The scope of the present work is limited to the general distribution of red fluorescence in shells. Of the spectral bands detected some at least are due to concomitant pigments of non-porphyrin character which have been separated chromatographically. Details of these, and of the chemistry of isolated shell porphyrins, will be published in forthcoming communications.

SUMMARY

1. The distribution of red fluorescence in molluscan shells is described. It is absent from land and fresh-water forms, and commonest in the Archaeogastropoda, appearing also in the Lamellariacea, certain Cypraeidae, the tectibranch opisthobranchs,

Umbraculum, the pearl oysters, and sporadically elsewhere. The limits of distribution coincide closely with the existing anatomical nomenclature.

2. Porphyrins in shells are commonly associated with a wide variety of other acid-soluble pigments.

3. Both these and the porphyrins are at present under fuller investigation, of which details will appear separately.

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Characterization of Sugar Components of Proteins

By ROSA FRIEDMANN, *Wellcome Physiological Research Laboratories, Beckenham*

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Carbohydrates form an integral part of many biologically active complexes and the problem of their estimation in the presence of protein is common. The classical methods are inapplicable to the small amounts generally involved, and numerous colour reagents have been used for the detection of various sugars. Their number and the modifications of the few reactions which have been developed on quantitative lines reflect the existing state of dissatisfaction. A critical investigation of the problem seemed desirable.

The ideal of a specific visible absorption assay for each sugar has not been realized, nor has it been possible to find a reagent giving equal colour intensities for equal quantities of sugar, irrespective of the configuration, so that current methods necessitate qualitative characterization before quantitative estimations can be attempted.

Colour reagents for sugars are either phenols or polyphenols with the substituents in the meta position (thymol, phloroglucinol, α - and β -naphthol, resorcinol, orcinol), or nitrogenous compounds, such as indole, skatole, diphenylamine, urea or guanidine. A range of thirty-two compounds of these two types was examined under standard conditions in concentrated sulphuric acid solution for colour formation with four representative hexoses, namely mannose, galactose, glucose and fructose. In every case colours were obtained, and distinction between mannose and galactose on the one hand, and glucose and fructose on the other hand, was possible. Mannose and galactose, however, showed similar absorption spectra. Fructose gave colour reactions more intensely and more quickly than the aldo sugars. The absorptions, especially in the case of the aldo sugars, were not intense enough to warrant a quantitative investi-

gation, particularly as many colours were not stable and sometimes the reagent itself gave a colour reaction in the absence of the sugar although the spectra of these 'blank' colours were usually distinct from those of the sugars.

EXPERIMENTAL

Orcinol method

The procedure was essentially that of Sørensen & Haugaard (1933). Orcinol, usually pink as supplied, was recrystallized

Orcinol method: the qualitative (A, B and C) and quantitative (D) determination of mannose +++●+++, galactose —x—, glucose —△— and fructose —□—. A, variation in percentage transmission with time (Ilford spectrum filter 602); B, variation in percentage transmission with time (Ilford spectrum filter 604); C, variation in the ratio of percentage transmission with Ilford spectrum filter 602 with time; D, variation in percentage transmission with the concentration of the sugars (Ilford spectrum filter 604).

A closer investigation of the more promising reactions led to the conviction that of the two reactions which have so far been developed on quantitative lines, those with orcinol and carbazole are outstanding, with skatole as a possible third. A detailed comparison was, therefore, restricted to these three methods.

from benzene until white crystals were obtained. A 1.6% solution (modification by Hewitt, 1937) keeps well when prepared precisely as described by Sørensen & Haugaard (1933), i.e. 1.6 g. of orcinol is dissolved in 50 ml. of distilled water, and the volume made up to 100 ml. with the grade of H_2SO_4 specified for the reaction. Filtration through a sintered-glass crucible over a thin layer of washed (30% H_2SO_4) supercel removed minute pinkish brown floccules

which appear to act as centres of decomposition and crystallization. Spontaneous crystallization may take place at temperatures below 16° and lower temperatures should be avoided. The reagent is light-sensitive and must be stored in the dark; it should be colourless and must be rejected when it acquires a pinkish-brown tint. In place of Sørensen's 2.0 ml., 2.5 ml. of the reagent were used.

