

fructosamine structure in natural products like mucoproteins and mucopolysaccharides.

The observation by Akabori (1933) that amino-acids when heated with furfuraldehyde or with (VII) undergo oxidative deamination and decarboxylation may account for the incomplete recovery of the amino-acid in an experiment in which fructose-phenylalanine was treated with acetic acid. With *p*-toluidine as the amine the loss in (VII) and in *p*-toluidine upon acetic acid treatment of the parent compounds (I) and (III) is explained by the presence among the reaction products of the Schiff's base (VI) and of considerable amounts of insoluble humin (Table I, Fig. 1). When treated with *N*-HCl the *N*-substituted fructosamines yield only small quantities of (VII) due to secondary reactions of (VII) induced by the mineral acid (Exp. 3).

N-Substituted fructosamines just like glucosamine and glucosylamine form a deep colour with *p*-DAB only after pretreatment with acetylacetone; the identity of the absorption bands of the coloured compounds from glucosamine and the *N*-alkyl-fructosamine suggests closely related chromophoric structures. Most probably, as proposed by Elson &

Morgan (1933), these chromophores are pyrrol derivatives.

SUMMARY

1. The *N*-substituted fructosamine (*isog*lucosamine) fructose-phenylalanine (*N*-(1'-carboxy-2'-phenylethyl)amino-1-deoxyfructose) has been prepared. The product, which showed no tendency to crystallize, was not completely free of impurities; the contaminants, however, were present in small amounts only.

2. The compound's characteristic reactions were also given by *N-p*-tolyl-D-fructosamine, a crystalline *N*-arylfructosamine. The outstanding property of *N*-substituted fructosamines tested is the ease with which upon mild acid treatment (2*N*-acetic acid) they form 5-hydroxymethylfurfuraldehyde, in the case of the phenylalanine compound up to 63% of the theory.

3. Since under the same conditions even the acid-labile D-fructose does not yield appreciable amounts of 5-hydroxymethylfurfuraldehyde, its production by dilute acetic acid may be used as a tracer for *N*-substituted fructosamines in natural products.

REFERENCES

- Akabori, S. (1933). *Ber. dtsh. chem. Ges.* **66**, 143.
 Bremner, J. M. & Kenten, R. H. (1951). *Biochem. J.* **49**, 651.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Cremer, H. D. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Gottschalk, A. (1943). *Aust. J. exp. Biol. med. Sci.* **21**, 139.
 Gottschalk, A. (1951). *Nature, Lond.*, **167**, 845.
 Gottschalk, A. & Partridge, S. M. (1950). *Nature, Lond.*, **165**, 684.
 Haworth, W. N. & Jones, W. G. M. (1944). *J. chem. Soc.* **667**.
 Kuhn, R. & Birkofer, L. (1938). *Ber. dtsh. chem. Ges.* **71**, 621.
 Kuhn, R. & Dansi, A. (1936). *Ber. dtsh. chem. Ges.* **69**, 1745.
 Kuhn, R. & Weygand, F. (1937). *Ber. dtsh. chem. Ges.* **70**, 769.
 Micheel, F. & Klemer, A. (1951). *Chem. Ber.* **84**, 212.
 Mitts, E. & Hixon, R. M. (1944). *J. Amer. chem. Soc.* **66**, 483.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Pigman, W., Cleveland, E. A., Couch, D. H. & Cleveland, J. H. (1951). *J. Amer. chem. Soc.* **73**, 1976.
 Weygand, F. (1939). *Ber. dtsh. chem. Ges.* **72**, 1663.
 Weygand, F. (1940). *Ber. dtsh. chem. Ges.* **73**, 1259.

Studies of Sebum

3. METHODS FOR THE COLLECTION AND ESTIMATION OF SMALL AMOUNTS OF SEBUM

BY I. S. HODGSON-JONES AND V. R. WHEATLEY

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

(Received 30 January 1952)

In the course of investigations into the nature and functions of sebum it was necessary to measure the 'sebum levels' (i.e. the amount of sebum which covers 1 sq.cm. of skin) and rates of secretion of different areas of the human body. This necessitated the collection of samples of sebum from an accurately measured area of skin and the estimation of the quantity of sebum thus obtained. The methods

that have been used for the collection of sebum have been reviewed elsewhere (Wheatley, 1952); of these only one can be used for the quantitative collection of sebum from a measured area of skin. This method entails the extraction with a fat solvent of an area of skin demarcated by means of a cup or cylinder held against the skin by manual pressure, or by suction applied to an outer chamber, and has

already been widely used with satisfactory results. The fat solvent used differs widely, but ether is the most popular, and is used either alone (Emanuel, 1936; Kvorning, 1949) or mixed with ethanol (Butcher & Parnell, 1947) or chloroform (Carrié, 1936).

