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Interaction of Amino Compounds with Monosaccharides

A Spectrophotometric Study of the Colour with Ehrlich's Reagent

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The differing results on the glucosamine content of the jelly coat of the sea-urchin egg obtained by Krauss [1,2], Vasseur [3], and Vasseur and Immers [4] prompted us to undertake a more careful study of this matter.

Among the hydrolysis products of the jelly coat substance are about 45 per cent of reducing sugars, 1.6 per cent of lysine, and glycine, but no hexosamine [3,4]. As has been noted previously [5,6] a mixture of a reducing sugar with lysine or glycine gives a positive colour reaction for hexosamine when treated according to the procedure described by Elson and Morgan [7]. In contradistinction to glucosamine, a mixture of reducing sugar and, for example, lysine gives a red colour with Ehrlich's reagent after heating with sodium carbonate buffer alone [4], *i.e.* in the absence of acetylacetone. The optimum pH values for glucosamine and for the sugar — amine mixture differ also. This enabled us to give a possible method for the elimination of this error [8]. A more detailed account on these investigations is given in this paper.

METHOD

In a "Hysil" test tube (150 × 15 mm), marked at 5 ml, 0.5 ml of the sample was mixed with 0.5 ml of the buffer solution (usually *M* sodium carbonate — sodium bicarbonate), containing acetylacetone if desired. A larger volume of this mixture was prepared when the pH of the solution was to be measured. The pH was determined with a glass electrode (Radiometer PHM 11a, Copenhagen), the pH values between 9.0 and 11.5 being corrected for the sodium ion content of the solution.

The mixture (1.0 ml) was heated in the stoppered tube in a vigorously boiling water bath for an appropriate time, then cooled and 2 ml of 95 per cent ethanol was added, and the contents were mixed. To this mixture was added 0.5 ml of Ehrlich's reagent (0.8 g *p*-dimethylaminobenzaldehyde — Eastman Kodak, three times recrystallised from 95 per cent ethanol — in 30 ml of 95 per cent ethanol plus 30 ml of concentrated hydrochloric

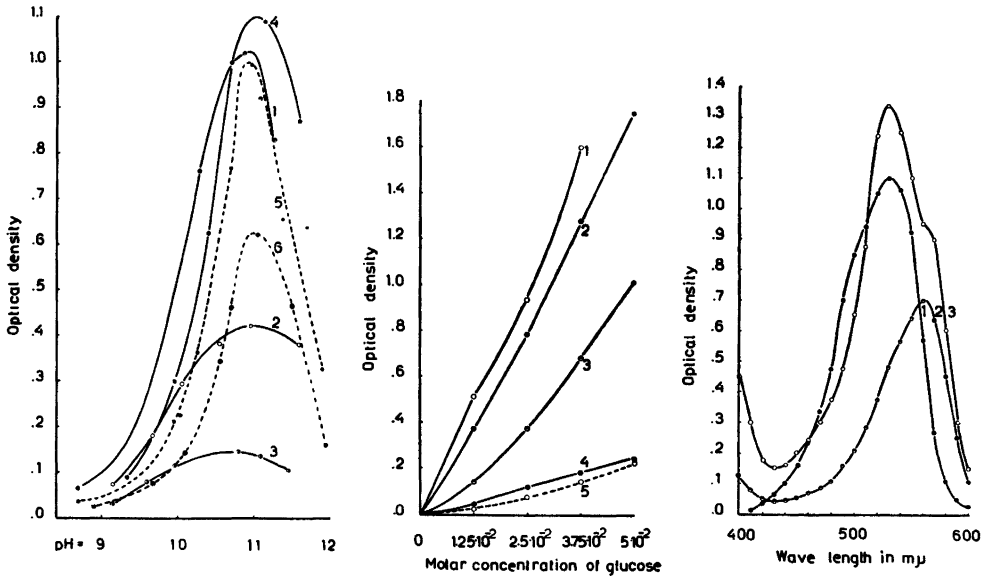

Fig. 1. Influence of pH during the heating on the colour formation with Ehrlich's reagent. Mixture of 0.50 ml of M *carbonate buffer plus 0.25 ml of 0.05* M *glucose solution and 0.25 ml of 0.025* M *solution of: lysine · 2 HCl (curve 1), β-alanine (curve 2), glycine (curve 3), ethylamine · HCl (curve 4). Mixture of 0.50 ml of* M *phosphate buffer plus 0.25 ml of 0.05* M *glucose solution and 0.25 ml of 0.025* M *solution of: lysine · 2 HCl (curve 5), ornithine · 2 HCl (curve 6). Optical density at 570 mμ, after 30 minutes.*