As qualitative characterization in this method is based on the varying rates of colour formation, necessitating the determination of colour development before completion of the colour-forming reaction, rigid standardization is all important. Results obtained with reaction times of 3-7 min., which contribute most of the characteristic part of the time curve for any particular sugar (see Fig. 1), are reproducible only under optimum conditions of timing and temperature control. We aimed at an accuracy of ± 2 sec., (see also Rimington, 1940), and a maximum temperature variation of $\pm 0.3^\circ$. Since the orcinol colours are light-sensitive (Sørensen & Haugaard, 1933; Hewitt, 1937; Rimington, 1940), particularly those obtained with reaction times of 3-7 min., reproducible time curves are obtainable only when the solutions are shielded from light, not only during cooling before the tubes are read, but also during the heating on the water bath and while readings are taken on the absorptiometer. The reaction bath, of circular shape (Sørensen & Haugaard, 1933) with the tubes arranged around the periphery, was totally enclosed, an efficient stirrer being introduced through a hole in the centre of a sliding lid. Evaporation was minimized by floating a film of liquid paraffin on the surface of the water, the level of which was kept constant. The reaction was terminated by immersion in an ice bath. After 20 min. in the dark, the tubes were transferred to wooden boxes with well-fitting sliding lids, and after a further 30 min. the colours were read in a photoelectric absorptiometer of the slide-back voltmeter type of conventional design (Müller, Garman & Droz, 1943). Ilford spectrum filters 602 and 604 were used in place of S 53, and S 43 (Sørensen & Haugaard, 1933; Rimington 1940). When all these precautions were strictly adhered to, time curves for the qualitative characterization of sugars were reproducible from day to day.

The quantitative estimations are less sensitive to variations. The time curves, obtained by plotting percentage transmission against reaction time, become parallel to the abscissa after 15 min. (Fig. 1). Slight inaccuracies in timing or temperature control are thus not as important as in the steep part of the curve, and for the range 0.04-0.16 mg. hexose/ml., a straight-line relationship holds good; a convenient slope is obtained with Ilford spectrum filter 604 after 20 min. heating. In the dark, the colours remained stable for several hours. It is not necessary to repeat standard curves every day. A weekly check, repeated when reagents were renewed, was found to be sufficient.

We can confirm the observations of Sørensen (1936) and of Hewitt (1937) that the slightly coloured solutions obtained by heating the protein solutions with the reaction H_2SO_4 in the absence of orcinol, do not represent true 'blanks.' They are a source of error when used as blank corrections in the conventional sense, and should be omitted.

The influence of amino-acids on the reactions with the pure sugar solutions was tested by means of an amino-acid mixture of the following composition: glycine 30, alanine 40, glutamine 40, histidine 2, arginine 2, cystine 4, trypto-

phan and tyrosine 0.5 parts. From the percentage transmission figures given in Table 1, it can be seen that there is some slight interference which increases with increasing amounts of amino-acids. However, these slight deviations from the figures obtained with pure sugar solutions cancel out when filter 'ratios' are used for the characterization.

Table 1. *Orcinol method: effect of an amino-acid mixture on percentage transmission for four hexoses.*

(Concentration of the sugar solutions, 0.02%; time of heating, 7 min., i.e. time of largest contrast in colour response. Ilford spectrum filters 602 and 604. The figures are in each case the means of ten estimations.)

Sugar: amino-acid ratio	Percentage trans- mission with filters		Ratio 602/604
	602	604	
Mannose:			
Amino-acid mixture	43.5	45.0	0.97
1 : 1	43.2	45.0	0.96
1 : 4	42.8	43.8	0.98
1 : 40	45.0	46.0	0.98
Galactose:			
Amino-acid mixture	43.2	32.2	1.34
1 : 1	44.2	33.5	1.32
1 : 4	44.0	33.2	1.32
1 : 40	44.8	35.0	1.28
Glucose:			
Amino-acid mixture	61.2	61.8	0.99
1 : 1	59.2	60.5	0.98
1 : 4	61.0	61.2	1.00
1 : 40	61.2	61.3	1.00
Fructose:			
Amino-acid mixture	59.0	73.2	0.81
1 : 1	58.2	72.8	0.80
1 : 4	59.2	74.0	0.80
1 : 40	60.2	74.8	0.81

Carbazole method

Gurin & Hood's method (1939) was strictly adhered to. The degree of purity of the reaction acid and the carbazole reagent are critical for success. Occasionally, batches of H_2SO_4 (A.R.) were unsuitable because of an impurity giving rise to fluorescence.

