140. THE ISOLATION OF SMALL QUANTITIES OF GLUCOSAMINE AND CHONDROSAMINE

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THE widespread occurrence of amino-sugars as components of polysaccharides is becoming increasingly evident. The conclusion that an amino-sugar is present in a material under investigation has, however, in many instances, been based exclusively on the appearance of a positive colour reaction when the products of acid hydrolysis of the material under investigation are warmed with an alkaline solution of acetylacetone and subsequently treated with Ehrlich's *p*-dimethyl-aminobenzaldehyde reagent according to the method of Elson & Morgan [1933] or one of the modifications of this method [Nilsson, 1936; Palmer *et al.* 1937; Sørensen, 1938; Hewitt, 1938]. For purposes of preliminary investigation a positive colour may be accepted as a very strong indication that an amino-sugar is present, but this evidence should be confirmed by the isolation of the hydrochloride or some other suitable crystalline derivative of the amino-sugar before its presence in the material is finally accepted. The methods available for the isolation of small amounts of the naturally occurring amino-sugars, especially when these substances are contaminated with sugars or with the products of protein hydrolysis, cannot be considered satisfactory. A useful procedure for the isolation of glucosamine is that developed by Chargaff & Bovarnick [1937] in which the amino-sugar is converted into the sparingly soluble carbobenzyloxy-glucosamine which yields the free hexosamine on hydrogenation according to Bergmann & Zervas [1932]. This method does not appear to be suitable for the isolation of small quantities (10–20 mg.) of glucosamine, especially in presence of excess of other sugars or amino-acids. Furthermore, the carbobenzyloxy-derivative of chondrosamine has been found by us to be considerably more soluble in water than carbobenzyloxyglucosamine and is therefore quite unsuitable for the isolation of the former amino-sugar.

Irvine & Earl [1922] showed that glucosamine formed a sparingly soluble Schiff's base when treated in alkaline solution with salicylaldehyde and somewhat later Bergmann & Zervas [1931] described the corresponding anisaldehyde derivative. During an attempt to isolate the amino-sugar that was believed to be present in the specific polysaccharide of *Bact. dysenteriae* (Shiga) a number of similar Schiff's bases were prepared and examined [Morgan, 1938]. Of these only the *p*-nitrobenzylidene and the cinnamylidene derivatives were found to be suitable for the isolation of small quantities of glucosamine. The corresponding derivatives of chondrosamine, owing to their solubility in water, were of little use for the isolation of this amino-sugar. Neuberger [1938] employed the Schiff's base derived from 2:4-dihydroxybenzaldehyde for the isolation of glucosamine from the polysaccharide component of serum albumin. Unfortunately 2:4-dihydroxybenzylidenechondrosamine is also too soluble in water to be used for the isolation of small amounts of chondrosamine.

The ease with which Schiff's bases of this kind can be formed and subsequently decomposed with liberation of the amino-sugar in almost theoretical yield prompted us to investigate the properties of Schiff's bases derived from other aldehydes in the hope of finding a derivative that would enable small amounts (10–30 mg.) of glucosamine and chondrosamine to be isolated in good yield from crude protein or polysaccharide hydrolysis products.

The following aldehydes were employed: (1) *p*-nitrobenzaldehyde, (2) *p*-nitrocinnamic aldehyde, (3) 3-methoxy-4-hydroxybenzaldehyde (vanillin), (4) 2-hydroxynaphthaldehyde, (5) 2:4-dinitrobenzaldehyde, (6) 2-hydroxy-4-benzeneazobenzaldehyde (benzeneazosalicylaldehyde), (7) 2-hydroxy-4-*p*-nitrobenzeneazobenzaldehyde, (8) 3-methoxy-4-acetoxybenzaldehyde, (9) 2-methoxynaphthaldehyde, (10) 2-hydroxy-*p*-nitrobenzeneazonaphthaldehyde.