Fig. 2. Effect of carbon chain length of amino acids on the reaction with glucose. 0.5 ml of M *carbonate buffer of pH 10.90 plus 0.5 ml of mixture of amino acid (0.0125* M*) and glucose (from 0 to 0.05* M*). Curves: 1 — lysine, 2 — ornithine, 3 — β-alanine, 4 — α,γ-diaminobutyric acid, 5 — glycine. Optical density at 570 mμ, after 30 minutes.*

Fig. 3. Absorption spectra: curve 1 — a 0.00125 M *glucosamine · HCl solution treated with 1.96 per cent (v/v) acetylacetone in* M *carbonate buffer, at pH 9.55; curve 2 — a mixture of 0.025* M *glucose and 0.0025* M *lysine · 2 HCl treated with* M *carbonate buffer at pH 10.90; curve 3 — a mixture of 0.025* M *glucose and 0.0025* M *lysine · 2 HCl treated with 1.96 per cent (v/v) acetylacetone in* M *carbonate buffer at pH 10.50. Readings 30 minutes after the addition of Ehrlich's reagent.*

acid, sp.gr. 1.19) and then ethanol to the 5 ml mark. The solution was thoroughly mixed and the colour was measured after certain time intervals with a Beckman spectrophotometer model DU, wavelengths 400 to 600 mμ, slit width 0.015 to 0.04 mm, using a 10 mm Corex cell.

EXPERIMENTAL

1. *Influence of pH and buffer during heating on the formation of coloured products.* Glucose was heated with different amino compounds at various pH values in the presence or in the absence of acetylacetone. The buffer used in

most experiments was *M* sodium carbonate — bicarbonate, but 0.5 *M* sodium phosphate (tribasic-dibasic) also was tried, giving essentially the same results (see Fig. 1). The optimum pH is about 10.8 to 11.2 for the glucose — amine mixtures either with or without acetylacetone. On the other hand, for glucosamine the optimum pH is about 9.5 to 9.6 [9].

2. *Influence of the structures of the amino compound and carbohydrate on the ability to give coloured products.* Of the α-amino acids lysine and ornithine give by far the most intense colours [4]; glycine, arginine, and asparagine give a much weaker coloration, and all the other α-amino acids tested, including alanine, give no colour when tested separately with carbohydrate (without acetylacetone). A weak colour is given also by α, γ-diaminobutyric acid (Fig. 2, curve 4), and β-alanine gives a colour intermediate in intensity between the former and ornithine (Fig. 2, curve 3). Acetamide and hydroxylamine do not give any colour under these conditions.

Only primary amines give a positive reaction; secondary and tertiary amines do not react under these conditions (see Table 1).

Table 1. Optical density at 570 mμ of the coloured product of glucose with primary, secondary, and tertiary methylamine in M *carbonate buffer at pH 10.95.*

0.25 ml 0.025 *M*	0.25 ml glucose			
	0.05000 *M*	0.02500 *M*	0.01250 *M*	0.00625 *M*
$CH_3NH_2 \cdot HCl$	1.600	.880	.317	.085
$(CH_3)_2NH \cdot HCl$	.067	.045	.050	.000
$(CH_3)_3N \cdot HCl$	.075	.025	.015	.000

These results demonstrate that (1) the amino group must be unsubstituted, *i.e.* only primary amino groups react; (2) the colour is more intense when the amino group is remote from carboxyl, hydroxyl, and other electron-attracting groups, *i.e.*, a stronger coloration develops when the carbon chain separating the carboxyl and the amino groups of the amino acid is lengthened (Fig. 2).