Purification of carbazole may be effected conveniently by chromatographic adsorption. Savory and Moore's alumina, standardized according to Brockmann, was used as the adsorbent and benzene as solvent. The impurities were retained on top of the column, even in the case of very impure commercial preparations. The narrow deeply coloured band on top may be separated into a deep blue, a yellow, a pink and a brown zone by subsequent washing with benzene. Washing was discontinued when the brown zone reached the bottom of the column and the carbazole was recovered from the eluate in 80% yield. One recrystallization from ethanol gave a white stable product. Less pure preparations become unsuitable for use within a few days. Even slight impurities caused the appearance of pink and green

tints in the blanks, making qualitative characterization impossible and introducing erratic errors in the quantitative estimations.

The rate of colour formation was found to be approximately the same for all four hexoses, regardless as to whether the reaction was carried out at 60, 80 or 100°, except for fructose where maximum colour development is obtained after 5 min. at 100°. Time curves are, therefore, not sufficiently distinctive for a qualitative characterization, but the differences in the specific absorptions between the various sugars are greater than in the orcinol method (Fig. 2) and Gurin & Hood (1939) have made use of filter 'ratios'. The set of Ilford spectrum filter ratios used by us is indicated in Table 2.

Table 2. *Carbazole method: comparison of percentage transmission ratios obtained with mannose, galactose and glucose in the presence and absence of 40 times as much amino-acids.*

Ilford spectrum filter ratio	604	604	607	607
...	601	602	604	601
Mannose	1.76	1.09	1.54	2.78
Mannose + amino-acids	1.75	1.11	1.50	2.78
Galactose	1.22	0.92	1.64	2.01
Galactose + amino-acids	1.22	0.88	1.67	2.04
Glucose	0.71	0.64	2.02	1.44
Glucose + amino-acids	0.74	0.66	2.02	1.51

Amino-acids containing an aromatic nucleus interfere with the reaction (Gurin & Hood, 1939), and hexose figures obtained with the carbazole method are for this reason slightly higher than those obtained with the orcinol method (see Table 4). Table 2 shows that amino-acids interfere little with the qualitative characterization. If the indicated range of ratios is used, differences due to amino-acids cancel out. The same amino-acid mixture as in the orcinol method was used.

Skatole method

Colour reactions of sugars with skatole were first described by Weehuizen (1907) who used hydrochloric acid. Dische & Popper (1926) investigated the reaction, using sulphuric acid. They did not, however, extend their investigations because skatole itself was found to give a red colour in the absence of sugar. We found that a deep purple was formed within 4 min. on a boiling water bath and that the same colour is eventually reached on prolonged standing at a lower temperature. Although this colour is quite distinct from the 'sugar' colours (Fig. 2), it is fairly intense. A specific absorption filter could not be devised, but it proved possible to reduce this blank value considerably by working at 80° instead of at 100° and choosing suitable concentrations of the acid and the reagent. Fig. 3 shows that the optimum concentrations of sulphuric acid and skatole are 85 (v/v) and 0.5% (w/v) respectively, giving intense colour formation with the hexose and relatively little colour in the blank.

The skatole colours are light-sensitive, and have to be protected as in the orcinol method. In the dark,

after heating times of 15 min., the colours are stable for several hours. Solutions which have been heated for 5–10 min. darken more quickly, and should

Fig. 2. Photometric absorption curves for mannose $\bullet\bullet$, galactose $\times$ —, glucose $\cdots\Delta$ and fructose $\square$ —, with the orcinol (top), carbazole (middle), and skatole (bottom) methods. (Hilger medium quartz spectrograph).

always be read exactly 1 hr. after removal from the water bath. Temperature control and timing is as critical as in the orcinol method and for the same reasons.

