CCXLVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF GLUCOSAMINE AND CHONDROSAMINE.

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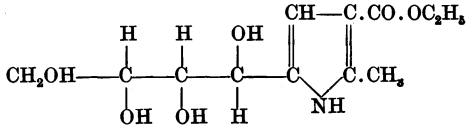
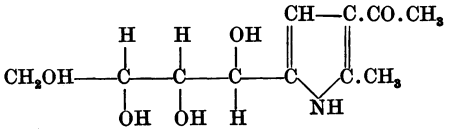
A COLORIMETRIC method for the determination of glucosamine has recently been described by Zuckerkandl and Messiner-Klebermass [1931]. According to their procedure the test-solution, which should contain between 1 and 4 mg. glucosamine hydrochloride, is evaporated to dryness and treated with a freshly prepared solution of sodium methoxide. The free glucosamine base is then cautiously acetylated with acetic anhydride to yield the *N*-monoacetyl derivative which can be estimated by means of the intense reddish-purple colour that develops when, after a preliminary treatment with dilute alkali, *p*-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) is added. We have found this method unsatisfactory. The acetylation of glucosamine which yields the *N*-monoacetyl derivative, under the conditions described by Zuckerkandl and Messiner-Klebermass does not appear to be quantitative and, moreover, rapid fading of the colour that develops on the addition of the *p*-dimethylaminobenzaldehyde reagent increases the difficulty of colorimetric estimation. That Zuckerkandl and Messiner-Klebermass were aware of the difficulties involved in the use of the method for quantitative purposes is evident from their description of the reaction. They state that the error of estimation is, at the most, 6 %.



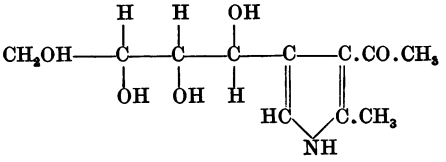
I.

Ethyl ester of 2-methyl-5-tetrahydroxybutylpyrrole-3-carboxylic acid.



II.

3-Acetyl-2-methyl-5-tetrahydroxybutylpyrrole.



III.

3-Acetyl-2-methyl-4-tetrahydroxybutylpyrrole.

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A colorimetric method for the determination of glucosamine has therefore been elaborated which avoids the process of evaporation to dryness and subsequent conversion of the glucosamine into its *N*-monoacetyl derivative and which gives rise to very stable coloured solutions. The method depends upon the colour which develops when pyrroles are condensed with *p*-dimethylaminobenzaldehyde. The conversion of glucosamine into the pyrrole derivatives (I) and (II), by the action of ethyl acetoacetate and acetylacetone respectively has been described by Pauly and Ludwig [1922]. When glucosamine hydrochloride is boiled in alkaline solution with either ethyl acetoacetate or acetylacetone the resulting solutions, on treatment with Ehrlich's reagent in the presence of alcohol, develop a stable red colour. The colour obtained with the condensation product of acetylacetone (II) was found to be the more intense and for this reason was selected as a basis for the colorimetric determination described below.

Experimental.

Reagents. (1) *Acetylacetone solution.* The reagent is made by dissolving 1 cc. of acetylacetone in 50 cc. of 0·5 *N* sodium carbonate solution; the acetylacetone dissolves readily on shaking. The reagent should be kept in an ice-chest when not in use and should be prepared fresh every 4 or 5 days.

(2) p-*Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (0·8 g.) which has been twice recrystallised from dilute alcohol is dissolved in alcohol (30 cc.) and concentrated hydrochloric acid (30 cc.) added. The reagent possesses a pale yellow colour and keeps indefinitely.

(3) *Glucosamine hydrochloride standard.* An aqueous solution of glucosamine hydrochloride saturated with chloroform and containing 10 mg. of the hydrochloride in 1 cc. is prepared. From this solution suitable dilutions can be made when required. The standard solution should be kept between 0 and 4°.