In the presence of acetylacetone, ornithine — glucose give a more intense colour than lysine — glucose (*cf.* Immers and Vasseur [8]).

On the other hand, the structure of the monosaccharide does not seem to be equally essential. Glucose, galactose, mannose, fructose, arabinose, xylose, fucose, and rhamnose have all the same action. The behaviour of lactic aldehyde and other similar compounds would be of greatest interest for studying the mechanism of this reaction.

3. *Character and stability of the colour.* a) *Under ordinary conditions*, i.e. *in strongly acid medium.* The absorption curves (see Fig. 3) for glucosamine (curve 1), glucose — lysine without acetylacetone (curve 2), and glucose — lysine with acetylacetone (curve 3) are all different. The glucosamine curve has its maximum at 530 mμ. The glucose — lysine curve without acetylacetone has one maximum, at 560 mμ, but in the presence of acetylacetone two maxima, one at 530 mμ and a smaller one at about 570 mμ. These curves were obtained by measuring the optical density 30 minutes after the addition of Ehrlich's reagent.

When the solutions are kept at room temperature, the character of the colour changes (see Figs. 4 to 6) and the intensity decreases. The maximum of the glucosamine curve (Fig. 4) shifts from 530 mμ initially to 510 mμ after 21 hours *. The presence of the compound giving the absorption at 510 mμ is evident even after 20 minutes, just as the 530 mμ compound is still present after 21 hours. In each case the presence of the minor component is indicated by a bulge on the curve. The colour intensity decreases slowly with time.

In the absorption curve of the glucose — lysine mixture without acetylacetone two maxima, one at 570 mμ and the other at 510 mμ, gradually displace the maximum at 560 mμ (Fig. 5). The intensity of the 570 mμ peak is lowered on keeping at room temperature, whereas the intensity of the 510 mμ peak seems to increase slightly.

The absorption of the glucose — lysine solution with acetylacetone does not show any of these pronounced changes with time (Fig. 6). The main effect seems to be a diminution of the colour intensity. However, the peak at 530 mμ fades more rapidly than that at 570 mμ.

b) *At different pH.* The colour intensity of the products formed is sensitive to differences in pH. The colour is most stable in strongly acid media, below pH 1.3. When the solution is made less acid, the colour gradually fades and disappears completely at a pH of approximately 8.5 (Table 2).

Table 2. Colour intensity of glucose — lysine chromophore without acetylacetone at various pH values.

pH	1.00	2.32	2.85	3.60	5.40	6.35	8.70
Optical density at 570 mμ	.867	.540	.485	.393	.269	.152	.030

* After the experimental part of this work was completed, the paper by Schloss [10] appeared, containing *inter alia* a similar statement.

