Examination of Lipids in Human Sebum by Disk Chromatography

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The disk-chromatography technique of Hack (1953) has been used for the analysis of human sebum or, more accurately, skin-surface fat. This rapid method, with a number of detection methods for individual components, makes it possible to survey the qualitative composition of the lipid part of different natural materials.

METHODS

Collection of sebum. Samples of the sebum were obtained (Miescher & Schönberg, 1949) from the forehead of healthy white adults 16–60 years of age. Samples were analysed from 21 persons (11 men, 10 women) not suffering from skin diseases. To collect the sample, a strip of filter paper (Whatman no. 1, 3 cm. × 12 cm.), previously defatted by extraction with boiling light petroleum, was placed on the skin of the forehead lightly cleaned by a cotton wad soaked in ethyl ether. The paper was attached to the skin and kept there for 2 hr., then replaced by a new one for another 2 hr. The strips of paper with samples of sebum were kept at -10° in an atmosphere of nitrogen, then on the same day extracted in a micro-extractor (Bloor, 1943) with 20 ml. of boiling chloroform-methanol (4:1, v/v). The extract, after removal of the solvents, was weighed and stored at -10° in an atmosphere of nitrogen. On the average 21.5 mg. of sebum was obtained, i.e. 0.596 mg./cm.².

Preparation of chromatogram. For analysis the sample was dissolved in the above-mentioned mixture of chloroform-methanol, at a concentration of 1 mg./0.2 ml. About 30 μ l. of this solution was chromatographed as described by Hack (1953) on a 6 cm. diameter disk of Whatman no. 1 paper, previously extracted by a mixture of chloroform-methanol (4:1, v/v) and eluted first by chloroform-methanol mixture to a distance of 0.8 cm. (subsequently called 'the inner circle'), then by acetone to 1.5 cm. ('the middle circle') and finally by methanol to 2.5 cm. ('the outer circle'). Before addition of each further eluent the chromatogram was dried in a current of nitrogen. When separation was finished the disk chromatogram was cut into parts, each of which was treated separately by different reagents for detection of individual components.

Reactions used for identification of individual components. As an orientational test for the distribution of lipids in the chromatogram, elution by a solution of Sudan II in 50% ethanol has proved very useful. Staining is visible after about 30 min. The tests described by Hack (1953) for neutral fats (Nile blue), for cholesterol (acetic and sulphuric acid), for double bonds (osmic acid), for amino lipids (ninhydrin) and for phosphoric esters (ammonium

molybdate and H_2S) were used unmodified. In the test for plasmalogens (hydrolysis with $HgCl_2$ followed by detection of the liberated carbonyl group) the use of saturated aqueous 2:4-dinitrophenylhydrazine was found preferable to that of Schiff's reagent, and in the test for choline lipids (reduction of $K_2Cr_2O_7$ at 60°) detection of the chromium ion by immersion in a 1% ethanolic solution of diphenyl-carbazide was found preferable to the development of a haematoxylin lake as employed by Hack. Diphenyl-carbazide gives immediately a red-violet stain, which, however, is soluble in water, so that the chromatogram must be evaluated very rapidly.

In addition, free acids have been detected by the reduction of their silver salts (Inouye & Noda, 1952; Kobrle & Zahradník, 1954). The chromatogram is immersed for 3 min. in 10% $AgNO_3$, then rinsed thoroughly in water and dried at 140° for 10 min. The presence of free acids is detected by a dark-brown colour.

To corroborate the results obtained we tested the following samples belonging to groups of materials that are supposed to exist in the sebum. Lecithin (N.A.F.A., Denmark), stained yellow, dissolved in chloroform, was chromatographed by the method described above. Treatment with haematoxylin produced a grey lake in the inner circle. Reaction with ammonium molybdate produced a blue inner circle. Triolein (Merck) was stained pink by Nile blue in the middle circle (eluted by acetone). Free oleic acid (Merck) was eluted by methanol and resulted in a dark-brown stain of the outer circle after reduction of silver salt. Osmic acid immediately stained it black. Cholesterol (Lachema ČSR) was eluted by acetone in the middle circle, producing a pink stain with a mixture of sulphuric and acetic acid. Cholesteryl oleate (kindly given by Dr Č. Michalec, Caroline University, Prague) also remained in the middle acetone circle and was stained the same colour as cholesterol.