Apparatus. The reactions were carried out in an enclosed water bath at $80 \pm 0.3^\circ$. Timing was carried out with a stop-clock, and the colours were measured photoelectrically in 1 cm. all-glass cells.

Reaction acid. 85% (v/v) H_2SO_4 (A.R.), obtained by adding 8.5 vol. ice-cold conc. acid to 1.5 vol. of water, with external ice cooling.

Fig. 3. Top, effect of variation in sulphuric acid concentration on percentage transmission; —□— reaction of skatole with sulphuric acid in the absence of sugar; —■— reaction of mannose with skatole in sulphuric acid. Bottom, effect of variation in skatole concentration on percentage transmission; —○— reaction of skatole with sulphuric acid in the absence of sugar; —●— reaction of mannose with skatole in sulphuric acid. (Ilford spectrum filter 604, time of heating at $80 \pm 0.3^\circ$: 15 min.)

Skatole. Commercial preparations contain a yellow and a pink impurity. The former may be removed by recrystallization from light petroleum, the latter by recrystallization from aqueous ethanol until the base is obtained in a pure white state. A 0.5% (w/v) solution in 96% ethanol, which must be colourless, keeps practically indefinitely when stored in the cold.

Procedure. 0.04–0.25 mg. carbohydrate in 1 ml. of solution was measured into wide boiling tubes (3.5×20 cm.) and the solution cooled in an ice bath. 0.2 ml. of the skatole reagent was added with a micropipette, followed by 9 ml. of the reaction H_2SO_4 . Quick mixing was achieved by means of a flattened glass rod. The reaction was allowed to proceed at 80° in the dark and was interrupted at suitable time intervals by plunging in ice water. A standard time of 30 min. in the dark was allowed before removing the tubes to the light-protected boxes. Readings were taken after a further 30 min.

Qualitative characterization

1. **Specific absorption.** A comparison with Fig. 2 shows that the differences in the absorption spectra of four representative hexoses are greater than in the orcinol method and that the absorption curves are

similar to those obtained with carbazole. As in the latter method, qualitative characterization would be possible by means of a suitable set of percentage transmission 'ratios'.

2. **Rates of colour formation.** As with orcinol, the rates of colour formation (Fig. 4) vary with the configuration of the sugar, and allow better distinctions to be drawn between the sugars than the use of differences in specific absorptions. The light filters 601 and 604 were chosen for the construction of the time curves because their transmission ranges coincide roughly with the peak ranges in Fig. 2.

3. **Use of ratios.** By plotting a ratio of the percentages of light transmission obtained with the filters 601 and 604 against reaction time, characteristic curves were obtained for the various sugars which were independent of concentrations over the range where Beer's law is obeyed (Fig. 4).

Quantitative estimations

Quantitative estimations are again less sensitive to experimental variation than qualitative characterization, for the same reason as in the orcinol method. Beer's law is obeyed within the range of 0.04–0.24 mg. hexose/ml. Although the colours obtained after heating for 20 min. were more stable, a period of 15 min. was chosen because interference from the 'blank' colour becomes pronounced after this time. Filter 604 proved more suitable than 601.

Influence of amino-acids and glucosamine

In the presence of amino-acids, the initial mixing in the ice bath has to be done very gradually, or tints differing from those of the pure sugar solutions are obtained, and the shape of the time curves becomes distorted. If yellow tints are observed during the mixing in the ice bath, the particular tubes must be discarded. The amino-acid mixture used in the previous experiments was again employed.

Fructose showed a greater initial rate of colour formation than the aldoses. An orange-red colour formed immediately on mixing in the ice bath, although the intensity reached in the water bath at 80° was not much greater than that obtained with the aldoses. The initial velocity of the reaction was such that the different stages could not be reproduced accurately during the gradual mixing of the reagents which is necessary in the presence of amino-acids, and fructose, although readily recognized, cannot be estimated in their presence. As in the other two methods, glucosamine, in quantities up to 40 times those of the hexoses, did not influence the reaction.

