containing only about a third of the total $FeCl_3$-reducing substances. The acidic fraction can be removed by extracting an ethereal solution of sebum with aqueous 1% potassium hydroxide.

Table 4. *Tocopherol determinations on sebum after chromatography and acetylation*

	Unsaponifiable $FeCl_3$-reducing substances	
	Sebum (mg./g. sebum)	Sebum plus α-tocopherol* (mg./g. sebum)
(*a*) Before passing through column		
Before acetylation	0·44	0·85 (recovery 82%)
After acetylation	0·22	0·29
(*b*) After chromatography		
Unadsorbed:		
Before acetylation	0·19	0·46 (recovery 54%)
After acetylation	0·08	0·08
Adsorbed:		
Before acetylation	0·15	0·15
After acetylation	0·0	0·0

* 0·5 mg. α-tocopherol added/g. of sebum.

The unsaponifiable $FeCl_3$-reducing substances were further investigated. The chromatographic method of Tošić & Moore (1945) was used, and determinations were made of the $FeCl_3$-reducing power of the adsorbed and unadsorbed fractions before and after they had been acetylated (tocopherol esters do not reduce ferric ions). In this chromatographic method α-tocopherol is not adsorbed by the column, whereas most of the β-

tocopherol is adsorbed and many interfering substances are completely adsorbed. The results (Table 4) show that the α-tocopherol content of these sebum samples was probably not greater than 0·2 mg./g. sebum, even if allowance is made for a loss of 50 % of the vitamin in the saponification and chromatographic separation. These results suggest that only a trace of vitamin E is present in sebum, but that other $FeCl_3$-reducing substances are present in the unsaponifiable fraction.

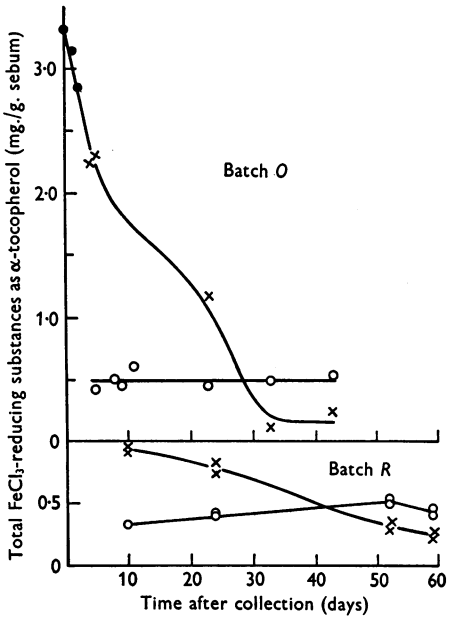

Fig. 2. The effect of autoxidation on the total (×) and non-saponifiable (○) $FeCl_3$-reducing substances of two batches (*O* and *R*) of human sebum. Earlier determinations of the total (●) $FeCl_3$-reducing substances of batch *O* were made on the acetone solution of sebum.

Since appreciable amounts of $FeCl_3$-reducing substances are present in sebum it was thought that they might serve as antioxidants. The effect of autoxidation on the reducing capacity of sebum was therefore studied.

Two batches of sebum were kept at room temperature in closed desiccators containing air. Samples were removed at intervals and determinations of total and unsaponifiable $FeCl_3$-reducing substances were made, the method of Tošić & Moore (1945) being used for the saponification. With one batch of sebum (*O*) the amount of $FeCl_3$-reducing substances in the acetone solution of sebum was determined, without removal of the acetone, immediately after collection and at intervals during the next 3 days.

The results (Fig. 2) show that the acidic $FeCl_3$-reducing material is rapidly oxidized, while the non-saponifiable substance remains constant for many days. The observation that, after prolonged storage of the sebum, the amount of unsaponifiable $FeCl_3$-reducing material is apparently greater than the total, may be accounted for by the liberation of $FeCl_3$-reducing substances during the saponification or to the presence of interfering substances in untreated sebum, or to both factors.