The methods used for the subsequent estimation of the sebum thus collected are, however, open to criticism. Emanuel (1936) and Butcher & Parnell (1947) have used a nephelometric method. Chromate oxidation methods have been used and vary from the original Bang method (Carrié & Neuhaus, 1951) to the more elegant manometric method of Kirk, Page & Van Slyke (1934) used by Kvorning (1949); while a fatty acid titration method has been used by Carrié & Ottofrickestein (1942). In no case has the method been standardized with samples of sebum; instead, either an arbitrary standard was used or an indirect calculation made. While the present investigation was in progress Herrmann & Prose (1951) drew attention to the inaccuracies of these procedures and advocated direct weighing of the sebum sample. A more detailed study has here been made of the methods available, and their reliability assessed by comparison with direct weighing.

MATERIALS AND METHODS

Collection of sebum. The method adopted was almost identical with that used by Kvorning (1949). A glass cylinder (generally of 3 cm. diam.) was pressed firmly against the skin. Fat-free solvent (5 ml., previously twice redistilled in all-glass apparatus), was pipetted into this cylinder and allowed to remain in contact with the skin for 60 sec. At the end of this period the solvent was transferred to a clean test tube by means of a teat pipette, the cylinder and area of skin were then washed with a further 3 ml. of solvent and the washings added to the first extract. The solution of sebum, which also contained cellular debris and dirt, was filtered through a sintered-glass microfilter and was then ready for the subsequent estimation. CCl_4 was selected as the most suitable solvent after preliminary trials with acetone, ether and CHCl_3 .

Methods of estimation. (a) *Gravimetric method.* After filtration the solution of sebum in CCl_4 was evaporated to dryness in a weighed 10 ml. conical microflask and the flask and its contents were dried *in vacuo* over conc. H_2SO_4 for 2 hr. or more and then reweighed on a micro- or semimicrobalance. (A blank determination should be made with each series of weighings.) A determination was then made on the residue of sebum by one of the following methods.

(b) *Acid-number method.* The residue of sebum was dissolved in 2 ml. CHCl_3 and the solution titrated with 0.005 N-NaOH using cresol red as indicator and stirring by means of a stream of N_2 (cf. Moyle, Baldwin & Scarisbrick, 1948).

(c) *Iodine-number method.* A modification of the method of Yasuda (1931) was used. The sebum was dissolved in 2-5 ml. CCl_4 , 1 ml. 0.04 N-pyridine dibromide reagent (freshly made by diluting a 0.1 N stock solution with glacial acetic acid) was then added, the mixture shaken and allowed to stand for 15 min. At the end of this period 1 ml. 3% KI solution was

then added and the liberated I_2 titrated with 0.005 N- $\text{Na}_2\text{S}_2\text{O}_3$ solution.

(d) *Chromate-oxidation method.* The method of Bloor (1928, 1947) was used without modification. The silver dichromate reagent (3 or 5 ml.) was added to the microflask: this was then placed in a boiling-water bath for 15 min. It was then removed from the bath, 3 ml. water were carefully added and the flask was allowed to stand for a further 10 min. to cool. The contents were then transferred to a 175 ml. conical flask with 75 ml. water: 1 ml. 10% KI solution was then added and the liberated I_2 titrated with 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$ solution.

(e) *Turbidimetric method.* The method used by Butcher & Parnell (1947) was adapted for an ordinary test tube type of photoelectric colorimeter. The sebum was saponified by heating in a water bath with 1 ml. 1% ethanolic KOH, the solution was carefully evaporated almost to dryness, diluted to 7 ml. with water and then acidified by the addition of 0.2 ml. conc. HCl. The mixture was then thoroughly shaken and the turbidity read in an EEL photoelectric colorimeter (Evans Electro Selenium Ltd.) using an Ilford no. 608 spectrum-red filter. The optical density thus obtained refers to a thickness of solution of approx. 1.4 cm.

