5. All the evidence obtained was consistent with the conclusion that the activity of the fractions was due to the presence in them of a fluorotricarboxylic acid as postulated in the hypothesis.

Our thanks are due to Prof. M. Stacey, F.R.S., for advice

upon the analysis of fluorine, to Dr C. S. Hanes and Dr F. A. Isherwood for their help in the paper chromatography. We are also grateful to Prof. T. Thunberg for drawing our attention to the results of Dr J. Mårtensson, to Prof. J. H. Burn, F.R.S., for a specimen of Synthalin B, and to Miss V. Bustin for technical assistance.

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The Separation and Identification of Small Amounts of Mixed Amino Sugars

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(Received 22 September 1950)

The amino sugars occur as components of mucopolysaccharides, mucoids and glycoproteins. In some materials the amount and nature of the amino sugar present has been satisfactorily determined after hydrolysis of the material with strong acid, the amino sugar being isolated as the crystalline hydrochloride. In many instances, however, the yield of amino sugar is small compared with the amount estimated to be present by the colorimetric procedure of Elson & Morgan (1933) or by one of the many modifications of this method now in use (Nilsson, 1936; Palmer, Smyth & Meyer, 1937; Sørensen, 1938; Hewitt, 1938; Blix, 1948; Immers & Vasseur, 1950). A low yield of crystalline hydro-

chloride makes a decision as to whether the original material contains more than one kind of amino sugar difficult and the only two quantitative colorimetric methods available (cf. Elson & Morgan, 1933; Dische & Borenfreund, 1950) fail to distinguish glucosamine from chondrosamine.

An examination by means of paper chromatography of the mucoids which carry the human blood-group characters indicates the presence of two amino sugars, glucosamine and chondrosamine, in each group substance (Aminoff & Morgan, 1948; Aminoff, Morgan & Watkins, 1950). In view of the fact that a single, apparently homogeneous, mucoid had not previously been shown to contain two amino sugars it was desired to isolate crystalline derivatives of the two hexosamines.

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There are available a number of methods for the isolation of 10–30 mg. amounts of glucosamine, but in most instances the corresponding derivatives of chondrosamine are too soluble in water and are quite unsuitable for the isolation of small quantities. The isolation of chondrosamine by the method of Jolles & Morgan (1940) from hydrolysis products of mucoid materials containing only 20 or 30 mg. of a mixture of both the amino sugars was not entirely satisfactory in the presence of amino-acids. A new method which would allow the quantitative isolation of each amino sugar from a mixture of both and in the presence of amino-acids was therefore developed.

The success which had attended the use of 1:2:4-fluorodinitrobenzene (FDNB) for the identification of the free amino groups in proteins and peptides (Sanger, 1945, 1949 *a*, *b*; Porter & Sanger, 1948) suggested that the amino groups in the naturally occurring glucosamine and chondrosamine could likewise be employed to yield coloured *N*-2:4-dinitrophenyl (DNP) derivatives, which could then be separated by one of the well known chromatographic procedures.

The structural difference between DNP glucosamine (I) and DNP-chondrosamine (II) resides in the configuration of C-4 in these amino sugars. This difference, however, does not in itself bring about a sufficient change in properties to allow of a quantitative separation of these derivatives by simple crystallization, but a separation can be achieved by utilizing the difference in the relative positions of the hydroxyl groups attached to C-3 and C-4. Glucosamine possesses a 3:4-*trans*-diol structure, whereas the corresponding groups in chondrosamine have a *cis*-configuration. Complex formation between boric acid and the *cis*-hydroxyl groups in carbohydrates is well known (Böeseken, 1949), and this property of complex formation is now used as a basis for the development of a method which accomplishes the separation of the DNP derivatives of glucosamine and chondrosamine when these amino sugars are present in small quantities in the products of the acid hydrolysis of mucoids. A preliminary account of the method has already been published (Annison, James & Morgan, 1950).

EXPERIMENTAL

All melting points given below are uncorrected unless otherwise stated. C, H and N analyses were made by Drs Weiler and Strauss, Oxford.