Procedure. The determination is best carried out in test-tubes graduated at a volume of 10 cc. The solution to be estimated, which should contain between 0·5 and 3·0 mg. of glucosamine hydrochloride, is pipetted into the tubes and the acetylacetone reagent (1 cc.) added from a pipette; the sides of the tubes are then washed down with 1 cc. of water. At the same time standard solutions of glucosamine hydrochloride are measured out and treated in the same manner. The tubes are heated for 15 minutes in a boiling water-bath in which the level of the water is kept just above the level of the liquid in the tubes. The upper portion of the test-tubes should project out of the water-bath in order to avoid any serious loss of acetylacetone by evaporation. After heating, the tubes are cooled and alcohol is added to within about 2 cc. of the 10 cc. graduation mark. During the addition of alcohol a precipitate frequently forms but it is rapidly dissolved on the subsequent addition of the Ehrlich reagent (1 cc.); alcohol is then added to make the volume up to 10 cc. The colours develop quickly and reach their full intensity in 15–20 minutes. During this time there is a slow evolution of carbon dioxide which renders colorimetric comparison difficult, and it has been found convenient to compare the colours after the solutions have stood for at least 30 minutes at room temperature. When compared with a stable artificial colour standard the red colour which develops shows no fading over a period of several hours.

The colorimetric comparison is reliable only when the intensities of the tints of the unknown solution and the standard solution are approximately the same. It has been found that a comparison of colours is difficult if the glucosamine contents of the solutions differ by more than 25 %. The accuracy of the method

is readily ascertained from the results shown in Table I where it will be seen that the error of estimation is less than 5 % if the amount of glucosamine hydrochloride is within the range 0·75–3·0 mg. and the colour intensity of the standard glucosamine hydrochloride solution does not differ by more than 25 % from that of the unknown.

Table I.

Glucosamine hydrochloride				Glucosamine hydrochloride			
Standard solution mg.	Present mg.	Found mg.	Percentage error	Standard solution mg.	Present mg.	Found mg.	Percentage error
0·25	0·50	0·475	−5·0	2·00	1·80	1·84	+2·2
„	„	0·530	+6·0	„	„	1·80	0·0
0·75	„	0·509	+1·8	„	2·20	2·17	−1·3
„	„	0·520	+4·0	„	„	2·19	−0·5
„	1·00	1·02	+2·0	„	2·50	2·50	0·0
„	„	1·00	0·0	„	„	2·45	−2·0
1·00	0·80	0·830	+3·7	2·50	2·25	2·24	−0·5
„	„	0·827	+3·3	„	„	2·21	−1·8
„	1·20	1·21	+0·8	„	2·75	2·80	+1·8
„	„	1·22	+1·6	„	„	2·78	+1·1
„	1·40	1·35	−3·5	„	3·00	3·07	+2·3
„	„	1·39	−0·7	„	„	2·02	+0·7
1·50	1·20	1·22	+1·6	3·00	2·50	2·50	0·0
„	„	1·23	+2·4	„	„	2·54	+1·6
„	1·80	1·80	0·0	„	3·50	3·40	−3·0
„	„	1·79	−0·6	„	„	3·32	−5·1
				„	4·00	3·75	−6·2

This method can also be used for the determination of chondrosamine. Equal amounts of glucosamine hydrochloride and chondrosamine hydrochloride give rise to colours identical in tint and intensity. A few of the results obtained by using standard solutions of chondrosamine hydrochloride are given in Table II; it will be seen that the limits of accuracy of the determination as given for glucosamine hydrochloride also hold for chondrosamine hydrochloride.

Table II.

Chondrosamine hydrochloride			
Standard solution mg.	Present mg.	Found mg.	Percentage error
1·50	1·00	0·992	−0·8
1·50	1·00	1·00	0·0
1·50	2·00	2·00	0·0
2·00	2·50	2·42	−3·2

The influence of foreign substances.

In order to ascertain whether the presence of sugars has any influence upon the determination of glucosamine a number of estimations have been made in which glucose, galactose, fructose or arabinose was added to the glucosamine solution before estimation. The results of these tests are given in Table III and it will be seen that in no case had the added substance any appreciable influence upon the accuracy of the estimation. Similarly the presence of glycine, alanine or histidine does not interfere with the determination.

For obvious reasons the method cannot be used when certain pyrrole or indole derivatives are present; these substances, however, can be readily detected since they will condense with *p*-dimethylaminobenzaldehyde in acid

Table III. *The influence of foreign substances upon the accuracy of the estimation.*