RESULTS

The use of a simple glass cylinder, as advocated by Kvorning (1949), was found to be entirely satisfactory as a method of collecting sebum and, provided that sufficient care was taken, the use of suction device (as recommended by Emanuel, 1936) to keep this cylinder pressed against the skin was found unnecessary. Earlier work (MacKenna, Wheatley & Wormall, 1950) has shown that sebum is only slightly soluble in ethanol and acetone, rather more soluble in ether, and readily soluble in chloroform and carbon tetrachloride. The last two solvents appeared to be the most suitable for use in the cylinder, and of these carbon tetrachloride is more inert and is less irritant to the skin. Ether proved particularly unsuitable; not only does it require to be kept peroxide-free, but it also was found to remove significant amounts of non-lipid material from the skin. In contradistinction to the findings of Herrmann & Prose (1951) it was found necessary to filter the sebum solution before proceeding with the analysis, for with some samples the amount of horny-layer scales removed by filtration would have apparently raised the sebum level by as much as 30 $\mu\text{g.}/\text{sq. cm.}$

Samples of sebum were collected from various areas of the body from a number of normal subjects and also from patients with certain skin diseases. The sample was first weighed, then an estimation was performed by one of the other methods described; a random selection of sebum samples was studied by each method. Dot diagrams were drawn in which the weight of sebum was plotted against the titration figure or colorimeter reading, and the reliability of each method assessed. The following results were obtained.

Acid-number method. Thirty samples were estimated by this method with the results shown in Fig. 1. It was found that 1 mg. of sebum was equivalent to 0.163 ± 0.083 ml. of $0.005N$ - NaOH . These results show that the acid number of sebum varies widely and that this method cannot satisfactorily be used to estimate sebum. The amount of

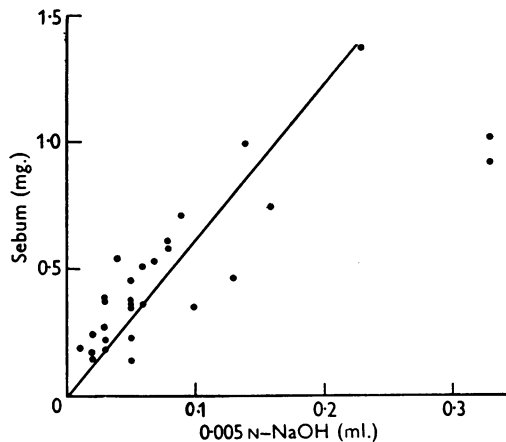


Fig. 1. Attempt to estimate sebum by direct titration with alkali (acid-number method).

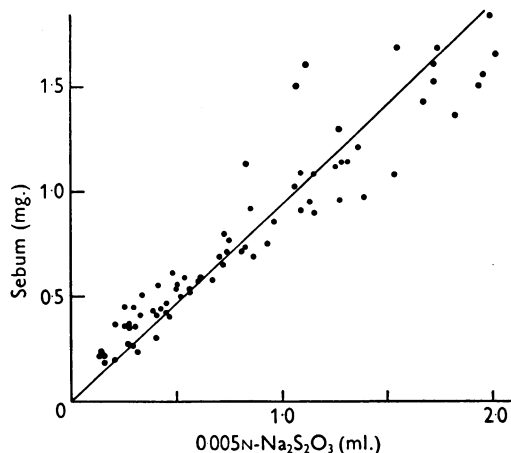


Fig. 2. Results obtained when the iodine uptake of sebum is used as a method of estimation (iodine-number method).

alkali required in the titrations was very small and for this reason accurate results were not possible. In view of these discouraging results it was not considered worth while to investigate a method in which the total fatty acids liberated after saponification were titrated.

Iodine-number method. The results of seventy-five determinations by this method are shown in Fig. 2. With this method it was found that 1 mg. sebum $\equiv 1.06 \pm 0.22$ ml. of $0.005N$ - $\text{Na}_2\text{S}_2\text{O}_3$, and the

method had an average error of $\pm 15.8\%$. The iodine number of sebum appears to vary widely and results obtained in the present method indicate a range of 35–90, with an average of 67. It was found, moreover, that the results from one area of the body (namely the forearm) showed the same range of variation as did those from all areas of the body; this suggests that the variations are due to differences in the composition of sebum from person to person rather than differences in composition over the surface of the body. The method appears, therefore, to have some uses in the study of a given subject after a calibration has first been made with samples of sebum from the person concerned.

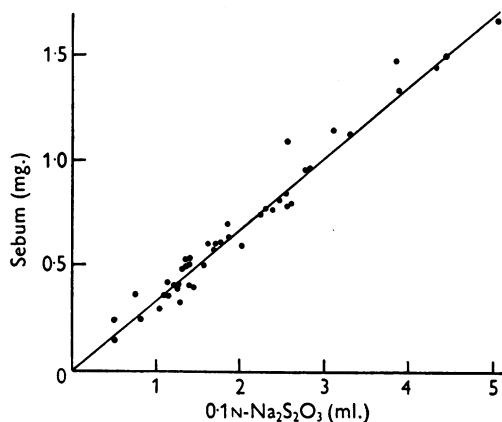


Fig. 3. Calibration curve for the estimation of sebum by the chromate-oxidation method.