In some preliminary investigations we have fed considerable amounts of mixed tocopherols (the equivalent of 100–300 mg. of α-tocopherol/day, for up to 42 days) to two subjects, in an attempt to increase the excretion of vitamin E in the sebum. It was thought that any evidence of increased secretion of the vitamin might be of interest in view of the fairly frequent administration of relatively large doses of tocopherols to patients suffering from certain skin diseases. The amount of sebum collected from the forearms of these subjects was insufficient for chromatographic treatment of the unsaponifiable matter, and this investigation was limited to a determination of the total $FeCl_3$-reducing substances in the untreated sebum. These values showed considerable fluctuation from day to day, with each of the subjects studied, but the general conclusion drawn from the results was that the administration of large amounts of tocopherols did not lead to any significant increase in the excretion of $FeCl_3$-reducing substances.

Vitamins other than tocopherols

Many samples of fresh sebum collected from individual subjects and also a mixed sample from fourteen subjects were tested for vitamins A, K, B_1 and C and provitamin D, using about 100 mg. of sebum for each test. Tests were also made on specimens containing added vitamin, so that an upper limit could be put to the amount of vitamin that is detectable by these chemical tests under the conditions of our experiments. The results obtained are shown in Table 5, together with the results of the vitamin E determinations. Sebum thus appears to contain little, if any, of the vitamins included in this list. It was not considered, however, that these colorimetric methods were sufficiently reliable or sensitive for these results to be accepted without confirmation, and at our request Prof. R. A. Morton kindly agreed to examine the ultraviolet absorption spectra of several batches of sebum. The results of

Table 5. *Colorimetric tests for vitamins in sebum*

Vitamin	Calculated limit of detection (per g. sebum)	Amount found (per g. sebum)
A	10 i.u.	None
Pro-D_2 and D_3	0·3 mg.	None
E	0·01 mg.	0·2 mg.
K	0·1 mg.	None
B_1	1·5 μg.	None
C	0·05 mg.	None

these studies, a preliminary report of which has already been made (Festenstein & Morton, 1951), confirm our observations that vitamins A and K, β-carotene and provitamins D_2 and D_3 cannot be detected in human sebum, and that only a trace, if any, of vitamin E is present (cf. also Festenstein & Morton, 1952).

DISCUSSION

In a previous investigation (MacKenna *et al.* 1950) it was found that human sebum contains about 32% of unsaponifiable matter, including 5% of squalene. The results recorded here show that the chief constituents of this unsaponifiable matter are hydrocarbons (30–46%), normal-chain aliphatic alcohols (14–20%) and cholesterol (14–19%). The remainder consists of oxidation products of squalene and several other unidentified substances, including a 'non-cholesterol' sterol-like compound.

Squalene accounts for 30–40% of the hydrocarbons, and the remainder of this fraction is mainly normal paraffins, though evidence suggesting the presence of another unsaturated hydrocarbon besides squalene has been obtained. The presence of hydrocarbons in sebum is of special physiological interest. It is possible that this elimination of hydrocarbons merely represents the excretion of metabolically useless substances, but there is also the possibility that some of them, in particular squalene, are important metabolites. A more complete examination is now being made of hydrocarbon excretion in the sebum by normal individuals and by those suffering from various skin disorders, in order to obtain information about the possible functions of these compounds in skin metabolism. Squalene is present also in ovarian dermoid cyst lipids and other sebum-like materials (Dimter, 1932; Sobel, 1949) and the structural relationship between this hydrocarbon and carotenoids, some of the fat-soluble vitamins (e.g. A and D) and the sterols and steroids should not be overlooked.

Squalene ($C_{30}H_{50}$) contains six double bonds, and it is fairly readily oxidized (Chapman, 1917). Oxidation yields several products, but Bolland & Hughes (1949) found that the chief product formed in the early stages of oxidation by oxygen at 55° is a diperoxide in which four atoms of oxygen are combined with 1 squalene molecule. Little is known, however, of other oxidation products, though it is believed that some have a cyclic structure. Oxidation of squalene is accelerated by ultraviolet irradiation (Farmer & Sutton, 1942), and we were not surprised to find that part of the squalene in human sebum is present in an oxidized form. Sebum forms a thin film on the skin, with an average thickness of about 0·7 μ., calculated from our yields from the forearm, and at temperatures approaching 37°, and with occasional exposure to ultraviolet rays, oxidation of squalene and other unsaturated compounds in sebum will undoubtedly occur. Our results indicate that untreated human sebum contains several oxidation products of squalene, but because of the small amounts present and the relative lack of information about the chemical nature of the oxidation products, we have not yet been able to identify these compounds.