As a control measure Hack's analysis of lyophilized human blood serum was also repeated. Comparison was also made with an extract of human-heart muscle, which is known to contain phosphatides. The heart muscle was extracted also with a mixture of chloroform-methanol (4:1, v/v).

RESULTS

Samples of lipids of the sebum obtained and analysed by the method described above gave the following results.

(1) Treatment with Nile blue showed a wide pink middle circle, a dark-blue inner circle and a fainter blue outer one. Comparison with model samples indicated in the pink middle circle the

presence of neutral fats, most likely triglycerides, the blue inner circle showed the presence of lecithin and the outer circle of free acids.

(2) Sudan II gave similar results. All three circles were stained orange-red, the middle one being the widest.

(3) Treatment with potassium dichromate and haematoxylin or diphenylcarbazide stained the outer and the inner circle. The staining of the inner circle indicated the presence of choline lipids, as shown by comparison with the sample of lecithin. The stained outer circle indicated the presence of phosphatides soluble in methanol. The more convenient method of detection by diphenylcarbazide gave the same results.

(4) Plasmalogens were detected after HgCl_2 hydrolysis in the outer circle.

(5) The reaction for cholesterol and its esters was less pronounced than the other reactions because the paper was liable to disintegrate very rapidly, often before the reaction could take place. Most pronounced staining was produced in the middle circle.

(6) Double bonds were detected by osmic acid in the inner circle, very clearly in the middle circle and less clearly in the outer one.

(7) Detection of free acids by silver produced a dark-brown ring in the outer circle (free acids are eluted by methanol).

(8) The ninhydrin reaction gave positive results in the outer circle.

(9) Esters of phosphoric acid were detected in the inner and outer circles.

If light petroleum was used for extraction, instead of a chloroform-methanol mixture, the reactions for phospholipids were negative. This supports the hypothesis that phosphatides occur in the sebum as components of lipoproteins. We have not succeeded, however, in using this principle for a separate extraction of phospholipids. The result of reactions for phospholipids depended also on the quantity of the sebum. Whereas 20–50 μg . was sufficient to yield positive results for detection of other components (glycerides, cholesterol, acids), as much as 100–150 μg . was needed for detection of phospholipids. The results of the analyses on sebum, extract of lyophilized human blood serum

and extract of human heart muscle are summarized in Table 1.

DISCUSSION

Disk chromatography has separated the lipids in the skin surface film into three parts according to the three eluents employed.

Inner circle. This was stained blue by Nile blue, orange by Sudan II and the reaction for phosphatides was positive indicating the presence of lecithin. Osmic acid indicated the presence of double bonds in this group. They are either double bonds in the acid chains of lecithin, or in hydrocarbons (squalene) whose presence in the sebum has been repeatedly proved (MacKenna & Wheatley, 1950; Rothman, 1952; Hodgson-Jones & Wheatley, 1952; Horáček & Černíková, 1958).

Middle circle. The substances eluted by acetone contained the triglycerides, which are stained pink by Nile blue and orange by Sudan II. The intense reaction with osmic acid indicated the presence of double bonds in the acid residues. This circle also showed a positive reaction for cholesterol and its esters.

Outer circle. The group of substances eluted by methanol gave positive results to Nile blue (blue stain) and Sudan II, as well as the reactions for plasmalogens, free acids, free amino groups and double bonds. The reaction for plasmalogens was comparatively intense.

Our orientational method has detected in the sebum, in addition to the known triglycerides, free acids, cholesterol and its esters, and the presence of phosphatides (choline lipids and plasmalogens), which by other analytical methods have so far escaped attention (MacKenna & Wheatley, 1950; Rothman, 1952; Hodgson-Jones & Wheatley, 1952; Horáček & Černíková, 1958). Phosphatides are present in the sebum probably only in small quantities.