Chromate-oxidation method. Estimations were made on forty-five samples of sebum by this method with results as shown in Fig. 3. The results gave a calibration factor for sebum of 1 mg. $\equiv 2.94 \pm 0.33$ ml. of $0.1N$ - $\text{Na}_2\text{S}_2\text{O}_3$, and indicated that the method was very sensitive and had an average error of $\pm 9.0\%$. A similar calibration value was obtained with samples of sebum collected from the whole forearm by the method described by MacKenna *et al.* (1950). When cholesterol was oxidized under the conditions of analysis described here, a factor of 1 mg. $\equiv 3.85$ ml. of $0.1N$ - $\text{Na}_2\text{S}_2\text{O}_3$ was obtained, compared with the theoretical value of 3.92 ml. (Bloor, 1928). This indicates that oxidation was practically complete, but the results obtained with sebum indicate an apparent carbon content of 64%, compared with 77% for human plasma lipids (Kirk *et al.* 1934).

Turbidimetric method. The results of forty-five estimations by this method are shown in Fig. 4. The calibration curve is not linear, an observation which has been confirmed by performing a series of estimations with different weights of the same sample of forearm sebum. The method has an average error of

$\pm 11.6\%$; as described here it is not very sensitive, but the sensitivity can be increased by using a nephelometer instead of an ordinary photoelectric colorimeter. The average turbidity produced by sebum was found to be 102% of that produced by triolein under these conditions of analysis.

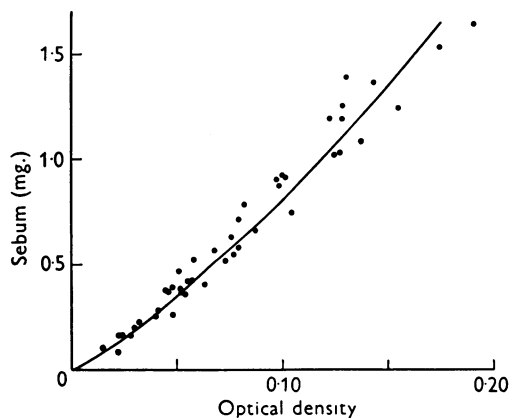


Fig. 4. Calibration curve for the turbidimetric estimation of sebum.

Gravimetric method. The weight of sebum collected has been used as standard against which the results obtained by the other methods have been compared. Such a gravimetric method is not completely free from error, even excluding weighing errors, since it is assumed that all the material in the carbon tetrachloride solution is lipid. Under certain conditions samples may be contaminated by such substances as urea and lactic acid which originate from sweat. Such contamination is probably negligible under most conditions of collection, and it is thought unlikely that the criticism applies to the results presented here.

The results of estimations of the sebum levels of a number of different areas of the body, obtained by the gravimetric method, are shown in Table 1. These results are in general agreement with those obtained by other workers and confirm the finding

that the sebum level is highest in the scalp, decreases over the body, and is lowest in the extremities.

DISCUSSION

Of the methods available for the estimation of small amounts of sebum, direct weighing of the sample appears to be the method of choice; as this is tedious and requires a more sensitive balance than the ordinary analytical type, such a method cannot always be used. Alternative methods are turbidimetric (or nephelometric) estimation, and estimation of the total organic matter by chromate oxidation. Both of these methods give results that are sufficiently accurate for most purposes, but it is desirable that the method to be used should first be calibrated using samples of pure sebum. Samples large enough for such calibration can easily be collected from the forearm by the method described in the preceding paper of this series (MacKenna, Wheatley & Wormall, 1952). Where such a calibration is not practicable the following recommendations are made. (a) In the turbidimetric method triolein can conveniently be used as a standard since it has been found to produce practically the same turbidity as sebum. (b) Provided that the conditions of oxidation described here are adhered to, a factor of 2.94 ml. of 0.1N-K₂Cr₂O₇/mg. sebum can be used for the chromate-oxidation method. This factor corresponds to a carbon content of only 64% and it is suggested that under our conditions of analysis the hydrocarbon fraction of sebum is not oxidized. This does not occur in all chromate-oxidation methods, for when the more drastic conditions of the Kirk, Page & Van Slyke (1934) method are used hydrocarbons appear to be satisfactorily oxidized (cf. Kvorning, 1950). It is therefore necessary to calibrate chromate methods using slightly different conditions of oxidation from those described here.