No evidence has been obtained that human sebum contains 'isocholesterol' (lanosterol and agnosterol). These triterpene alcohols apparently occur only in the sebum of certain animals, notably the sheep, goat, llama and dromedary (Lederer & Kiun, 1945). Our observation provides a further example of the considerable differences between the sebum of different species of animals.

Human sebum contains appreciable amounts of substances capable of reducing ferric chloride under the conditions usually adopted in the Emmerie & Engel (1938, 1939) colorimetric method for determining vitamin E, or in various modifications of this method (cf., for example, Emmerie, 1940, 1949; Tošić & Moore, 1945). On the basis of these results, and in view of the fact that this method is widely used for the determination of the vitamin E content of blood, body fats and foods, it was concluded (MacKenna *et al.* 1950) that human sebum contains significant amounts of this vitamin. Our further investigations, in which we have used several methods for excluding non-tocopherol reducing substances, have shown, however, that the amount of vitamin E in sebum is in fact small; it is probably not greater than the amount present in adipose-tissue fat and other body fats. The problem of the chemical assay of vitamin E is 'complicated by the plurality of tocopherols having different activities, by the interference of other inactive compounds, and by the instability of tocopherols during the manipulations' (Eden & Booth, 1950). Thus it seems probable that a reliable value for the vitamin E content of human sebum will only be possible when a large amount of sebum can be collected for the more specific but laborious bioassay. It is of interest, however, that Festenstein & Morton (1951, 1952) find that our samples of human sebum contain little, if any, vitamin E detectable by ultraviolet absorption. We have not yet established the nature of the non-tocopherol substances reducing ferric chloride present in human sebum, but the main constituent of this group is saponifiable and is apparently of acidic (or phenolic) nature. It seems probable that this compound (or compounds) may have a valuable antioxidant effect in sebum, thus retarding the oxidation of important unsaturated fatty acids and any vitamin E present.

An exhaustive examination of human sebum for fat-soluble vitamins other than vitamin E has not been possible, but chemical tests have failed to reveal the presence of any of these vitamins in amounts which would be significant. The absence of vitamin A (and β-carotene) is interesting in view of the function of this vitamin in maintaining normal keratinization of the skin. Our observation in this respect is consistent with the finding of Cornbleet & Popper (1942) that vitamin A cannot be detected in the epidermis of man though significant amounts are present in the adjacent fatty tissues.

Our inability to detect any provitamin D_2 and D_3 in human sebum is of special interest. Provitamin D_3 (7-dehydrocholesterol) has been isolated from pig skin by Windaus & Bock (1937) and has been shown, by ultraviolet spectrum methods, to be present in chicken skin (Koch & Koch, 1941) and in frog ovaries (Morton & Rosen, 1949). Hou (1930) has suggested that in the rat vitamin D is formed in the sebum. Helmer & Jansen (1937) have shown that irradiated human surface skin fat and also the surface skin fat from irradiated human subjects possess antirachitic activity. There is therefore a strong indication that in man vitamin D may be formed in the sebum, but it is difficult to reconcile this theory with the absence of provitamins D_2 and D_3 from human sebum unless the provitamin is another, as yet unidentified, substance. From this point of view the unidentified 'sterol-like' substance in fraction IV is of interest since it gives a positive Rosenheim reaction. Some of the properties of this compound are similar to those recorded for 7α-hydroxycholesterol, but as yet we have been unable to precipitate it with digitonin. A further study of this compound is being made as part of a wider investigation on the mode of formation of vitamin D by the skin.

SUMMARY

1. A study has been made of the unsaponifiable matter of human sebum. Chromatographic analysis yielded hydrocarbons (30–46 % of the unsaponifiable matter), cholesterol (14–19 %), normal-chain aliphatic alcohols (about 20 %) and some unidentified substances including oxidation products of squalene.

