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Studies of Sebum

4. THE ESTIMATION OF SQUALENE IN SEBUM AND SEBUM-LIKE MATERIALS

By V. R. WHEATLEY

Departments of Biochemistry and Dermatology, Medical College of St Bartholomew's Hospital, London, E.C. 1

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Early in the present investigations it was found that squalene is a normal constituent of human sebum (MacKenna, Wheatley & Wormall, 1950); at about the same time Sobel (1949) showed that squalene is also a component of human ear wax, smegma and hair fat. In order to study the metabolism of this compound and its possible function in maintaining the health of the skin, it was necessary to devise a suitable method of estimation that could be applied to those small amounts of sebum that can be obtained from small, measured areas of skin.

Squalene is a rather inert substance and only two methods of estimation are available. First, it has a high iodine number (370) and an iodometric method of analysis was devised by Fitelson (1943) to estimate the squalene content of edible oils. Secondly, it gives a brown coloration in the Liebermann-Burchard test; this test has the disadvantage that colour develops slowly, though it can be made to develop more rapidly at slightly raised temperatures and if acetic anhydride is used as solvent instead of chloroform (Sobel, 1949). The hydrocarbon fraction of a lipid can be separated by chromatography on alumina after saponification and either the iodometric method or the colour test applied. These methods have been investigated and the conditions for best recovery established.

The previous paper in this series (Hodgson-Jones & Wheatley, 1952) dealt with the collection and estimation of sebum.

EXPERIMENTAL

Methods for estimating pure squalene

Colorimetric method

As described by Sobel (1949), this method consists of treating the sample of squalene (0·7 mg.) with 2 ml. of acetic anhydride and 5 drops of H_2SO_4. The solution thus obtained is placed in a water bath at 38° for 30 min.; 5 ml. of $CHCl_3$ are then added. The resulting colour is an indefinite brown with greatest absorption at 400 mμ. but no well-defined maxima. It was observed that the colour did not completely develop in 30 min. and also that an increase in temperature hastened the rate of colour development; this effect was therefore studied in greater detail.

Effect of temperature on the rate of colour development. Samples of squalene (0·7 mg.) were treated with acetic anhydride (2 ml.) and H_2SO_4 (5 drops), then placed in a thermostat bath within the range 19–68°. At intervals duplicate samples were removed, 5 ml. $CHCl_3$ were added and the resulting colours read in the colorimeter (Evans Electroselenium portable model fitted with Ilford spectrum blue filter no. 621). Blank determinations were made with reagents incubated for the same length of time. Five series of determinations were performed at different temperatures and the results obtained (Fig. 1) show that the rate of colour development increases with increasing temperature, and even at the highest temperature studied does not appear to have reached a maximum in 30 min. At higher temperatures the blank reading becomes significant, and above 68° an intense colour develops rapidly in the blank, obscuring any colour produced by squalene in the test. The most convenient conditions for colour development appeared to be

2 hr. at 38°, provided that the temperature was accurately controlled.

Calibration curve. A series of determinations was performed on quantities of squalene in the range 0·25–2·0 mg., the colour being allowed to develop at 38° for 2 hr. The results obtained showed that the colour was directly proportional to concentration but that agreement between duplicates was poor; in one case they differed by 25%.

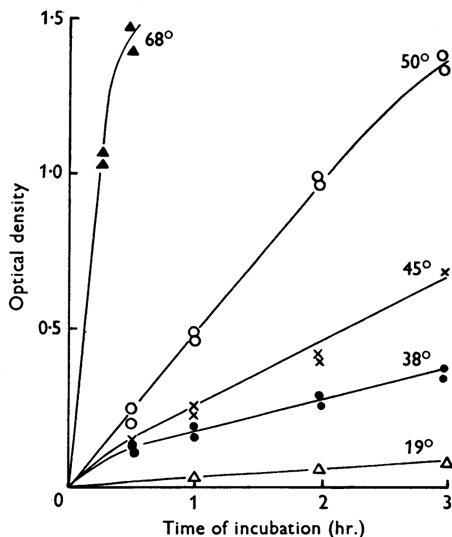

Fig. 1. Effect of temperature on the rate of colour development when squalene (0·7 mg.) is treated with acetic anhydride and H_2SO_4. Optical density refers to a depth of solution of about 1·4 cm. See text.