Preparation of the *N*-2:4-dinitrophenylamino sugars

N-2:4-Dinitrophenyl-D-glucosamine. D-Glucosamine hydrochloride (0.001 mol.) dissolved at 0° in 4 ml. 0.4M-potassium borate pH 9.8 was immediately treated with a freshly prepared solution of FDNB (0.005 mol.) in ethanol (150 ml.) also at 0°. The mixture was shaken vigorously, allowed to stand for 48–72 hr. at 0° and then taken to dryness under reduced pressure at this temperature. The DNP derivative was extracted with ether and the extract taken to dryness and crystallized from (a) 20% (v/v) aqueous acetone, (b) acetone-light petroleum (b.p. 60–80°) or (c) methanol-benzene. DNP-glucosamine was obtained as orange-yellow needles, m.p. 202–204° (corr.). $[\alpha]_{5461}^{20} + 65 \pm 3^\circ$ in 80% (v/v) ethanol (c, 1). (Found: C, 42.1; H, 4.3; N, 12.4. $C_{12}H_{15}O_5N_3$ requires C, 41.8; H, 4.4; N, 12.2%.)

N-2:4-Dinitrophenyl-D-chondrosamine. This was prepared in the same manner. Crystallized from acetone it yielded pale-yellow needles, m.p. 184–186° (corr.). $[\alpha]_{5461}^{20} + 84 \pm 3^\circ$ in 80% (v/v) ethanol (c, 1). (Found: C, 42.0; H, 4.4; N, 12.4. $C_{12}H_{15}O_5N_3$ requires C, 41.8; H, 4.4; N, 12.2%.)

Separation of the *N*-2:4-dinitrophenylamino sugars

It seemed probable that a complex formed between boric acid and the *cis*-hydroxyl groups of DNP-chondrosamine would possess a partition coefficient favouring its solubility, compared with that of the corresponding derivative of glucosamine, in the aqueous phase of a partition chromatogram. Attempts to use aqueous boric acid as the stationary phase on columns of silica gel and kieselguhr with different organic solvent systems, however, gave no separation of the DNP derivatives. Attention was then turned to partition chromatograms having alkaline 0.1M-borate buffer as the stationary phase, but again no useful separation of the two DNP derivatives was observed when the pH of the borate solution was lower than 9.0. When, however, the pH of the buffer was raised to about 9.7 and the solvent systems 20% (v/v) *n*-propanol-cyclohexane or 20% (v/v) amyl alcohol-chloroform were used, a separation was accomplished.

Replacement of the sodium borate-sodium hydroxide buffer by a glycine-sodium hydroxide or by a carbonate-bicarbonate buffer of the same pH and strength did not give rise to a separation of the DNP-amino sugars on the column; only a single broad band was observed which moved at approximately the same rate as DNP-glucosamine. The behaviour of the DNP-amino sugars in the presence of borate suggested strongly the formation of a complex between the DNP-chondrosamine and the borate ion. An increase in the concentration of the borate ion without change in pH caused a greater separation of the DNP-amino sugar bands. To obtain a useful

increase in the concentration of borate ion above 0.1M at 1–3°, however, it was necessary to change from sodium to potassium borate, and columns holding 0.2M-potassium borate buffer as the stationary aqueous phase gave a very useful separation of the DNP-amino sugars. In most of the experiments described below 0.2M-potassium borate-potassium hydroxide buffers at pH values between 9.7 and 9.9 were used.

It was observed that when kieselguhr columns were used at room temperature (18–22°) the two yellow bands arising from the DNP-amino sugars faded somewhat during their passage through the column, presumably owing to the occurrence of a significant amount of alkaline decomposition of the DNP derivatives. Accordingly, the columns are best used between 1 and 3° in a thermostatically controlled cold room. Under these conditions no significant decomposition of the DNP-amino sugars occurs, excellent separation is achieved and very satisfactory yields of the derivatives are obtained from the column.