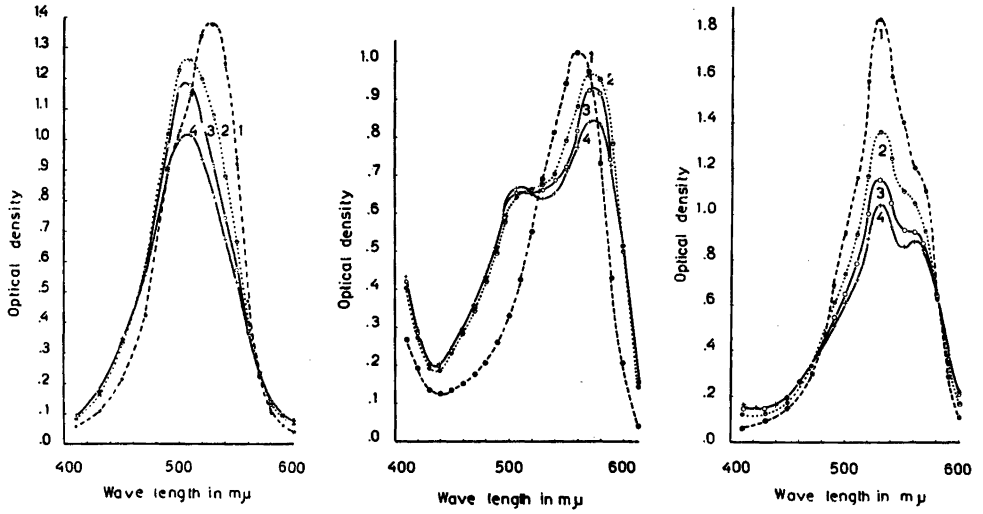

Fig. 4. Change of absorption spectrum of the chromophore of 0.0015 M *glucosamine · HCl treated with 1.96 per cent acetylacetone in* M *carbonate buffer at pH 9.55 and developed with Ehrlich's reagent, on keeping at room temperature. Curves: 1 — after 20 minutes, 2 — 21 hours, 3 — 45 hours, 4 — 68 hours.*

Fig. 5. Change of absorption spectrum of the chromophore of 0.025 M *glucose and 0.0025* M *lysine · 2 HCl treated with* M *carbonate buffer at pH 11.00 and developed with Ehrlich's reagent, on keeping at room temperature. Curves: 1 — after 20 minutes, 2 — 18 hours, 3 — 25 hours, 4 — 42 hours.*

Fig. 6. Change of absorption spectrum of the chromophore of 0.025 M *glucose and 0.007* M *lysine · 2 HCl treated with 1.96 per cent acetylacetone in* M *carbonate buffer at pH 10.80 and developed with Ehrlich's reagent, on keeping at room temperature. Curves: 1 — after 20 minutes, 2 — 21 hours, 3 — 45 hours, 4 — 68 hours.*

When this colourless solution is re-acidified, the colour reappears, the intensity being higher in more acid solution, just as before. The solution may be kept alkaline for many hours without losing the capacity to develop the red colour when re-acidified.

However, there is a difference in reactivity of the coloured products towards alkali. The colour derived from glucosamine or from glucose — lysine with acetylacetone disappears immediately upon addition of excess sodium hydroxide, even when the coloured sample has been standing for many hours. The colour from a glucose — lysine mixture without acetylacetone, on the other hand, does not disappear completely on the addition of alkali, provided that the colour has been developed some 18 hours or more before, and the solution thereafter has been kept at room temperature. There is, however, a decrease in intensity. Only the absorption maximum at 570 mμ is evident after this treatment.

4. *Behaviour of the glucose — lysine chromogen without acetylacetone when kept at different pH values before the addition of Ehrlich's reagent.* Samples of glucose (0.05 *M*, 1 part) and lysine (0.025 *M*, 1 part) were heated with carbonate buffer (*M*, pH 10.95, 2 parts) in the usual manner. To 1.0 ml aliquots were added (1) 0.50 ml of distilled water; (2) 0.20 ml of 6 *N* HCl and 0.30 ml of distilled water; (3) 0.30 ml of 6 *N* HCl and 0.20 ml of distilled water; (4) 0.50 ml of 6 *N* HCl. The pH values after these additions were 10.9, 6.7, 3.35, and < 1.2, respectively.

The samples were allowed to stand for different lengths of time, 0, 4, 21, 45, and 71 hours, before the colour was developed with Ehrlich's reagent. Thereafter the absorption curve between 400 and 600 mμ was measured for each sample after 40 minutes, 22 hours, 46 hours, and 71 hours. Lack of space does not allow the publication of all these curves, but Table 3 gives some data

Table 3. Change in optical density at 510 and 570 mμ of the glucose-lysine chromophore without acetylacetone when the chromogen was kept at different pH values before the addition of Ehrlich's reagent or p-dimethylaminobenzaldehyde (p-DAB).