The first four aldehydes reacted readily with glucosamine and gave Schiff's bases that were sparingly soluble in water. Aldehydes (3) and (4) gave rise to sparingly soluble derivatives of chondrosamine as well as of glucosamine; (8) and (9) failed to react with the amino-sugars under the conditions employed whereas (10) was so insoluble in the usual organic solvents that its solution could only be accomplished when inconveniently large volumes of solvent were employed. The Schiff's base, 2-hydroxy-4-benzeneazobenzylidenechondrosamine derived from aldehyde (6), was too soluble in water to be of use.

EXPERIMENTAL

The method employed for the preparation of the various Schiff's bases was similar in all the examples given and for this reason the experimental details will only be described in one instance.

2-Hydroxynaphthylideneglucosamine. A solution of glucosamine hydrochloride (20 mg.) in 0.5 ml. water is treated with sodium acetate (2 mol.) and gently shaken until the solid has dissolved. The mixture at this stage should be just acid to litmus. An excess of 2-hydroxynaphthaldehyde (50 mg.), prepared according to Duff & Bills [1934], is dissolved in methyl alcohol (3 ml.) and is added to the amino-sugar. After thorough mixing and keeping for 2–3 hr. in a dark place, the solution, which develops a bright yellow colour, is taken to dryness *in vacuo* at 10–15°. The excess aldehyde is then removed from the reaction products by extraction with dry chloroform or ether in which the Schiff's base of the amino-sugar is insoluble. After removing the remaining chloroform the residue is extracted at 0° with three 0.5 ml. portions of distilled water in order to remove inorganic salts. It is essential that this process should be carried out as near to 0° as possible as at higher temperatures the Schiff's base becomes appreciably soluble in water. The three washings are mixed, concentrated to about 0.5 ml. and set aside at 0° for several hours. A further amount of the Schiff's base usually separates in the form of bright yellow crystals and is collected in the bottom of a centrifuge tube in the usual manner. This additional quantity of base, after thoroughly washing with ice-cold water, is added to the main portion. The material, which consists of almost pure 2-hydroxynaphthylideneglucosamine, is dried *in vacuo* and recrystallized from 1 to 2 ml. of warm methyl alcohol; the Schiff's base separates in tufts of fine needles, m.p. 202–203° (decomp.), and the yield, which at this stage is about 70%, can be increased to 90–95% by the cautious addition of acetone to the alcoholic solution. The substance mutarotates; the initial $[\alpha]_{5461} + 274^\circ$ (in methyl alcohol) changing to $+217^\circ$ in 18 hr. (Found: C, 61.10; H, 5.71; N (Dumas), 4.15%. $C_{17}H_{19}O_6N$ requires C, 61.26; H, 5.71; N, 4.20%). The substance is soluble in methyl alcohol and in pyridine, slightly soluble in cold ethyl alcohol and acetone

and insoluble in ether or chloroform. In aqueous alcoholic suspension the Schiff's base, when treated in the cold with a few drops of conc. HCl, dissolves and a few minutes later separates in the form of a crystalline hydrochloride. The yellow needles sinter at about 200° but do not melt. (Found: Cl, 9.27%. $C_{17}H_{22}O_7NCl$ requires Cl, 9.15%.)

2-Hydroxynaphthylidenechondrosamine. M.P. 175–178° (decomp.). Somewhat more soluble in methyl and ethyl alcohols than the corresponding glucosamine derivative. Initial $[\alpha]_{5461} + 287^\circ$ (in methyl alcohol) changing to $+258^\circ$ in 18 hr. (Found: C, 59.80; H, 5.63; N (Dumas), 4.20%. $C_{17}H_{19}O_6N$ requires C, 61.26; H, 5.71; N, 4.20%.) The hydrochloride of the Schiff's base did not separate from solution as described above for the glucosamine compound.

p-Nitrobenzylideneglucosamine. Colourless crystals from ethyl alcohol, M.P. 182–184° (decomp.). (Found: C, 50.5; H, 5.33; N (Dumas), 9.05, 9.16%. $C_{13}H_{16}O_7N_2$ requires C, 50.00; H, 5.13; N, 8.97%.) Sparingly soluble in water. Insoluble in acetone, ether and chloroform.

p-Nitrobenzylidenechondrosamine. Colourless crystals from ethyl alcohol, M.P. 175–176° (decomp. 177°). (Found: C, 49.83; H, 5.24; N (Dumas), 9.02%. $C_{13}H_{16}O_7N_2$ requires C, 50.00; H, 5.13; N, 8.97%.)