After the present studies had been completed a paper by Jones, Spencer & Sanchez (1951) appeared, in which the authors describe a very sensitive surface film method of estimating sebum. We hope to make a more detailed study of this method which will, no

Table 1. *Some sebum levels of various sites of the body obtained by the gravimetric method of estimation*

Site of body	No. of subjects	No. of estimations	Sebum level ($\mu\text{g./sq.cm.}$)	
			Range	Average
Forehead	17	22	97-340	212 \pm 73
Chest	21	24	44-237	120 \pm 61
Back	27	40	21-268	106 \pm 56
Abdomen	13	41	25-227	67 \pm 45
Axilla	8	12	30-237	84 \pm 59
Arm	38	63	9-146	58 \pm 34
Groin	4	4	50-105	75 \pm 28
Leg	11	13	18-82	36 \pm 19

doubt, prove very useful in certain investigations owing to its high degree of sensitivity.

The wide variations of both the acid number and the iodine number indicate variations in the composition of sebum. Squalene is the most unsaturated constituent of sebum and small changes in its concentration would produce wide fluctuation in the iodine number. This and other variations in the composition of sebum are now being studied in order to obtain further information about certain skin diseases.

SUMMARY

1. Methods available for the collection and estimation of sebum have been studied in order to assess their reliability.

2. The cup method of collection has been found satisfactory when carbon tetrachloride is used as solvent.

3. Direct weighing of the sebum sample is recommended, but reliable results can be obtained with either the nephelometric or the chromate-oxidation method provided that the method has previously been calibrated by means of samples of sebum.

The authors wish to thank Dr R. M. B. MacKenna for his interest in this work and Prof. A. Wormall for much helpful advice and criticism. They also wish to thank the medical students of this College from whom sebum specimens were obtained. One of us (I. S. H.-J.) is in receipt of a research scholarship from the British Medical Association.

REFERENCES

- Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
 Bloor, W. R. (1947). *J. biol. Chem.* **170**, 671.
 Butcher, E. O. & Parnell, J. P. (1947). *J. invest. Derm.* **9**, 67.
 Carrié, C. (1936). *Arch. Derm. Syph., Wien*, **173**, 604.
 Carrié, C. & Neuhaus, H. (1951). *Arch. Derm. Syph., Wien*, **192**, 261.
 Carrié, C. & Ottofrickestein, H. (1942). *Arch. Gewerbepath. Gewerbehyg.* **11**, 345.
 Emanuel, S. (1936). *Acta derm.-venereol., Stockh.*, **17**, 444.
 Herrmann, F. & Prose, P. H. (1951). *J. invest. Derm.* **16**, 217.
 Jones, K. K., Spencer, M. C. & Sanchez, S. A. (1951). *J. invest. Derm.* **17**, 213.
 Kirk, E., Page, I. H. & Van Slyke, D. D. (1934). *J. biol. Chem.* **106**, 203.
 Kvorning, S. A. (1949). *Acta Pharmacol., Kbh.*, **5**, 248.
 Kvorning, S. A. (1950). *Acta Pharmacol., Kbh.*, **8**, 20.
 MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
 MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1952). *Biochem. J.* **52**, 161.
 Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
 Wheatley, V. R. (1952). *Livre Jubilaire 1901-1951 de la Société belge de Dermatologie et de Syphiligraphie*, p. 90. Bruxelles: Imprimerie Medicale et Scientifique.
 Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

The Preparation and Properties of β -Glucuronidase

4. INHIBITION BY SUGAR ACIDS AND THEIR LACTONES

By G. A. LEVVY

Rowett Research Institute, Bucksburn, Aberdeenshire

(Received 13 February 1952)

Karunairatnam & Levvy (1949) showed that animal β -glucuronidase was strongly inhibited by saccharate and feebly by mucate solutions. These observations were confirmed by Mills & Paul (1949), who claimed greater inhibition by saccharate than had been obtained by Karunairatnam & Levvy. Spencer & Williams (1951) found, on the contrary, that the action of saccharate was weaker than had originally been stated. Campbell (1949), in a histochemical study of β -glucuronidase, found that the enzyme in frozen mouse-kidney sections was almost completely inhibited by 10^{-3} M-saccharate.

Other enzyme systems have been compared with β -glucuronidase on the basis of their response to the

presence of saccharate. Thus, Karunairatnam & Levvy (1949) found that the glucuronide-synthesizing system in mouse-liver slices was not appreciably affected by saccharate. This finding was put forward in support of the view that β -glucuronidase is not involved in the biosynthesis of glucuronides. Satisfactory evidence to exclude the possibility that the inhibitory factor did not penetrate the intact cell was not available at that time, but has since been obtained by Campbell (1949) in his histochemical work, and by Karunairatnam (1950), who showed that hydrolysis of phenolphthalein glucuronide by mouse-liver slices was completely inhibited by 10^{-2} M-saccharate.