Preparation and use of the column. Hyflo-Super Cel (40 g.) is treated with 20 ml. of the aqueous phase from the system 0.2M-potassium borate-KOH (100 ml.)—30% (v/v) amyl alcohol- CHCl_3 (1000 ml.) equilibrated at 2°. The kieselguhr is stirred vigorously to form a free-flowing powder and then just sufficient of the organic layer of the solvent system is added to form a thin slurry. A small part of this is poured into a tube (2.2 × 60 cm.), the base of which is drawn in to support a stainless steel plate which is perforated with holes 2 mm. in diameter and carries a disk of filter paper. The remaining part of the slurry is then introduced into the tube in small portions at a time and tightly packed by means of a perforated stainless steel disk of the type described by Howard & Martin (1950). Packed by this procedure to a height of about 40 cm. a column is obtained which gives perfectly straight fronts with no channelling and can be used for 20–30 mg. amounts of mixed DNP-hexosamines. The DNP preparation to be examined is mixed with a small amount of the organic solvent phase, poured on to the top of the column and allowed to become absorbed in the first few mm. of the filling. The chromatogram is then developed under gravity with the selected solvent system in the usual way. Any DNP-amino-acids present in the mixture are retained at the top of the column. The fastest moving band consists of coloured impurities which arise during the formation of the DNP derivatives. The second yellow band is DNP-glucosamine and the third, and slowest moving component, DNP-chondrosamine. In practice it has been found convenient to collect the two DNP-amino sugars together and, after removing the solvent at about 2°, to redissolve them in a fresh quantity of the organic phase and run them a second time on a new column. In this way it is possible to obtain a wide separation of the yellow DNP-hexosamine bands together with the complete elimination of coloured impurities and DNP-amino-acids, if they are present in the original material. The concentration of the alcohol in the organic phase can be increased to 35–40% (v/v) to speed up the movement of the DNP derivatives on the column.

Determination of the N-2:4-dinitrophenylhexosamines. The concentration of the DNP-amino sugar in each fraction issuing from the column was at first determined by measuring the light absorption of a unit depth of the eluate in a photoelectric colorimeter using a blue filter. A calibration curve

Fig. 1. The ultraviolet absorption spectra of DNP-glucosamine and DNP-chondrosamine (0.0025% solutions in ethanol). —○—○—, DNP-glucosamine; —●—●—, DNP-chondrosamine.

Fig. 2. The optical density-DNP-amino sugar concentration relationship over the range 3–12 μg./ml. —○—○—, DNP-glucosamine; —△—△—, DNP-chondrosamine.

of concentration against colorimeter reading was constructed for each of the DNP derivatives and used to determine the approximate distribution of the DNP-hexosamines in the eluate. In later experiments where the separation of the amino-sugar derivatives appeared to be quite complete, as judged from the wide separation of the yellow bands on the column, the whole of the material in the band was collected as one fraction, taken to dryness, redissolved in ethanol and estimated in the Uvispek spectrophotometer. Absorption curves of the two hexosamine derivatives in solution in ethanol (Fig. 1) showed that a maximum absorption occurred at 3550 Å. for both compounds. The relationship between

concentration within the range 3–12 $\mu\text{g./ml.}$ and extinction at this wavelength for the authentic DNP-hexosamine derivatives in the same solvent was obtained (Fig. 2), and in this way the concentrations of these substances in the eluates from the columns were determined.

Removal of the solvent from the fractions. In order to prevent decomposition of the DNP-amino sugars at the alkaline reaction employed, it is necessary during removal of the solvents to keep the temperature as near 0° as possible. This is accomplished by allowing the solvents to be drawn off rapidly and completely at pressures below 10 mm., a procedure which is greatly facilitated by employing a rotating magnetic stirrer to keep the sides of the flask covered with a thin film of solvent and so increase the effective surface area of the solvent during evaporation. The solutions should be protected from direct sunlight during manipulation.

Fig. 3. Separation of artificial mixture of DNP-amino sugars. Concentration of DNP-amino sugars in eluate fractions from 30% (v/v) amyl alcohol- CHCl_3 -0.2M-potassium borate (pH 9.9) kieselguhr column. Area enclosed by curve A, DNP-glucosamine. Area enclosed by curve B, DNP-chondrosamine.