3-Methoxy-4-hydroxybenzylideneglucosamine. Colourless crystals, M.P. 184° (decomp.). Sparingly soluble in all usual solvents. $[\alpha]_{5461} + 64^\circ$ (in pyridine). For analysis the substance was dissolved in pyridine and precipitated with ether. (Found: C, 53.78; H, 6.04; N (Dumas), 4.51%. $C_{14}H_{19}O_7N$ requires C, 53.67; H, 6.07; N, 4.48%.)

3-Methoxy-4-hydroxybenzylidenechondrosamine. M.P. 153–155°. (Found: C, 52.96; H, 6.4; N (Dumas), 4.73%. $C_{14}H_{19}O_7N$ requires C, 53.67; H, 6.07; N, 4.48%.)

p-Nitrocinnamylideneglucosamine. M.P. 187° (decomp.). Crystallized from pyridine-ether. Initial $[\alpha]_{5461} + 57.6^\circ$ (in pyridine) changing to $+41.5^\circ$ overnight. (Found: C, 53.61; H, 5.33; N (Dumas), 8.29%. $C_{15}H_{18}O_7N$ requires C, 53.26; H, 5.30; N, 8.28%.)

p-Nitrocinnamylidenechondrosamine. M.P. 172–173°. (Found: C, 53.41; H, 5.58; N (Dumas), 8.15%. $C_{15}H_{18}O_7N$ requires C, 53.26; H, 5.30; N, 8.28%.)

All melting points are uncorrected. The C and H determinations were made by Drs Weiler and Strauss of Oxford.

In a series of comparable experiments the yields of the different Schiff's bases were determined. The results of some of these experiments are shown in Table 1. It will be seen that with the exception of *p*-nitrocinnamylidenechondrosamine all the Schiff's bases examined were suitable when amounts of amino-sugar as large as 50 mg. were employed. Experience showed, however, that when less than this was used the yield in most instances diminished rapidly with decreasing amount of amino-sugar. Vanillin and 2-hydroxynaphthaldehyde, however, were found to give excellent yields of the Schiff's base even when the amount of amino-sugar used fell to 10 mg. The vanillin derivatives were colourless whereas the corresponding 2-hydroxynaphthaldehyde bases were bright yellow in colour. The sparing solubilities of 2-hydroxynaphthylidene-glucosamine and -chondrosamine suggested the use of these compounds as the basis of a method for the isolation of glucosamine and chondrosamine from mixtures containing simple sugars and amino-acids.

The influence of foreign substances upon the final yield of amino-sugar

Some experiments were carried out to determine the influence of certain sugars on the yield of the amino-sugars when these were separated by means of

Table 1. *The yields of Schiff's bases*

Amino-sugar	Amount of amino-sugar hydrochloride mg.	Aldehyde employed	Yield of Schiff's base %
Glucosamine	100	<i>p</i> -Nitrocinnamaldehyde	85, 81
	50	<i>p</i> -Nitrobenzaldehyde	85
	50	3-Methoxy-4-hydroxybenzaldehyde	85, 90, 92
	50	2-Hydroxy-1-naphthaldehyde	96, 90
	10	" "	76
Chondrosamine	50	<i>p</i> -Nitrocinnamaldehyde	39
	50	<i>p</i> -Nitrobenzaldehyde	63, 70
	40	3-Methyl-4-hydroxybenzaldehyde	53, 58
	45	2-Hydroxy-1-naphthaldehyde	84, 79
	30	" "	85, 88
	20	" "	78

Table 2. *Showing the influence of simple carbohydrates on the yield of amino-sugars*