Effect of analytical manipulations. In an attempt to study the recovery from alumina columns it was found that, while for amounts of squalene of 1–2 mg. recovery was good (95–101%), with smaller amounts of squalene the apparent recovery exceeded the theoretical value (123% with 0·5 mg.). This was presumably due to oxidation of the squalene, since it has already been observed that oxidized squalene on treatment with acetic anhydride and H_2SO_4 develops a brown colour rapidly even at room temperature (cf. MacKenna, Wheatley & Wormall, 1952). The method is therefore sensitive to even the slightest oxidation, which can easily occur during the simplest analytical manipulations.

In view of the obvious defects of the colorimetric method attention was directed to the iodometric method.

Iodometric method

For convenience the iodine-number method developed for the estimation of small amounts of sebum was used (Hodgson-Jones & Wheatley, 1952). This proved reliable and sensitive (1 ml. 0·005 N-$Na_2S_2O_3 \equiv$ 0·171 mg. squalene). The method gave good results with known amounts of squalene, and did not appear to be sensitive to possible slight oxidation during analytical manipulations as was the colorimetric method.

Estimation of squalene in the presence of lipids

Removal of interfering substances by chromatography

The colorimetric and iodometric methods are both non-specific and can only be applied either to pure squalene, or at best to the hydrocarbon fraction isolated from a lipid, provided that other unsaturated hydrocarbons are known to be absent from the material. Removal of interfering substances is best achieved by chromatography on alumina after first saponifying the lipid. Chromatography without preliminary saponification fails to remove interfering substances. The conditions of chromatography were therefore investigated.

The effect of the activity of the Al_2O_3 on completeness of separation of squalene from its oxidation products and from saturated aliphatic alcohols was first studied using 10 cm. (15 g.) columns of Al_2O_3 and 0·5–1·0 g. materials. It was found that, when light petroleum is used as eluant, squalene is separated completely from other substances (except hydrocarbons) on grade II Al_2O_3 (Brockmann & Schodder, 1941); with grade III Al_2O_3 it is separated completely from oxidation products and almost completely from aliphatic alcohols (the first 50 ml. eluate contains practically pure hydrocarbons), but with grade IV Al_2O_3 no separation is obtained. Grade II Al_2O_3 is much too active for use with small amounts of squalene (0·5 mg.), but grade III is sufficiently active to remove both oxidation products and aliphatic alcohols when analysing small amounts of lipid.

The effect of the length of the column was also studied. Shorter columns (5 cm.; 5 g. Al_2O_3) were satisfactory in all respects and there was no advantage in using a longer column. When small amounts of squalene (0·5 mg.) are used on these columns, recovery is almost complete in 25 ml. eluate, and complete in 50 ml.

Losses on saponification and extraction

Extraction of squalene from the saponification mixture with light petroleum was first studied. It was initially planned to use the saponification method of Tošić & Moore (1945) and extraction was studied under these conditions. From a mixture of pyrogallol (2 ml. of 1% solution in ethanol), KOH (0·1 ml. of 20%) and squalene (0·5 mg.) after addition of 2 ml. water, three extractions with 5 ml. portions of light petroleum gave a recovery of only 92% but better recovery (96%) was obtained by using three portions of 10 ml. Negligible recovery (8%) was obtained if the extraction was carried out in the presence of soaps at pH 9 (5 mg. oleic acid added before extraction), but good recovery (87%) was obtained if the mixture was acidified with HCl before extraction. It was therefore necessary to acidify the saponification mixture before extraction; the fatty acids which also were extracted were removed completely at the chromatographic stage of the analysis.