The chondrosamine-borate complex. It was observed that after removal of the solvent mixture from the DNP-chondrosamine-containing fractions emerging from the column, and recrystallization of the material from an acetone-light petroleum mixture, the yellow needles obtained melted with decomposition at temperatures between 210 and 235° for different preparations, whereas DNP-chondrosamine not run on the column melted at 182° . Recrystallization of the material with the high melting point from an aqueous solvent system, such as 25% methanol, gave crystals which melted at 181° and showed no depression of melting point when mixed with authentic DNP-chondrosamine. It seems probable that the high melting material is a DNP-chondrosamine-borate complex which is readily dissociated on crystallization from aqueous solvents, but no direct experiments to confirm this suggestion have been made.

Standardization of separation technique. The main procedure for the separation of DNP-amino sugars was standardized by using mixtures of known weights of the substances. The separation achieved, when a mixture of about 15 mg. of each of the DNP-amino sugars was run at $1-3^\circ$ in 30% (v/v) amyl alcohol- CHCl_3 on a standard column buffered with borate at pH 9.9, was determined colorimetrically from the amount of material in successive fractions (2.4 ml.) emerging from the column, collected by means of a mechanical fraction cutter (James, Martin & Randall, 1950). The results are given in Fig. 3. The substance, obtained by pooling the material present in all fractions taken between 130 and 180 ml. of eluate and which comprised the fastest running band, was identified after recrystallization as DNP-glucosamine (m.p. 199°). The second band, emerging as fractions collected over the range 220–350 ml. of eluate, gave rise to DNP-chondrosamine (m.p. $180-182^\circ$) after recrystallization from 25% (v/v) aqueous ethanol. In a series of similar experiments the recovery of each DNP-amino sugar was 90–95% of the amount put on the column.

Yield of N-2:4-dinitrophenylamino sugar from amino-sugar hydrochloride. The yield of DNP-amino sugar from a known amount of the amino-sugar hydrochloride was determined using the method of conversion to the DNP-derivative and its recovery from the 'standard' column already described. Mixtures of amino-sugar hydrochlorides were treated in the same way and the recovery of each DNP-amino sugar from the column determined colorimetrically. The results are summarized in Table 1 and indicate that yields of the order of 80% DNP-amino sugars, in terms of the original amino-sugar hydrochloride used, may be expected from mixtures of 20 mg. amounts of the amino-sugar hydrochlorides.

Table 1. Yield of N-2:4-dinitrophenylamino-sugar from amino sugar hydrochloride

Amino-sugar hydrochloride		Wt. of amino-sugar hydrochloride used (mg.)	Yield of DNP derivative (% of theoretical)
Glucosamine	a	24.6	75
	b	25.4	79
	c	25.6	81
Chondrosamine	a	20.9	74
	b	20.1	75
Mixed glucosamine and chondrosamine	a	23.5	81
	b	19.2	82
Mixed glucosamine and chondrosamine	a	25.8	77
	b	24.6	81

In the first attempts to procure additional evidence in support of the conclusions reached as to the nature of the amino sugars present in the biological materials examined, the individual DNP derivatives isolated from the material were added to a mixture of known weights of authentic DNP-glucosamine and DNP-chondrosamine and subjected to chromatographic analysis on the standard column. The results obtained when 10 mg. of material obtained from hog gastric mucoid (Morgan & King, 1943) and believed to be DNP-chondrosamine was added to DNP-glucosamine (12 mg.) and DNP-chondrosamine (12 mg.) and the mixture

run through the column are given in Fig. 4 from which it will be seen that the concentration of the eluate fractions corresponding to DNP-chondrosamine alone show an increased height. A similar result was obtained when 9 mg. of material believed to be DNP-glucosamine, which had likewise been

Fig. 4. Concentration of DNP-amino sugars in eluate fractions from 20% (v/v) amyl alcohol- CHCl_3 -0.1M-sodium borate (pH 9.9) kieselguhr column. $\text{--}\bigcirc\text{--}\bigcirc\text{--}\bigcirc\text{--}$, DNP-glucosamine (12 mg.) and DNP-chondrosamine (12 mg.); $\text{--}\triangle\text{--}\triangle\text{--}\triangle\text{--}$, same as $\text{--}\bigcirc\text{--}\bigcirc\text{--}\bigcirc\text{--}$, but with 10 mg. DNP-chondrosamine, isolated from hog gastric mucin, added.

isolated from hog gastric mucoid, was added to the same mixture of DNP-amino sugars. In this instance, however, only the amount of DNP-glucosamine recovered was increased, the amount of DNP-chondrosamine remaining constant at 12 mg.