Amino-sugar hydrochloride employed	Wt. mg.	Carbohydrate added	Amino-sugar hydrochloride recovered Yield (%)
Glucosamine	30	Galactose (20 mg.) + fructose (22 mg.)	70
	10	Galactose (5 mg.) + fructose (5 mg.)	73
	10	Galactose (10 mg.)	72
	5	Galactose (5 mg.)	42
Chondrosamine	30	Galactose (33 mg.)	57*
	30	Galactose (7 mg.) + fructose (8 mg.)	84
	20	Galactose (24 mg.)	65*
	20	Galactose (5 mg.) + fructose (5 mg.)	74
	10	Galactose (5 mg.) + fructose (5 mg.)	72
	10	Galactose (10 mg.)	72

* Schiff's base and amino-sugar hydrochloride collected by filtration.

the 2-hydroxynaphthylidene derivative. The results of a few of these experiments are given in Table 2. It will be seen that even a large excess of added sugar has no appreciable influence on the yield of amino-sugar finally obtained. Other experiments showed that 50 mg. of glucosamine and chondrosamine could be recovered in 80 and 73% yield respectively after admixture with 200 mg. of a mixture made up from 50 mg. of each of the following sugars: glucose, fructose, rhamnose and galactose.

The influence of an amino-acid mixture on the yield of the amino-sugar hydrochloride has also been investigated. Under the experimental conditions described the amino-acids form Schiff's bases with 2-hydroxynaphthaldehyde but the separation of these compounds from the Schiff's base of the amino-sugar is easily accomplished owing to the ready solubility of the amino-acid derivatives in chloroform. The Schiff's bases of the amino-sugars are insoluble in this reagent. The influence of the amino-acids on the separation is shown in Table 3. In these experiments an amino-acid mixture, derived from the acid hydrolysis of gelatin, was added in amount equivalent to 50% of the quantity of amino-sugar employed in the determination. It will be seen that this quantity of amino-acid mixture has no appreciable influence on the final yield of amino-sugar hydrochloride. Considerably larger quantities of amino-acids caused the yield to decrease but this falling off in yield could be partly checked by using as solvent for the amino-acid Schiff's bases a mixture of chloroform and carbon

Table 3. *Showing the influence of protein hydrolysis products on the yield of amino-sugars*

Amino-sugar hydrochloride employed	Wt. mg.	Hydrolysed gelatin added mg.	Amino-sugar hydrochloride recovered Yield (%)
Glucosamine	40	20	91
	30	15	77
	20	10	82
	10	5	70
	30	50*	61
Chondrosamine	40	20	73
	30	15	75
	20	10	70

* Hydrolysed casein

tetrachloride (3 : 1) in place of pure chloroform. One experiment using a casein hydrolysis product is also given. The purity of the amino-sugar hydrochloride finally isolated was checked in most of the experiments by the determination of N and optical rotation.

Application

A few experiments were undertaken involving the isolation and identification of the amino-sugar from biological products which contained both sugars and amino-acids.

The following method was found to be satisfactory. The material under investigation is hydrolysed with HCl or H_2SO_4 until the amount of amino-sugar, as estimated by the colorimetric method, reaches a maximum. About 20–30 mg. of amino-sugar are usually sufficient for the method as here described. The acid is almost completely removed by repeated evaporation *in vacuo* at room temperature or by means of baryta according to the nature of the acid employed. If considerable darkening has occurred during the hydrolysis the slightly acid products are treated with 5–10 mg. of charcoal and filtered through a thin layer (1–2 mm.) of charcoal on a small conical filter. The filter is then washed three times with a few drops of distilled water and the filtrate and washings are concentrated to a syrup *in vacuo* in a pointed centrifuge tube measuring about 12×1.5 cm. The walls of the tube are finally washed down with about 0.5 ml. of distilled water. From this stage in the process to the final isolation of the amino-sugar hydrochloride the various steps in the method are all carried out in the centrifuge tube. The acidity of the syrup is now reduced, by means of added KHCO_3 if necessary, until the solution no longer reacts blue with Congo red. The solution must at no time react blue with litmus paper. Sodium acetate crystals (150–200 mg.) are now added and dissolved in the solution by gentle shaking. The 2-hydroxynaphthaldehyde is added in considerable excess in the form of a solution in methyl alcohol (3–4 ml.). The excess of aldehyde employed must depend on the amount of amino-acids present in the mixture but four or five times the quantity calculated for the amino-sugar present can safely be added. The contents of the centrifuge tube are now thoroughly mixed and set aside in a cool, dark place for 2–3 hr. The solution, which becomes bright yellow during this period, is evaporated at room temperature *in vacuo* to about 0.5 ml. The semi-solid mass is treated with a few drops of methyl alcohol and set aside at 0° overnight. The contents of the centrifuge tube are now taken to dryness *in vacuo* below 20° and the solid mass thoroughly extracted three times with about 6 ml. of freshly distilled chloroform. This process removes the excess aldehyde and the Schiff's bases of the amino-acids and leaves behind the Schiff's