Time in hours before the addition of Ehrlich's reagent or p-DAB	Measurement 40 minutes after the addition of							
	Ehrlich's reagent						p-DAB	
at pH	10.90		3.35		<1.2		<1.2	
wavelength, mμ	510	570	510	570	510	570	510	570
0	.685	2.00	.630	1.90	.580	1.05	.650	1.38
4	.652	1.90	.560	1.56	.420	.660	.430	.710
21	.710	2.00	.452	1.07	.317	.320	.340	.330
45	.627	1.80	.384	.740	.273	.205	.297	.210
71	.665	2.00	.318	.488		.185		.183
Time in hours before the addition of Ehrlich's reagent or p-DAB	Measurement 46 hours after the addition of							
	Ehrlich's reagent						p-DAB	
at pH	10.90		3.35		<1.2		<1.2	
wavelength, mμ	510	570	510	570	510	570	510	570
0	.958	1.50	.880	1.25	.730	.583	.940	.785
4	.945	1.54	.795	1.08	.625	.390	.790	.510
21	.995	1.58	.660	.720	.525	.270	.685	.305
45	.915	1.38	.620	.530	.500	.232	.670	.255

for the changes in intensity at the essential wavelengths, 510 and 570 mμ (*cf.* Fig. 5). The variations in the individual data are rather high in this particular experiment owing to the large number of operations which had to be performed simultaneously or in quick succession.

On keeping, the samples of pH 10.9 showed very little if any change in the intensity of the colour and the character of the curves, provided that the measurements were carried out at the same time after the addition of Ehrlich's reagent. The curves obtained after 40 minutes were all of the kind shown in Fig. 5, curve 1 (with but one maximum at ca. 560 mμ), and the curves obtained 46 hours after the addition of Ehrlich's reagent were similar to curve 4 in Fig. 5 (with one maximum at 570 mμ and another at 510 mμ). Thus, keeping at room temperature at the same pH as that at which the heating of the sample had been carried out, does not affect the curve.

On the other hand, when the pH of the sample is lowered after the heating at pH 10.9 and the sample is allowed to stand at this lower pH before Ehrlich's reagent is added, the character of the curves changes. This change is the more pronounced the more the sample has been acidified. The intensity of the colour is decreased considerably. The most interesting feature is, however, the appearance of the 510 mμ peak in the 40 minutes curves and the dominance of this peak in the 46 hours curves. By changing the pH of the sample to a very low value (< 1.2), after the heating has been carried out at pH 10.9, and allowing the sample to remain at this pH one or two days at room temperature, the 570 mμ component disappears almost completely, whereas the 510 mμ component becomes the dominating chromogen. These two components may thus be obtained almost free from each other and this should facilitate a closer study of their structures (*cf.* Schloss [10]).

These experiments show that the 570 mμ component is stable in alkaline solution but unstable in acid solution. It seems very probable that the 570 mμ component is transformed partly to the 510 mμ component in acid media. On the other hand, in alkaline media the 510 mμ component is less stable, and seems to be transformed partly to the 570 mμ component (see section 3b).

5. *Effect of concentration of the two components (sugar and amine) on the colour intensity.* Different mixtures of glucose and lysine in carbonate buffer of pH 10.9 without acetylacetone were treated in the usual way. The concentration of one of the components was kept constant and the concentration of the other was varied (Table 4). It thus becomes evident (1) that glucose is degraded to a greater extent than is lysine, and (2) that the degradation of glucose is less pronounced when higher concentrations of lysine are present simultaneously. Thus, the reaction between lysine and glucose will compete with the degradation of glucose, so that when lysine has reacted with glucose, the

Table 4. Effect of concentration of glucose and lysine on the colour intensity, optical density at 570 mμ.

Concentration of lysine, molarity	Concentration of glucose, molarity				
	$3.125 \cdot 10^{-3}$	$6.25 \cdot 10^{-3}$	$1.25 \cdot 10^{-2}$	$2.5 \cdot 10^{-2}$	$5.0 \cdot 10^{-2}$
$3.125 \cdot 10^{-3}$	.000	.050	.225	.620	1.105
$6.25 \cdot 10^{-3}$	.020	.080	.280	.770	1.57
$1.25 \cdot 10^{-2}$	.030	.110	.385	.860	1.90
$2.5 \cdot 10^{-2}$	.045	.180	.365	.880	1.90
$5.0 \cdot 10^{-2}$	.055	.165	.450	.920	2.00

latter will not be liable to be degraded to the same extent as when present alone or together with a lower concentration of lysine.