The isolation of N-2:4-dinitrophenylglucosamine and N-2:4-dinitrophenylchondrosamine from A substance

A preparation (100 mg.) of blood-group A substance isolated from ovarian cyst fluid according to the method described by Aminoff *et al.* (1950) was hydrolysed for 16 hr. at 100° with 0.5N-HCl in an atmosphere of N_2 . The hydrolysis products were then taken to dryness *in vacuo* over solid KOH and H_2SO_4 and dissolved at 0° in 5 ml. of 0.4M-potassium borate buffer (pH 9.7). If necessary, the pH was adjusted to this value by the addition of NaOH. A solution of FDNB (500 mg.) in 100 ml. of cold ethanol was added to the borate solution, the mixture shaken vigorously, allowed to stand at 0° for 2-3 days and finally taken to dryness *in vacuo* at $0-3^\circ$. A portion (1 ml.) of the aqueous phase of the solvent system and kieselguhr (2 g.) were then added to the dried DNP derivatives and the whole, after thoroughly mixing until an even-coloured yellow powder resulted, was transferred to the top of a 40 g. kieselguhr column prepared as already described. Any yellow material remaining in the reaction flask was dissolved in a few drops of the 30% (v/v) amyl alcohol- CHCl_3 solution and added to the column.

The DNP-amino-acids were retained at the top of the column or at most moved very slowly, as at pH values above 9 they exist entirely in the ionized form. The first and most rapidly moving band consisting of coloured by-products of the reaction was rejected. The second and third bands

which comprised the DNP-glucosamine and DNP-chondrosamine derivatives, respectively, were collected together and taken to dryness *in vacuo* at $1-3^\circ$. The yellow residue was dissolved in a few ml. of the organic phase, transferred to the top of a fresh column and the chromatogram developed in the usual way with the amyl alcohol- CHCl_3 solvent system at $1-3^\circ$. Under the conditions described the DNP-amino sugar derivatives were well separated on the column and the eluate could be easily handled to yield the individual DNP-amino sugars.

The DNP-glucosamine, after eliminating the amyl alcohol- CHCl_3 solvent mixture and recrystallizing from 15% (v/v) aqueous acetone, melted at $199-201^\circ$. Mixed m.p. with authentic DNP-glucosamine, $199-202^\circ$. $[\alpha]_{5461}^{20} + 61 \pm 3^\circ$ in 80% (v/v) ethanol (c, 1). (Found: C, 42.5; H, 4.8; N, 11.8. Calc. for $\text{C}_{12}\text{H}_{15}\text{O}_9\text{N}_3$: C, 41.8; H, 4.4; N, 12.2%.) The DNP-chondrosamine-containing eluate was similarly treated and the material obtained recrystallized from 80% (v/v) aqueous acetone and finally from acetone at -10° . Fine pale-yellow needles were obtained which melted at 181° . Mixed m.p. with authentic DNP-chondrosamine 180° . $[\alpha]_{5461}^{20} + 86 \pm 4^\circ$ in 80% (v/v) ethanol (c, 1). (Found: C, 42.4; H, 4.5; N, 11.9. Calc. for $\text{C}_{12}\text{H}_{15}\text{O}_9\text{N}_3$: C, 41.8; H, 4.4; N, 12.2%.) The 100 mg. sample of A substance used gave 22.9 mg. DNP-glucosamine and 25.6 mg. of DNP-chondrosamine. Glucosamine : chondrosamine ratio, 1.00 : 1.13. A duplicate experiment gave the ratio, 1.00 : 1.12.