Substance added		Glucosamine hydrochloride: Present mg.	Glucosamine hydrochloride: Found mg.	Percentage error
	mg.			
Glucose	2·5	2·0	1·97	−1·5
	5·0	,,	2·02	+1·0
Galactose	5·0	,,	2·03	+1·5
	10·0	,,	1·99	−0·5
Fructose	2·5	,,	2·02	+1·0
	5·0	,,	2·01	+0·5
Arabinose	2·5	,,	1·98	−1·0
	5·0	,,	2·00	0·0
Glycine	2·5	,,	2·00	0·0
	5·0	,,	1·97	−1·5
Alanine	2·0	,,	2·03	+1·5
	2·0	,,	1·98	−1·0
Histidine	1·0	,,	2·00	0·0
	1·0	,,	2·03	+1·5
Tryptophan	1·0	,,	2·04	+2·0
	1·0	,,	2·03	+1·5

solution to yield coloured solutions without previous heating with the acetylacetone reagent. The presence of tryptophan does not affect the accuracy of the estimation because the colour which this amino-acid is known to give with Ehrlich's reagent does not develop in the presence of hydrochloric acid of the strength used in the determination; the tryptophan reaction requires a much greater concentration of acid. *N*-Acetylglucosamine and *N*-acetylchondrosamine, if present, will also yield a reddish-purple coloration with Ehrlich's reagent, but only after heating in alkaline solution. The coloration produced with these two compounds, however, is not due to the formation of pyrrole derivatives, but to the elimination of the elements of water, followed by ring-closure with the formation of a disubstituted oxazole [Elson and Morgan, 1933, 2], which condenses with *p*-dimethylaminobenzaldehyde to yield an intense reddish-purple coloured solution [Elson and Morgan, 1933, 1].

Although 1-aminoglucose is not known to occur naturally and is, therefore, unlikely to be present in the acid hydrolysis products of either glucoproteins or nitrogen-containing polysaccharides it is of interest that this aminohexose, prepared according to the method of Ling and Nanji [1922] condenses readily with acetylacetone, under the conditions described above for the estimation of glucosamine and chondrosamine, to yield a coloured solution almost identical in tint and intensity with that produced by equal quantities of these compounds. In this case the acetylacetone presumably combines with 1-aminoglucose to give the compound (III) which on treatment with Ehrlich's reagent gives the usual pyrrole colour.

The influence of the concentration of hydrochloric acid on the rate of development of the colour[1].

Four colorimetric determinations were made, using 1·0 mg. of glucosamine hydrochloride, in which the concentration of hydrochloric acid present in the final solution was the only factor that was varied. The results, as shown in

[1] (*Note added November 21st.*) In the presence of considerably higher concentrations of hydrochloric acid ($> 2N$) it has been found that many carbohydrates and amino-acids, after being heated with acetylacetone in alkaline solution, react with *p*-dimethylaminobenzaldehyde and give rise to red-coloured solutions.

Table IV. *The influence of concentration of hydrochloric acid on the rate and intensity of colour development.*

Final concentration of hydrochloric acid %	Time of colour development (minutes)						
	5	10	15	20	30	40	60
	Colorimeter readings (mm.)						
2·5	34·5	32·5	30·0	28·7	26·0	25·3	**24·5**
5·0	27·0	24·9	24·6	**24·3**	24·3	24·4	24·3
10·0	**24·7**	24·8	25·0	24·8	24·9	25·2	25·4

Table IV, were obtained by matching the colours which developed in the presence of the various hydrochloric acid concentrations, after definite intervals of time, against a stable artificial colour standard. It will be noted that in the presence of 5 % hydrochloric acid the colour develops rapidly and is constant in intensity after 20 minutes. Moreover, this concentration of hydrochloric acid gives rise to the greatest depth of colour and has therefore been selected as the most suitable concentration of acid for use in the preparation of the *p*-dimethylaminobenzaldehyde reagent.

The influence of the time of heating on the colour development.

Two tests were carried out in the same manner as those already described except that the glucosamine hydrochloride solutions were heated with the acetylacetone reagent for different periods of time. It will be observed from the colorimeter readings given in Table V that the maximum colour intensity

Table V. *The influence of the time of heating with the acetylacetone reagent on the subsequent colour development.*

Time of heating at 100° (minutes)	Colorimeter readings (mm.)	
	Exp. No. 1	Exp. No. 2
10	25·0	24·2
15	18·1	17·8
20	23·0	27·2

develops after the solutions have been heated for 15 minutes. It has been found that the colours obtained after heating solutions of glucosamine hydrochloride with the acetylacetone reagent for 20 minutes or longer, are of a slightly different tint from those obtained with solutions which have been heated for 10 or 15 minutes.

SUMMARY.

A method is described for the colorimetric estimation of glucosamine and chondrosamine. By heating these substances in alkaline solution with acetylacetone they can be converted into pyrrole derivatives which on treatment with *p*-dimethylaminobenzaldehyde give rise to very stable red-coloured solutions. Within limits the method gives a good degree of proportionality between the hexosamine content and the colour intensity.

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