Application

When determining the hexose composition of an unknown mixture, it will first be necessary to remove as far as possible any admixed pigment. The sugar concentration must then be brought within the

working range of the given methods, i.e. 0.04–0.20 mg./ml. By comparing the tints, or better the absorption spectra, given by the unknown with the orcinol, carbazole and skatole reagents with those obtained with a number of different pure sugar

approximations in which all three methods play their part.

A purified follicle-stimulating fraction of pregnant mares' serum (P.M.S. W III) assaying 250 i.u./mg. protein was analyzed by all three methods. Fig. 5

Fig. 4. Skatole method: the qualitative (A, B and C) and quantitative (D) determination of mannose +++●+++, galactose —×—, glucose ---△--- and fructose —□—. A, variation in percentage transmission with time (Ilford spectrum filter 601); B, variation in percentage transmission with time (Ilford spectrum filter 604); C, variation of the ratio $\frac{\text{percentage transmission with Ilford spectrum filter 601}}{\text{percentage transmission with Ilford spectrum filter 604}}$ with time; D, variation in percentage transmission with the concentration of the sugars (Ilford spectrum filter 604).

solutions of similar concentration, a preliminary working hypothesis may be arrived at. The carbazole 'ratios' may then be used for a rough characterization which can be carried out quickly. The indicated possibilities can then be further investigated by means of time curves according to the orcinol and skatole methods. The approximate qualitative composition may thus be determined by a series of

gives a comparison of the ratio-time curves of this sample with that of mannose, galactose and an equimolar mixture of these two sugars obtained with the orcinol and skatole methods. In both cases, the shape of the curve obtained with the P.M.S. sample is consistent with the assumption that the carbohydrate component of this serum fraction is made up of equal amounts of mannose and galactose. The

results obtained with the carbazole method using four different percentage transmission ratios, in place of Gurin's (1942) one ratio, are also consistent with this view (Table 3). Fructose is certainly absent since no colour is formed with skatole in the cold. Although it is conceivable that the presence of glucose might

The carbazole and skatole values for hexose tend to be higher than those obtained with orcinol, but analysis shows that these differences are statistically insignificant as far as the skatole and orcinol methods ($P=0.4$) are concerned, and only at the verge of significance with respect to the carbazole

Fig. 5. A comparison of the ratio-time curves (see Figs. 1 and 4) for mannose —●—, galactose —×—, an equimolar mixture of mannose and galactose ---○---, and a purified fraction of pregnant mares' serum (P.M.S. W III) ---△---, as obtained with the orcinol method (A) and the skatole method (B).

pass unnoticed in the orcinol method in the presence of a mixture of mannose and galactose (see Fig. 1 C), it would be obvious in the skatole method (Fig. 4 C) and can also be excluded. In view of the distinction between mannose and galactose in the orcinol method, it is reasonably certain that the sugar component of P.M.S. W III is not pure mannose or pure galactose.

The proteins of normal horse serum and of the sera of horses immunized against the toxins of *Corynebacterium diphtheriae*, *Clostridium perfringens* and *Cl. tetani* were purified by several precipitations with ammonium sulphate and finally dialyzed against running tap water to remove any residual uncombined carbohydrates. There was no difference either by the skatole, orcinol or carbazole method, in the ratio-time curves of these sera, which were again consistent with an equimolar mannose-galactose composition.

The sugar component of the P.M.S. sample was determined quantitatively 12 times by each of the three methods. An equimolar mixture of mannose and galactose was used for the standard curve. Table 4 shows that the accuracy and reproducibility is of the same order of magnitude for all three methods, but that the orcinol method gives a lower coefficient of variation than the other two methods.

Table 3. Carbazole method: comparison of percentage transmission ratios obtained with a purified follicle-stimulating fraction of pregnant mares' serum (P.M.S. W III), mannose, galactose and an equimolar mixture of mannose and galactose.