SUMMARY

1. Hack's (1953) rapid method of disk chromatography was used for orientational analysis of lipids of the sebum obtained from the skin of the forehead of healthy white adults.

Table 1. Examination of various materials for lipids by disk chromatography

Material	No. of examinations	For methods see text.							
		No. of positive results for							
		Neutral lipids	Double bonds	Free acids	Phosphatides (choline lipids + plasmalogens)			Cholesterol	Amino groups
Extract of heart muscle	5	5	5	3	5	5	4		
Extract of blood-plasma lipids	5	5	5	4	4	5	5		
Skin-surface fat	21	21	21	20	15	18	19		

2. The detection of individual components has been extended by the use of additional reactions.

3. This method has detected, besides other previously demonstrated substances, the presence of phosphatides in the human sebum.

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The Distribution of 'Fall-out' Radio-strontium in a Sheep Skeleton

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Since the testing of nuclear weapons began, much work has been devoted to the study of strontium metabolism in animals and man and in particular to the deposition of this element in bone. The radioactivity in both animal and human bone resulting from the ingestion of 'fall-out' radio-strontium is being measured in a number of countries, including Britain. The levels of activity encountered in these surveys are far too low to permit radioautographic distribution studies to be made and, even when radiochemical analysis and the most sensitive counting techniques are used, relatively large weights of human bone are required for accurate determinations of ^{90}Sr .

The object of this work was to determine the pattern of ^{90}Sr and ^{90}Sr in various bones of a yearling sheep, to give more information on the skeletal distribution resulting from the continuous ingestion of contaminated vegetation. Although much work has been done with single administrations of radio-strontium, little is known about the distribution in bone resulting from continuous ingestion. Sowden & Stitch (1957) estimated stable strontium concentrations in various bones from individual human beings and their results suggest that, with a constant stable strontium level in the diet, the variability in the skeleton is not great.

In order that comparatively small sections of bone could be assayed accurately, it was necessary to have the highest possible radio-strontium concentration. Bryant, Chamberlain, Morgan & Spicer (1957) have shown that in certain areas of Britain a combination of high rainfall and calcium-deficient soil results in abnormally high ^{90}Sr /calcium ratios in herbage, and this is reflected in the bones of

sheep grazing this herbage. For this experiment a sheep from such a district was selected to facilitate the assay of radio-strontium. This was a yearling animal killed in July 1957.

METHODS

The method used for the determination of ^{90}Sr and ^{90}Sr in bone was based on that described by Bryant, Chamberlain, Morgan & Spicer (1956). After strontium carrier had been added to the ashed bone, successive separations with fuming nitric acid were used to remove the calcium. Possible radium contamination was removed by barium chromate and ferric hydroxide scavenging precipitates. The purified strontium was stored with yttrium carrier while the ^{90}Y equilibrated with the ^{90}Sr . Finally the yttrium was separated and the ^{90}Y radioactivity counted. From its activity that of the ^{90}Sr was calculated. The strontium was counted after the ^{90}Y had re-equilibrated and the ^{90}Sr - ^{90}Y contribution deducted to give the ^{90}Sr activity. Preliminary investigations showed that the average bone activity was about $200\mu\text{C}$ of $^{90}\text{Sr}/\text{g}$. of calcium, indicating that 0.5 g. of bone ash was adequate for analysis. Some determinations of stable strontium were made by the Spectrographic Section, Woolwich Outstation, Chemistry Division, A.E.R.E., using an emission-spectroscopy method, details of which will be published elsewhere.

Reagents

Carriers. Standard solutions containing strontium (10 mg./ml.), barium (5 mg./ml.) and yttrium (5 mg./ml.) as nitrates were used.

Other reagents. These were: ammonium dichromate, A.R. grade, 10% (w/v) aq. soln.; ammonium oxalate, A.R. grade, aq. soln. saturated at room temp.; ferric chloride, A.R. grade, 3% (w/v) aq. soln.; fuming nitric acid, A.R. grade, (d 1.5). Buffer solution contained acetic acid (A.R. grade; 50 ml.) and sodium acetate trihydrate (A.R. grade; 9.8 g.) per litre of aq. soln.