6. *Effect of pretreatment of the two components (sugar and amine) on the colour intensity.* Table 5 shows some of the results of two series of experiments, in which lysine and glucose were treated separately or together (0 or 20 minutes heating at pH 9.60) and then treated in the normal way with or without acetylacetone.

The results are very complex, but some conclusions may be drawn from the experiments. (1) The 570 mμ peak is the dominating one in the absence of acetylacetone (columns with even numbers) and the 530 mμ peak in the presence of acetylacetone (columns with uneven numbers). (2) The very low value for the non-heated glucose — lysine mixture with acetylacetone (columns 3 and 7) is increased very considerably when the mixture is heated before the acetylacetone is added (column 1). (3) The optical density without acetylacetone is nearly doubled when the mixture is heated for 20 minutes before the ultimate heat-treatment (columns 2, 4, 8). (4) When glucose and lysine have been heated separately, the optical density in the absence of acetylacetone is decreased (columns 6, 8), but in the presence of acetylacetone it is increased (columns 5, 7). (5) The optical density both with and without acetylacetone is barely influenced if lysine alone is heated before (columns 7, 9, and 8, 10, respectively), but there is a considerable decrease in optical density without acetylacetone (columns 8, 12) and an increase with acetylacetone (columns 7, 11) if glucose is heated separately before the ultimate heat-treatment.

There seems, therefore, to exist in the heated glucose solution a degradation product of glucose which in the absence of acetylacetone does not contribute to the formation of the chromogen, but which in the presence of acetylacetone has this property.

Table 5. Effect of pretreatment of glucose and lysine on the colour intensity.

Column no.	1	2	3	4	5	6	7	8	9	10	11	12
0.005 *M* lysine in *M* carbonate buffer, pH 9.60; no heating; ml (A)	—	—	—	—	—	—	.25	.25	—	—	.25	.25
0.05 *M* glucose in *M* carbonate buffer, pH 9.60; no heating; ml(B)	—	—	—	—	—	—	.25	.25	.25	.25	—	—
0.005 *M* lysine + 0.05 *M* glucose in *M* carbonate buffer, pH 9.60; no heating; ml (C)	—	—	.25	.25	—	—	—	—	—	—	—	—
As A but 20 min. heating; ml	—	—	—	—	.25	.25	—	—	.25	.25	—	—
As B but 20 min. heating; ml	—	—	—	—	.25	.25	—	—	—	—	.25	.25
As C but 20 min. heating; ml	.25	.25	—	—	—	—	—	—	—	—	—	—
M carbonate buffer, pH 9.60; ml	.25	.75	.25	.75	—	.50	—	.50	—	.50	—	.50
6 per cent acetylacetone reagent (in *M* carbonate buffer), pH 9.60; ml	.50	—	.50	—	.50	—	.50	—	.50	—	.50	—
20 minutes heating of final mixture	+	+	+	+	+	+	+	+	+	+	+	+
Optical density at 530 mμ Exp.1	.375	.310	.075	.192	.175	.110	.080	.185	.045	.205	.225	.085
» 2	.460	.380	.050	.195	.225	.108	.096					
Optical density at 570 mμ Exp.1	.470	.465	.060	.295	.145	.153	.065	.292	.043	.320	.185	.115
» 2	.620	.590	.045	.295	.205	.135	.085					

7. *Change of ultraviolet spectrum of glucose in sodium carbonate — bicarbonate buffer of pH 9.6, and of this buffer alone upon heating.* The ultraviolet spectrum of a glucose solution in carbonate buffer (pH 9.6) undergoes very complicated variations when the solution is heated for different lengths of time. In addition to the peak at ca. 265 mμ [11], a peak at 290 mμ appears after 20 minutes heating. This peak fades shortly afterwards. By analysing the ultraviolet spectrum of the carbonate buffer alone it could be demonstrated that this peak at 290 mμ must be attributed entirely to the buffer and not to any degradation product of glucose.