2. The hydrocarbon fraction contains 30–40 % squalene, and a normal-chain paraffin (possibly pentacosane).

3. The principal sterol present is cholesterol, but another sterol-like substance is also present.

4. No vitamin A, β-carotene, vitamin K, or provitamins D_2 and D_3 could be detected by chemical tests on sebum or the separated fractions.

5. Sebum contains appreciable amounts of ferric chloride-reducing substances. Part of this material is probably vitamin E, but not more than the equivalent of 0·2 mg. of tocopherol per gram of sebum. The principal reducing substance is an acidic (or phenolic) compound which may function as an antioxidant in sebum.

The authors wish to express their gratitude to Prof. R. A. Morton, F.R.S., and Mr G. N. Festenstein for their collaboration in the ultraviolet spectrum examinations, and to those medical students, technicians, research workers and teaching staff of our College who acted as subjects for these investigations. Thanks are also tendered to the British Rubber Producers' Research Association for generous samples of pure squalene, and to two members of their staff, Dr H. P. Koch and Miss J. M. Fabian, for kindly carrying out the infrared spectrum determinations. We are also indebted to Messrs Glaxo Laboratories Ltd. for a supply of mixed tocopherols. Part of the expenses of the investigation were covered by expenses grants to one of us (A.W.) by the Medical Research Council and the Central Research Fund of London University.

REFERENCES

Ameseder, F. (1907). *Hoppe-Seyl. Z.* **52**, 121.
Bolland, J. L. & Hughes, H. (1949). *J. chem. Soc.* p. 492.
Carr, F. H. & Price, E. A. (1926). *Biochem. J.* **20**, 497.
Chapman, A. C. (1917). *J. chem. Soc.* p. 56.
Cocks, L. C. (1933). *Analyst*, **58**, 203.
Cornbleet, T. & Popper, H. (1942). *Arch. Derm. Syph., Chicago*, **46**, 59.
Dimter, A. (1932). *Hoppe-Seyl. Z.* **208**, 55.
Eden, E. & Booth, V. H. (1950). *Food Manuf.* **25**, 279.
Emmerie, A. (1940). *Rec. Trav. chim. Pays-Bas*, **59**, 246.
Emmerie, A. (1949). *Ann. N.Y. Acad. Sci.* **52**, 309.
Emmerie, A. & Engel, C. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 1351.
Emmerie, A. & Engel, C. (1939). *Rec. Trav. chim. Pays-Bas*, **58**, 895.
Emmerie, A. & Engel, C. (1943). *Z. Vitaminforsch.* **13**, 259.
Farmer, E. H. & Sutton, D. A. (1942). *J. chem. Soc.* p. 139.
Festenstein, G. N. & Morton, R. A. (1951). *Biochem. J.* **48**, xxxix.
Festenstein, G. N. & Morton, R. A. (1952). *Biochem. J.* **52**, 168.
Heilbron, J. M. (1936). *Dictionary of Organic Compounds*, 1st ed., vol. 2. London: Eyre and Spottiswoode.
Helmer, A. C. & Jansen, C. H. (1937). *Stud. Inst. Divi Thomae*, **1**, 207.
Hou, H. C. (1930). *Chin. J. Physiol.* **4**, 345.
Irreverre, F. & Sullivan, M. X. (1941). *Science*, **94**, 497.
Koch, E. M. & Koch, F. C. (1941). *Poult. Sci.* **20**, 33.
Lederer, E. & Kiun, T. P. (1945). *Bull. Soc. Chim. biol., Paris*, **27**, 419.
Levene, P. A. & Taylor, F. A. (1924). *J. biol. Chem.* **59**, 905.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1951). *Biochem. J.* **48**, xxxviii.
Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.