The X-ray powder photograph of the DNP-chondrosamine isolated was found to be not significantly different from that given by an authentic specimen of DNP-chondrosamine and differed markedly from the corresponding derivative of glucosamine.

Glucosamine and chondrosamine have also been identified as components of the mucoids which carry the human blood group characters known as 'Lewis' (Le^a) and 'H' (so-called O property). Full details of this work will be given in subsequent publications.

DISCUSSION

The interaction between borate ions and organic compounds containing two *cis*-hydroxyl groups is well known (see Böeseken, 1949), and this property has been used as the basis for the development of a method for the separation and identification of small quantities (10-20 mg.) of the naturally occurring amino sugars. The elaboration of a suitable method involved (1) the conversion of the amino sugars into coloured derivatives, the behaviour of which could be readily observed on a kieselguhr column, and (2) the subsequent development of conditions that would give rise to the formation of a complex between the 3:4-*cis*-hydroxyl groups of chondrosamine and the borate ion. The DNP derivatives of the amino sugars are easily formed, possess a distinctive yellow colour, are very suitable for following the movement of the amino sugars during their passage through the column and are easy to identify.

The formation of a complex between the chondrosamine and the borate ion occurs at pH values of about 10 and is most probably due to the reaction of the 3:4-*cis*-diol of the amino sugar with the meta-

borate ion. It seems probable that the extent of complex formation depends on the amount of metaborate present, which in turn is determined by the alkalinity of the borate solution. Solutions more alkaline than pH 10 bring about decomposition of the DNP-amino sugars and at the same time decrease the concentration of the metaborate ion with the formation of BO_3^{--} and $\text{B}_4\text{O}_7^{--}$.

The experimental evidence seems to indicate that the separation of the two DNP-amino sugars depends on the formation of an easily dissociable complex of DNP-chondrosamine and the borate ion, which leads to an increase in the solubility of the chondrosamine in the aqueous phase of the partition chromatogram. This is indicated by the broadness of the DNP-chondrosamine band which is found to be considerably greater than expected from its retention volume. Furthermore, an increase in the concentration of the borate ion, without changing the pH of the system, brings about a greater separation of the two DNP-amino sugars, and no separation of the two DNP-amino sugars occurs when sodium hydroxide-glycine or carbonate-bicarbonate buffer of the same pH is used in place of the potassium hydroxide-borate buffer. Finally, the DNP-chondrosamine isolated directly from the eluate has a higher melting point than DNP-chondrosamine that has not been passed through the column. The high-melting material, which is most probably the DNP-chondrosamine borate complex, is converted to that of the authentic DNP-chondrosamine after simple recrystallization from aqueous organic solvents.

If the formation and separation of the DNP-derivatives of the amino sugars is carried out under the conditions described the yields of these substances are about 80% of the amino sugars em-

ployed. The application of the technique to the separation and identification of the amino sugars in biological materials will be given later, but a single example is described, the isolation of DNP-glucosamine and DNP-chondrosamine from 100 mg. of human blood-group A substance. This indicates that the method is suitable for the recovery and characterization of amino sugars present in complex macromolecules in which simple hexoses and methylpentoses as well as a high proportion of amino-acids are also present.

SUMMARY

1. A method is described for the isolation and characterization of small quantities (10–20 mg.) of glucosamine and chondrosamine when these sugars are found together and in the presence of other sugars and amino-acids, as in the human blood-group mucoids.

2. The method depends on the conversion of the amino sugars into their *N*-2:4-dinitrophenyl derivatives and the chromatographic separation of these substances using alkaline borate as the aqueous and 30% (v/v) amyl alcohol-chloroform as the organic phase.

3. The separation is probably due to the formation of a complex between the *cis*-diol of the dinitrophenyl derivative of chondrosamine and the metaborate ion.

Our thanks are due to Prof. A. R. Todd, F.R.S., who arranged for the X-ray powder photographs to be taken, to Dr A. J. P. Martin, F.R.S., for much helpful advice, and to Prof. E. Jorpes, Karolinska Institute, Stockholm, for a generous supply of chondrosamine hydrochloride. One of us (E. F. A.) is indebted to the Medical Research Council for a Studentship.

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