Ilford spectrum filter ratio	604	604	607	607
	601	602	604	601
Mannose	1.92	1.13	1.48	2.94
Mannose: galactose=1:1	1.55	1.04	1.56	2.50
P.M.S. W III	1.60	1.03	1.53	2.48
Galactose	1.27	0.93	1.58	2.00

Table 4. Statistical comparison of the three methods

(Twelve estimations of the sugar component of a follicle-stimulating fraction of pregnant mares' serum by each method.)

Method	Mean mg. hexose/g. protein	Standard deviation	Coefficient of variation (%)	Standard error
Orcinol	41.8	1.16	2.77	0.34
Carbazole	43.2	1.62	3.74	0.47
Skatole	42.3	1.67	3.95	0.49

$P_{\text{orcinol carbazole}} = 0.04.$
 $P_{\text{orcinol, skatole}} = 0.4.$
 $P_{\text{skatole, orcinol}} = 0.2.$

and orcinol methods ($P = 0.04$). There is no significant difference between the figures obtained by the carbazole and skatole methods ($P = 0.2$).

DISCUSSION

A large number of nitrogenous and phenolic compounds form distinct coloured products with carbohydrates. The reactions with orcinol and carbazole have been studied in detail (Sørensen & Haugaard, 1933; Gurin & Hood, 1939); both methods have been applied widely, but have also been subject to many criticisms. We have now added the skatole method as a third alternative. All the methods suffer from lack of precision and lack of specificity, unless rigidly standardized. Small variations in the concentration of the acid and the colour-forming reagent or minute amounts of impurities influence the intensity and tint of the resulting colour. A variety of carbonyl compounds other than carbohydrates also give intensely coloured products with these reagents (Table 5). The significance of all colour tests for the qualitative characterization of sugars has been questioned (Meyer, 1938, 1945).

Table 5. *Colour reactions of carbonyl compounds other than carbohydrates with orcinol, carbazole and skatole*

(Concentration of carbonyl compound: 1 mg./ml.)			
	Orcinol	Carbazole	Skatole
Formaldehyde	Bright orange	Deep blue precipitate	Dark brown precipitate
Benzaldehyde	Yellowish white precipitate	Raspberry red	Deep red
Vanillin	Blood red	Faint mauve	Deep clear ruby red
Acetone	Like blank	Like blank	Like blank
Blank	Nearly colourless	Faint grey	Faint pink

The present investigation shows that quantitative estimations are less subject to variation than qualitative characterizations; absolute quantitative figures may be obtained quickly and accurately (Table 4) provided the qualitative composition of the carbohydrate complex is known.

Qualitative characterization is possible within limits by adhering precisely to the given details of the methods and making use of all three methods in the analysis. The orcinol method distinguishes best between fructose and galactose (Fig. 1 C). Less than 20% galactose cannot be detected in the presence of 80% mannose and vice versa (Fig. 6). Only 1:1 mixtures of glucose and mannose may be resolved. With respect to qualitative characterization, the carbazole method has the attraction of greater speed, but there is less differentiation than in the orcinol and skatole methods; an equivalent mixture of

glucose and mannose cannot be distinguished from galactose (Gurin & Hood, 1939), and only 1:1 mixtures of any two sugars can be resolved (Table 6). The skatole method distinguishes best between glucose and mannose where admixtures of less than 20% are recognizable. An equimolar mixture of glucose and mannose cannot easily be differentiated from galactose (see Fig. 4 C). It will be remembered that fructose is readily distinguished from the aldoses.

Fig. 6. Orcinol method. Characterization of mannose and galactose in the presence of each other by means of ratio-time curves (see Fig. 1): —●— galactose alone; ● 80% galactose + 20% mannose; ○ 60% galactose + 40% mannose; ○ 40% galactose + 60% mannose; ○ 20% galactose + 80% mannose; —○— mannose alone.

Quantitative estimations are quickest with the orcinol method, since no special care needs to be taken over the initial mixing of the reagents, a critical point in the carbazole and skatole methods.