base of the amino-sugar and the inorganic salts. The addition of 2 ml. of freshly distilled ether to the chloroform extract before centrifuging will enable the insoluble Schiff's base of the amino-sugar to be packed down readily in the point of the centrifuge tube. Without the addition of ether the insoluble material tends to float to the top of the tube. The extracted residue is now freed from chloroform and treated at 0° with 1 ml. of cold distilled water. The inorganic salts dissolve and the Schiff's base of the amino-sugar remains as a bright yellow residue. The water is left in contact with the Schiff's base for several hours at 0° and in all three or four extractions are made. The combined aqueous extracts are concentrated to 1.0–1.5 ml. and again put aside at 0° for several hours. A further quantity of the Schiff's base which separates is removed by centrifuging, washed three times at 0° with distilled water and added to the main preparation. The crude Schiff's base, which usually melts about 10° lower than the pure compound, can now be recrystallized from warm methyl alcohol in order to identify the amino-sugar or it can be directly converted into the amino-sugar hydrochloride. This can readily be accomplished by suspending the Schiff's base in 1 ml. of 4*N* HCl and heating the centrifuge tube in a boiling water bath for 15 min. The yellow colour of the Schiff's base is destroyed and the white 2-hydroxynaphthaldehyde separates from solution. The free aldehyde is removed from the cooled solution by extraction with ether, three 5 ml. portions of ether being sufficient for this purpose. The combined ether extracts are then shaken with 1 ml. distilled water and the aqueous extract is added to the main acid solution of the amino-sugar hydrochloride. The acid solution is then freed from excess ether, centrifuged to remove any insoluble particles and the clear aqueous solution is transferred to a clean centrifuge tube by means of a capillary pipette. The residue is washed with two 1 ml. portions of water. The solution and washings are concentrated to a syrup, one drop of 2*N* HCl and several drops of methyl alcohol are added and the mixed solution is treated with acetone until a slight cloudiness appears. The solution is then set aside for several hours at 0°. Under these conditions glucosamine and chondrosamine hydrochlorides separate from solution in an almost pure condition and in excellent yield. A single recrystallization from aqueous ethyl alcohol usually gives a pure product.

By means of the above method glucosamine has been isolated in good yield and identified as the amino-sugar present in the ecto-skeleton of the tail segments of the Egyptian scorpion *Buthus quinquestratus*.

From 150 mg. of polysaccharide (kindly supplied by Dr Neuberger), isolated from crystalline egg albumin, it has been possible to isolate 76% of the amino-sugar that is present according to colorimetric estimation. The amino-sugar was identified as glucosamine hydrochloride. Neuberger [1938] has already described the isolation of about 60% of the amino-sugar estimated to be present in the polysaccharide by the colorimetric method and has identified the substance as glucosamine hydrochloride.

The method has enabled the chondrosamine hydrochloride derived from 100 mg. of a preparation of the calcium salt of chondroitin sulphuric acid to be isolated in pure crystalline condition and in a yield of 54% of the amount determined by the colorimetric method. The Schiff's base gave the following analytical figures: M.P. 172–174° [α]₅₄₆₁ + 258° (after 24 hr.). N, 4.3%. C₁₇H₁₉O₆N requires N, 4.20%. The amino-sugar hydrochloride gave [α]₅₄₆₁ + 108° changing to +99.6° after 18 hr. According to Jorpes and Bergström [1936] 50 g. of chondroitin sulphuric acid (2.08% N) gave 61% yield of chondrosamine hydrochloride when the method of direct crystallization of the hydrochloride was employed.