If the saponification mixture plus squalene was heated on a water bath before extraction, recovery of 82% was obtained; but when the saponification was carried out in the presence of a small amount of lipid (5 mg. neat's foot oil) recovery was 86%. Finally, saponification was studied with squalene added to sebum. When the Tošić & Moore method was used recovery of only 66% added squalene was obtained; if the pyrogallol was omitted (i.e. 2 ml. ethanol only used), a recovery of 73% was obtained. If methanol was used in place of ethanol (Emmerie, 1940) recovery was greatly improved (88%). These last conditions of saponification were therefore used.

PROPOSED METHOD

Reagents

Light petroleum (*boiling range* 40–60°). This was purified by washing with conc. H_2SO_4, then thoroughly with water, drying with K_2CO_3 and finally redistilling twice. The fraction boiling below 58° was collected; this was tested by evaporating 50 ml. to dryness on a water bath; the residue was dissolved in 5 ml. CCl_4 and the iodine no. determined as described below. The petroleum is suitable for use only if there is no difference between the test and blank.

Carbon tetrachloride. This was redistilled in all-glass apparatus.

Squalene standard. A solution in hexane (0·5 mg./ml.). The squalene is first freed from oxidized material by chromatography on Al_2O_3. The solution keeps for about 6 weeks.

Pyridine dibromide reagent. The stock solution (0·1 N) is prepared by dissolving 8 g. pyridine and 10 g. H_2SO_4 separately in 20 ml. portions of glacial acetic acid. The two solutions are mixed, and to the mixture a solution of 8 g. bromine in 20 ml. glacial acetic acid is added. The resulting solution is cooled and diluted to 1l. with acetic acid. This stock solution is diluted to 0·04 N with acetic acid as needed for analysis.

Potassium iodide. Aqueous solution, 3% (w/v), prepared freshly at least once a week.

0·005 N-*Sodium thiosulphate*. Prepared freshly as required.

Other reagents. Methanol, 10 N-HCl, 20% (w/v) aqueous KOH, 1% (w/v) aqueous starch solution (indicator).

Alumina, Brockmann grade III. Activated Al_2O_3 (Savory & Moore Ltd., London) is weakened by carefully spraying with water while thoroughly mixing. When tested by Brockmann's method (for concise description see Williams, 1946) there should be a clear-cut separation of Sudan yellow and Sudan red, but no trace of Sudan yellow should appear in the eluate. The Al_2O_3 should be further tested by performing a recovery experiment with the standard squalene solution. It is suitable if a recovery of more than 96% is obtained.

Apparatus

Chromatogram tubes 1–1·2 × 30 cm., plain tubes plugged with cotton wool. Stoppered test tubes (50 mm. long) with ground-glass necks (B14). 100 ml. flat-bottomed flasks (B19 necks with stoppers).

Procedure

Saponification. The sample (about 5 mg.), usually in $CHCl_3$ solution, is introduced into a stoppered test tube; the solvent is evaporated in a stream of N_2. The sample is then dissolved in 2 ml. methanol, 0·1 ml. 20% KOH is added, the tube stoppered and placed in a water bath at 75° (±1°) for 10 min. Blank determinations should be set up at the same time. At the end of the saponification period the tubes are cooled to room temperature.

Extraction. The contents of the tubes are diluted with water (2 ml.) acidified with 10 N-HCl (0·2 ml.) and then shaken with 10 ml. light petroleum. The mixture is allowed to separate and the petroleum layer transferred to a flask by means of a teated pipette. The extraction is repeated twice more with further 10 ml. portions of petroleum and after the third extraction the tube is washed with a further 5 ml. petroleum and this washing added to the combined extract. The extract is then carefully evaporated to dryness on a water bath, the last traces being removed by means of a stream of N_2. The residue is then dissolved in 10 ml. of petroleum.

Chromatography. The column is packed immediately before use in the following manner. The end is fitted with a small plug of cotton wool, a suspension is made of about 5 g. Al_2O_3 (enough to give a 5 cm. column) in petroleum, and this is poured into the chromatogram tube and allowed to settle. When all the petroleum has drained through the column the petroleum extract is poured on to the column and the eluate collected in a second flask. The extract flask and column are washed with two 10 ml. portions of petroleum and finally with 20 ml., each being added to the column after the preceding washing has just drained through. The petroleum eluate (50 ml.) is then evaporated to dryness on a water bath, the last traces being removed by means of a stream of N_2. The residue is then dissolved in 5 ml. of CCl_4.