Parker, W. E. & McFarlane, W. D. (1940). *Canad. J. Res.* **18** (B), 405.
Ricketts, C. R., Squire, J. R. & Topley, E. (1948). Personal communication.
Ricketts, C. R., Squire, J. R. & Topley, E. (1951). *Clin. Sci.* **10**, 89.
Rosenheim, O. (1929). *Biochem. J.* **23**, 47.
Schoenheimer, R. & Evans, E. A. (1936). *J. biol. Chem.* **114**, 567.
Sobel, A. E. & Snow, S. D. (1947). *J. biol. Chem.* **171**, 617.
Sobel, H. (1949). *J. invest. Derm.* **13**, 333.
Tošić, J. & Moore, T. (1945). *Biochem. J.* **39**, 498.
West, E. S., Hoagland, C. L. & Curtis, G. H. (1934). *J. biol. Chem.* **104**, 627.
Windaus, D. & Bock, F. (1937). *Hoppe-Seyl. Z.* **245**, 168.
Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

Spectrophotometric Studies on Human Sebum

By G. N. FESTENSTEIN and R. A. MORTON
Department of Biochemistry, The University of Liverpool

(*Received* 6 *November* 1951)

The absorption spectra of the samples investigated were examined between 230 and 500 mμ., with special reference to the ultraviolet region of the spectrum; in particular it was sought to establish evidence for vitamin E.

α-, β-, γ- and δ-tocopherols show an absorption band near 295 mμ., $E^{1\%}_{1\,cm.}$ about 80. α-Tocopherol acetate shows a maximum at 285 mμ., $E^{1\%}_{1\,cm.}$ about 40. MacKenna, Wheatley & Wormall (1950, 1951, 1952) have found sebum to contain appreciable amounts of $FeCl_3$-reducing substances; part of this material is stated to be vitamin E, the amount not exceeding 0·2 mg. tocopherol per gram of sebum.

The investigations to be described were carried out using sebum samples of about 1 g. A simple calculation shows that under such circumstances detection of tocopherol must be difficult: if the whole amount (0·2 mg.) of tocopherol can be concentrated in a volume of 4 ml. (in a 1 cm. cell) then the extinction value at 295 mμ. will only be 0·4 (by way of comparison as small a quantity as 0·009 mg. vitamin A will give a similar extinction value at its absorption maximum). The chromatographic separation must be such as to concentrate the tocopherol in one fraction. Furthermore, other absorbing materials will have to be eliminated for the maximum at 295 mμ. to be evident.

A preliminary report of this work has already been given (Festenstein & Morton, 1951). Two samples of sebum and sebum unsaponifiable material were investigated. In addition, a further sample of unsaponifiable material, which had been assayed for $FeCl_3$-reducing substances by MacKenna *et al.* was examined; also a 'sterol-like' fraction provided by the same workers.

EXPERIMENTAL

Chromatography. Separations were carried out using Spence's grade 0 alumina (weakened by controlled addition of water in some cases); chromatographic tubes of 9 and 14 mm. internal diameter were used, depending on the amount of material.

The sebum or sebum fraction was usually dissolved in the minimum amount of light petroleum (A.R. quality) and the column developed at first with this solvent alone; any residue was discarded. Mixtures of ether and light petroleum, ether, methanol and ethanol were used as eluants. Arbitrary fractions were collected, but where distinct bands appeared to be eluting the fractionation was extended. All fractions were taken to dryness and weighed before spectroscopic examination; in the later work particularly, evaporation of solvent was carried out in an atmosphere of CO_2 or N_2, to minimize oxidation of any tocopherol.

The ether used was peroxide-free (usually distilled over reduced Fe before use).

Spectroscopy. Absorption spectra were determined quantitatively using a Beckman photoelectric spectrophotometer. Ethanol and *cyclo*hexane were used as solvents. Readings were taken every 5 mμ. in the range 230–310 mμ., and every 10 mμ. in the range 310–500 mμ.

RESULTS

Sebum. The sebum itself showed mainly end absorption, though there was a suggestion of an inflexion between 270 and 300 mμ. (Fig. 1).

Chromatography resulted in a good fractionation (cf. Table 1), but no selective absorption obviously characteristic of vitamins E appeared in any fraction (Figs. 2 and 3). Since, however, fraction 2 (sample 1, Table 1) showed a faint inflexion near 290 mμ., it was rechromatographed (Table 2), and absorption again examined (Fig. 4). No real evidence is found for the presence of tocopherol in any of these fractions. At least a marked inflexion at 290 mμ. should be shown to be indicative of tocopherol.

In view of the low recoveries from the chromatography of the sebum (57 % even after elution with ethanol) it was decided to remove the free fatty acids prior to chromatography, lest these should prevent the elution of any tocopherol.