DISCUSSION

The application of the method that has been described to the isolation of glucosamine and chondrosamine will probably throw some light on the failure to isolate more than a small part of the amino-sugar estimated to be present in many biological products by the colorimetric method. Observations of this nature have been reported by Neuberger [1938] who could isolate 60 % only of the glucosamine estimated to be present in the polysaccharide of crystalline egg-albumin. Morgan [1938] failed to isolate any amino-sugar from the specific polysaccharide of *Bact. dysenteriae* (Shiga) although the colorimetric method indicated that at least 20 % of the total polysaccharide was composed of amino-sugar. By means of the Schiff's base described in the present paper a part of the amino-sugar in the specific polysaccharide has now been isolated and identified as glucosamine; further details of this work will be given in another paper. More recently, Landsteiner & Harte [1940] obtained a yield of glucosamine that represented only 47 % of the amino-sugar contained in the polysaccharide component of the mucin of hog's gastric mucosa. When taken together, these and other similar observations might be considered to suggest that a substance other than glucosamine and chondrosamine exists which gives rise to a positive colour reaction when treated with Ehrlich's reagent under the conditions laid down for the quantitative test [Elson & Morgan, 1933], and indeed, the work of Miles & Pirie [1939] gives some support to this idea. It may be, however, that in some instances at least, the poor yields of amino-sugar isolated are due solely to the difficulty of liberating and isolating small quantities of glucosamine and chondrosamine by the methods employed. The nature of the component sugars and the rates of hydrolysis of the different polysaccharide complexes are in most instances largely unknown. For this reason it is clearly essential that the exact conditions which give complete hydrolysis must be determined for each polysaccharide molecule investigated and that after hydrolysis the amino-sugar must be completely free from combination with its neighbouring sugar molecules and any substituents in the amino-group, such as acetyl or formyl, must be completely eliminated. If either of these hydrolytic processes is incomplete all methods that depend on the sparing solubility of a Schiff's base for the isolation of an amino-sugar will give poor yields and such results cannot be used as evidence for the existence of an unknown amino-sugar. When complete hydrolysis has been achieved there seems no reason why 70-80 % yields of the glucosamine or chondrosamine should not be obtained in spite of the presence of sugars and amino-acids.

SUMMARY

A method is described for the isolation of small quantities (10-30 mg.) of glucosamine and chondrosamine. The method depends on the sparing solubility in water of the Schiff's bases, 2-hydroxynaphthylidene-glucosamine or -chondrosamine. The presence of sugars and amino-acids does not appreciably decrease the yield of Schiff's base.

REFERENCES

- Bergmann & Zervas (1931). *Ber. dtisch. chem. Ges.* **64**, 975.
—— — (1932). *Ber. dtisch. chem. Ges.* **65**, 1192.
Chargaff & Bovarnick (1937). *J. biol. Chem.* **118**, 421.
Duff & Bills (1934). *J. chem. Soc.* 1305.
Elson & Morgan (1933). *Biochem. J.* **27**, 1824.
Hewitt (1938). *Biochem. J.* **32**, 1554.
Jorpes & Bergström (1936). *Hoppe-Seyl. Z.* **244**, 253.
Irvine & Earl (1922). *J. chem. Soc.* **121**, 2376.
Landsteiner & Harte (1940). *J. exp. Med.* **17**, 551.
Miles & Pirie (1939). *Biochem. J.* **33**, 1716.
Morgan (1938). *Helv. chim. Acta*, **21**, 469.
Neuberger (1938). *Biochem. J.* **32**, 1435.
Nilsson (1936). *Biochem. Z.* **285**, 386.
Palmer, Smyth & Meyer (1937). *J. biol. Chem.* **119**, 491.
Sørensen (1938). *C.R. Lab. Carlsberg*, **22**, 487.