When the heated samples were acidified, only one maximum (at 250 mμ) remained in the 220 to 320 mμ region. The increase of the optical density at 250 mμ indicates that during the first 20 to 30 minutes of heating (at pH 9.6) a compound is formed, which exhibits a maximum at 250 mμ in acid media and at 265 mμ in alkaline media. Thereafter another compound with maximal absorption at a lower wavelength than 220 mμ is formed.

GENERAL DISCUSSION

The colorimetric method for the determination of glucosamine as described by Elson and Morgan [7] may give erroneous results when simple carbohydrates and amino compounds are present simultaneously. This may often be the case when hydrolysates from biological material are analysed. In the present investigation it has been shown that when monosaccharides are heated in alkaline media (with or without the addition of acetylacetone) with certain amino acids or primary amines, the resulting solutions give red colorations with Ehrlich's reagent. The coloured products exhibit maximal absorption in the same part of the spectrum as the glucosamine reaction product. The intensity of the colour depends on the following: (1) the pH during the heating in alkaline medium; (2) the acetylacetone concentration; (3) the nature of the carbohydrate and amino compounds; (4) the pH after the addition of Ehrlich's reagent; (5) the concentration of the carbohydrate and the amine.

The optimal pH during the heating is 9.5 to 9.6 for the glucosamine reaction [9], but pH 10.8 to 11.2 (Fig. 1) for the sugar — amine mixtures. The optimal acetylacetone concentration for the sugar — amine mixtures is about 0.8 per cent (v/v) in the acetylacetone reagent (Fig. 7), *i.e.* about 0.4 per cent in the reaction mixture. For glucosamine, on the other hand, the colour intensity increases with the concentration of acetylacetone up to at least 3 per cent in the acetylacetone reagent (Fig. 7, curve 1). Elson and Morgan [7] use 1.96 per cent acetylacetone at pH 9.5. The same concentration and pH have been used in many of the modifications of this method [12,13,14]. Sørensen [9] and Blix [15], however, propose the use of higher concentrations of acetylacetone.

Our own investigations show that usually the sugar — amine error is reduced by using a higher acetylacetone concentration. Therefore, 4 to 5 per cent of acetylacetone at pH 9.5 to 9.6 is recommended. On the other hand, sugar — amine interaction can be detected most easily by carrying out analyses at other acetylacetone concentrations as well, and at another pH. It is advisable to test for the sugar — amine error by analysing a sample at pH 10.8 to 11.2 with 0.8 per cent acetylcetone in the acetylacetone reagent or even without acetylacetone and to read the sample both at 530 mμ, which is

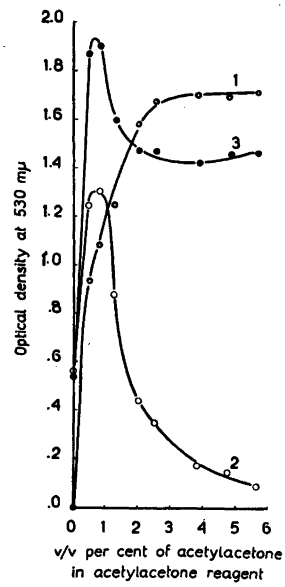

Fig. 7. Influence of acetylacetone concentration on the colour formation with glucosamine (curve 1; 0.0015 M*), glucose plus lysine (curve 2; 0.025* M *and 0.0025* M*, respectively), and a mixture of these (curve 3; 0.0015* M *glucosamine · HCl, 0.025* M *glucose, 0.0025* M *lysine · 2 HCl). Measurement of optical density at 530 mμ, 30 minutes after the addition of Ehrlich's reagent.*

the optimum for glucosamine and for the sugar — amine mixtures with acetylacetone, and at 570 mμ, the optimum for the sugar — amine mixtures without acetylacetone.