Iodometric estimation. To the CCl_4 solution is added 1 ml. 0·04 N-pyridine dibromide reagent; the solution is mixed and the flask stoppered and allowed to stand for 15 min. 1 ml. 3% KI is then added and the liberated iodine titrated with 0·005 N-$Na_2S_2O_3$. The amount of squalene in the sample can be calculated on the basis that 1 ml. 0·005 N-$Na_2S_2O_3 \equiv$ 0·171 mg. squalene.

RESULTS

Recovery experiments were performed in which varied amounts of squalene were added to samples of sebum of about 5 mg. The results (Table 1)

Table 1. *Recoveries of squalene added to sebum*

Sebum (mg.)	Squalene (mg.) Present	Added	Found	Additional squalene recovered (mg.)	(%)
5·04	0·377	0·142	0·480	0·103	73
6·64	0·247	0·165	0·370	0·123	75
4·25	0·257	0·167	0·375	0·118	71
4·76	0·277	0·222	0·477	0·200	90
2·93	0·256	0·234	0·475	0·219	94
6·30	0·207	0·258	0·431	0·224	87
4·25	0·257	0·318	0·476	0·219	74
6·64	0·247	0·327	0·492	0·255	75
4·76	0·277	0·390	0·576	0·299	77
2·93	0·256	0·450	0·614	0·358	80
6·30	0·207	0·471	0·621	0·414	88
				Average	80

showed that an average recovery of 80% was obtained under these conditions of analysis. Determinations were also performed of the squalene content of human sebum, obtained from the forearm by the method of MacKenna *et al.* (1950) and from the back by the swab method (cf. Rothman, 1950; carbon tetrachloride was used in place of ether), ear wax and ovarian dermoid cyst lipid. The results are shown in Table 2.

Table 2. *Squalene content of sebum and other materials*

Substance	No. of samples	Squalene (%) Range	Squalene (%) Average
Sebum (forearm)	8	3·3– 8·7	5·5
Sebum (back)	18	5·4–11·7	8·4
Ear wax	7	1·1–12·0	3·9
Dermoid cyst lipid	2	3·7, 10·2	—

The method therefore appears to be satisfactory when applied to human sebum and similar materials known to contain squalene. It will doubtless find application to other tissue lipids, but it must be stressed that the presence of squalene in the material must first be established by chemical isolation before the method is applied, and that other unsaturated hydrocarbons must be shown to be absent.

SUMMARY

1. A method of estimating unsaturated hydrocarbons is described which is applicable to small amounts (5 mg.) of sebum and similar materials, and which gives an average recovery of 80% of added squalene. After saponification, a hydrocarbon fraction is isolated chromatographically and the unsaturated material therein is determined iodometrically.

2. The method may be used for the determination of squalene in sebum and other lipids provided that the presence of squalene is first established and that no other unsaturated hydrocarbons are present.

3. Data on the Liebermann-Burchard reaction of squalene are recorded.

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A Note on the Influence of Energy Intake on Phospholipid Metabolism

BY T. W. WIKRAMANAYAKE,* H. N. MUNRO, D. J. NAISMITH AND W. C. HUTCHISON

Biochemistry Department, The University, Glasgow

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In a previous series of experiments (Munro, Naismith & Wikramanayake, 1953), it was observed that the amount of ribonucleic acid in the liver was largely determined by the amount of liver protein, but that the rate of synthesis of ribonucleic acid, as judged by ^{32}P uptake, was determined by energy intake. Campbell & Kosterlitz (1948, 1952) have demonstrated certain similarities between the metabolism of ribonucleic acid and phospholipid when rats are transferred from a protein-containing diet to a protein-free diet. In view of our findings with ribonucleic acid it was accordingly thought of interest to examine the influence of energy intake on phospholipid metabolism in the liver.

* Present address: Faculty of Medicine, University of Ceylon, Colombo.