Table 6. *Carbazole method: comparison of the percentage transmission ratios obtained for mannose, galactose and mixtures of the two sugars*

Ilford spectrum filter ratio	604		607	
	601	602	604	601
Mannose (M)	1.89	1.15	1.48	2.85
M : Ga = 80 : 40	1.67	1.07	1.48	2.46
M : Ga = 1 : 1	1.52	1.00	1.51	2.40
M : Ga = 20 : 80	1.34	0.97	1.57	2.04
Galactose (Ga)	1.22	0.93	1.57	1.92

Holzman, Macallister & Niemann (1947), using pure sugar solutions, have investigated the variables in the carbazole reaction. Following Seibert & Atno (1946), they added the carbazole in the sulphuric acid reagent, with better control of the amount of carbazole added, which they showed to be critical. However, the new reagent is not stable, and the precision of the modified procedure is the same as that of the original method (2–5% in pure sugar

solutions). Protein analyses made by Seibert & Atno (1946) using Gurin & Hood's (1939) carbazole method show variations of 5-6 % (three estimations), while we find a coefficient of variation of nearly 4 % (Table 4, twelve estimations). Staub & Rimington (1948) state that results obtained by the carbazole method are higher than those with orcinol, which appears to be borne out by our analysis, although the statistical significance of this difference is doubtful ($P=0.04$). Under the given precise conditions, the orcinol method gives more consistent results than the carbazole and skatole methods (coefficient of variation of 2.77 %, compared with 3.74 and 3.95 % respectively) and there is very little interference by protein (Table 1). Apart from any specific amino-acid interference, the high reaction temperature (carbazole method) and the high acid concentration lead in the carbazole and skatole methods to the production of more coloured decomposition products than can develop under the conditions of the orcinol reaction.

The skatole method is perhaps the most satisfactory qualitative method, since it combines the possibilities of the orcinol and carbazole methods for characterization, but it unfortunately also shares the disadvantages of both procedures. Fructose, although readily recognized, cannot be estimated quantitatively by this method in the presence of protein.

Some comment is needed on the nature of the sugar component of P.M.S., since our findings are at variance with those of Li, Evans & Wonder (1940) and Gurin (1942). Li *et al.*, using a modification of the orcinol method, claim that the carbohydrate component other than glucosamine is galactose, while Gurin's results with the carbazole method indicate galactose or an equimolar mixture of glucose and mannose. Rimington & Rowlands (1941, 1943, 1944), using the orcinol method, chose an equimolar mixture of mannose and galactose as the basis of the hexose estimations throughout their work on P.M.S., because the tints obtained with this mixture correspond closely to those obtained with P.M.S. preparations. Our more detailed investigations of this particular point by means of three different methods confirm their findings.

Summing up, it can be said that the main value of the colour reactions investigated lies in the following:

(1) To find quickly and accurately quantitative differences in the sugar contents when concentrating natural products. The nature of the sugar does not matter then as one of the fractions can serve as an arbitrary standard; by means of time curves it will be possible to decide whether any significant changes in the qualitative composition of the sugar component—whatever its nature—take place during concentration.

(2) To find quickly and accurately the absolute concentration of any mixture of sugars—however complicated—alone or in combination with protein, if the nature of the sugar component is known or the complex can be isolated.

(3) To give a rough indication of the sugars present. This may be a tedious process and it is almost impossible to analyze a mixture of more than two components. The proportions of the components can only be ascertained approximately and it is important to have some idea of the composition beforehand. The carbazole method appears less suitable for this purpose than the other two methods.

SUMMARY

1. Compounds of two types, either of a phenolic character or containing a reactive nitrogen group, have been investigated for colour formation with four representative hexoses resulting in the development of the skatole method for the qualitative and quantitative characterization of sugars.

2. The relative limitations of the orcinol, carbazole and skatole methods are compared and the necessary standardizations pointed out.

3. All three methods have been applied to the analysis of the sugar component of a fraction of pregnant mares' serum. The results obtained are consistent with an equimolar mannose-galactose composition (apart from glucosamine which is not estimated by the reactions used).

4. The sugar component of the blood proteins of normal and immunized horses have been analyzed similarly, and the results are again consistent with an equimolar ratio of mannose and galactose.