Curve 3 (Fig. 7) which relates to the colours produced by mixtures of glucosamine, glucose and lysine at different acetylacetone concentrations, indicates that the two reactions counteract each other to some extent. This becomes particularly evident at an acetylacetone concentration higher than 2 per cent, where the colour intensity is lower than that for glucosamine alone, thus giving rise to a negative error. The region where the glucosamine curve and the glucosamine — glucose — lysine curve intersect, depends on the concentrations of the interfering substances [8].

As to the chemical nature of the different chromogens, Gottschalk and Partridge [16,17] have recently suggested that on alkaline treatment a glucose — lysine mixture gives a Schiff's base, the enol form of which gives a dihydropyrazine condensation product. This in turn is assumed to form with *p*-dimethylaminobenzaldehyde the chromophore in the Ehrlich reaction.

Our results indicate, however, that the reactions may be still more complex. A Schiff's base is formed, when simple sugars react with almost all amino acids under alkaline conditions (*cf.* 11, 18 for literature). However, a coloured product with Ehrlich's reagent is formed only with a few of these amino acids and the degree of colour formation depends on the distance between the reacting amino group and electron-attracting groups which may be present.

Under the conditions of the glucosamine reaction, the 1 : 1 ratio calculated for the Schiff's base reaction cannot be expected to give the maximum colour, since some of the glucose is degraded, as shown in sections 5 and 7 (see also Frankel and Katchalsky [19]). A higher monosaccharide concentration, as compared with the amino compound, must therefore be present in order to give maximal colour. This is the case with hydrolysates of many mucoid and mucopolysaccharide preparations, the observed values of the hexosamine content of which will be in error for this reason.

Furthermore, the experiments reported in section 6 and those illustrated in Fig. 7 demonstrate that the presence or absence of acetylacetone is of very great significance. The maxima for the reaction products occur at different wavelengths in the different cases. When the coloured samples are allowed to stand at room temperature, these maxima shift, but the displacement is different for the different types of products.

Finally, the different behaviour towards alkali, reported in section 3b, indicates basic differences in these reactions.

SUMMARY

When monosaccharides are heated in alkaline media (pH 9 to 12) with certain amino acids (*e.g.* lysine) or primary amines, in the presence or in the absence of acetylacetone, red coloured products are obtained with Ehrlich's reagent. These interfere with the colorimetric determination of glucosamine by the method of Elson and Morgan [7].

This interference can be controlled by carrying out the colorimetric determination on the unknown sample parallel with the glucosamine standard (1) at pH 9.5 to 9.6 and at pH 10.8 to 11.2; (2) with different acetylacetone concentrations, *e.g.* 0, 0.8, and 4 to 5 per cent in the reagent; (3) by determining the absorption at 530 and 570 mμ.

Furthermore, the main results may be summarised as follows. The optimum pH for glucosamine is 9.5 to 9.6, for the sugar — amine mixtures 10.8 to 11.2.

The amino compound must have the amino group unsubstituted. The intensity of the colour is increased when the distance between the reacting amino group and electron-attracting groups (-COOH, >CO, $-\overset{|}{\underset{|}{C}}OH$, etc.), which might be present, is increased.

The absorption curve for glucosamine has its maximum at 530 mμ, but this shifts to 510 mμ on keeping. The glucose — lysine mixture without acetylacetone has its maximum at 560 mμ, but on keeping two maxima appear,

one at 570 and another at 510 mμ. The glucose — lysine mixture with acetylacetone has two maxima, at 530 and 570 mμ, respectively.

When the coloured, strongly acid solutions are made alkaline, the colour disappears completely, except for the glucose — lysine mixture without acetylacetone which has been standing for about a day or more at room temperature.

The 570 mμ component of the glucose — lysine mixture without acetylacetone is stable in alkaline but unstable in acid media, whereas the reverse is true for the 510 mμ component.

The degradation of glucose in alkaline solution is counteracted by its reaction with lysine to form the chromogen.

A degradation product of glucose is formed in alkaline solution which upon further heating with lysine and in the presence but not in the absence of acetylacetone contributes to the formation of the chromogen.

In a pure sodium carbonate — bicarbonate buffer of pH 9.6 a compound is formed upon heating, characterized by an absorption maximum at 290 mμ.

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