It is my pleasant duty to thank Dr G. E. Foster of The Wellcome Chemical Works, Dartford, for his kindness in giving me hospitality in his laboratory, where the curves for Fig. 2 were prepared.

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Fractionation of Oxidized Insulin

By F. SANGER (Beit Memorial Fellow), *Biochemical Laboratory, University of Cambridge*

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From the determination of the terminal residues of insulin it was suggested that the submolecule of molecular weight 12,000 is made up of four open peptide chains bound together by disulphide linkages (Sanger, 1945). Two of these chains have glycine and two have phenylalanine as terminal residues. By the action of performic acid it is possible to split insulin into its separate polypeptide chains by conversion of the cystine to cysteic acid residues (Sanger, 1947). The object of the present work was to separate the chains in a pure form, and the first problem was to find a suitable analytical method to follow the course of fractionation. Electrophoretic analysis gave rather variable results; this was largely due to the low molecular weight of the fractions which led to rapid spreading of the boundary by diffusion and did not allow preliminary dialysis. Later the method of end-group assay (Sanger, 1945; Porter & Sanger, 1948) was used in an attempt to obtain fractions containing only glycine or only phenylalanine terminal residues. This method of characterization does not necessarily indicate complete homogeneity, but the fractions are at least representative of the two types of chains in the insulin molecule. In this paper the preparation and some preliminary observations on the properties of two fractions are reported; the one, fraction *A*, containing only glycine terminal residues and no basic amino-acids; the other, fraction *B*, containing 97% phenylalanine terminal residues.

EXPERIMENTAL

Oxidation of insulin

The method was essentially that of Toennies & Homiller (1942), who showed that within 1 hr. cystine consumes the theoretical 5 atoms of oxygen, and that after 2 hr. a more general oxidation of a number of amino-acids takes place. It was thus desirable to use a time of oxidation which was the minimum required to split the S-S bridges completely.

Using paper chromatography (Consden, Gordon & Martin, 1946*b*) it was found that the reaction with free cystine was complete in 5 min., but with insulin 15 min. were required. The method finally used was as follows: 0.25 g. insulin was dissolved in 9 ml. formic acid, 1 ml. 30% (w/w) H_2O_2 was added, and the mixture allowed to stand for 15 min. at room temperature. Water (10 ml.) was added and the mixture evaporated *in vacuo* to a small volume (1–2 ml.). The oxidized protein was then precipitated by a large volume of acetone, centrifuged, washed with acetone till free of formic acid and dried in air; yield, 0.25 g.

Fractionation of the oxidized insulin

Of the various fractionation procedures tried, the most satisfactory were the conventional methods of precipitation by alteration of pH or addition of salt or ethanol. In the salting-out procedures, however, it was difficult to remove the salt since the peptides were too small to be dialyzed. This was overcome by using a volatile salt, ammonium acetate, which could be removed by evaporation.

Freshly oxidized insulin (1 g.) was dissolved in 12 ml. 0.1 M- NH_3 , and 0.1 M-acetic acid was added to bring the pH of the solution to 6.5. The precipitate was centrifuged down and used in the preparation of fraction *B*. The solution was used for the preparation of fraction *A*.

Fraction A. The above solution was brought to pH 4.5 with 0.1 M-acetic acid and, after removal of the precipitate (fraction *M*), was taken almost to dryness *in vacuo*. It was then transferred to a centrifuge tube with a minimum volume (about 3 ml.) of water, and an equal volume of 50% (w/v) ammonium acetate, which had been brought to pH 5.5 with glacial acetic acid, was added. This brought about the separation of a small precipitate which was discarded. The water and ammonium acetate were then removed by leaving the solution in a high vacuum over H_2SO_4 and NaOH till it reached constant weight. The residue was a white powder; yield, 0.25–0.32 g.

Fraction B. The material precipitated at pH 6.5 from 1 g. oxidized insulin was washed with 10 ml. 0.01 M-acetic acid, dissolved in 5 ml. 0.1 M-HCl and 40 ml. absolute ethanol added. This brought about the separation of a precipitate (fraction *X*) often in the form of a gel, which was centrifuged off and washed well with 80% ethanol. The combined