A Reaction for the Simple Sensitive Fluorimetric Assay of Heparin and 2-Amino Sugars

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1. 3,5-Diaminobenzoic acid reacted rapidly with the product from HNO_2 deamination of heparin, heparan sulphate and 2-amino-2-deoxyhexoses under very mild conditions (pH 3.0 and 37°C) to give stable fluorescent derivatives. 2. The fluorescence yield was rectilinearly related to the concentration of heparin etc. Less than $0.1\text{ }\mu\text{g}$ of 2-amino-2-deoxyhexose was easily measurable in standard cuvettes. 3. The deamination products of glucosamine and (particularly) galactosamine were labile in the HNO_2 reagent, with half-lives of 20–40 min at room temperature. At 0°C they were much more stable. The analogous product from heparin was not so labile. 4. Under the standard conditions, and at room temperature, relative fluorescence yields (D-glucosamine = 1.0) were: D-galactosamine, 0.75; D-gulosamine, 0.38; D-mannosamine, approx. 0.20. 5. Neutral sugars, chondroitin sulphates, DNA and *N*-acetylneuraminic acids did not react, nor did *N*-acetyl amino sugars or non-deaminated hexosamines. 6. It is suggested that the Dische–Borenfreund [Dische & Borenfreund (1950) *J. Biol. Chem.* 184, 517–522] indole method, the Kissane–Robins [Kissane & Robins (1962) *J. Biol. Chem.* 233, 184–188] DNA assay and the proposed amino sugar method are all examples of simple aldehyde reactions. The specificity of the proposed method is considerably greater than that of the Dische–Borenfreund procedure, partly because of the much milder reaction conditions. 7. The proposed method is very reproducible, about 50–100 times as sensitive as the Elson–Morgan reaction, and 10–50 times as sensitive as the Dische–Borenfreund procedures. It is also convenient; acid hydrolysates of amino sugar-containing compounds can be directly neutralized with sodium acetate solution.

The Elson–Morgan reaction is still, after almost 50 years, the most widely used procedure for the assay of 2-amino sugars. It is adequately specific, but not very sensitive. Amino-group reagents such as ninhydrin and naphthyl isothiocyanate (Scott, 1970) offer higher sensitivity, but additional manipulations are required to remove reactive non-hexosamines. The Dische–Borenfreund (1950) procedure, involving deamination of the amino sugar with HNO_2 , and spectrophotometric measurement of the resulting 2,5-anhydro sugar after reaction with indole, is specific, and in the version of Exley (1957) is claimed to be five times as sensitive as the Elson–Morgan assay. Perhaps because the Exley method emphasizes the use of special micro-equipment, and few investigations have otherwise been performed on the Dische–Borenfreund method, these procedures have not found favour. When Lagunoff & Warren (1962) discovered that the polymers heparin and heparan sulphate also react, a direct chemical method for the estimation of heparin became available, but sensitive to only about $15\text{ }\mu\text{g/ml}$ of sample. Because of interest in the role of heparan sulphate in cell membranes,

and in the biological activity of heparin, such a method would have wide applications, if the sensitivity were higher. A reagent for the determination of the deamination product, with a sensitivity ten to 100 times that of indole, is needed. Fluorimetric assays would meet this requirement.

The indole-reaction stage of the Dische–Borenfreund procedure resembles the indole method for DNA (Dische, 1955b). It is therefore relevant that a fluorescence method for DNA is performed under similar conditions, by using 3,5-diaminobenzoic acid (Kissane & Robins, 1962), on the basis of a reaction for aldehydes (Velluz *et al.*, 1948) in which the α -carbon atom is unsubstituted, as in deoxyribose. The product of the reaction on heating in strong mineral acid is a quinaldine, by analogy with the Doebner–Miller synthesis. The deamination product from 2-amino sugars should not react, since the α -carbon atom is substituted as part of a furan ring. However, under very mild conditions, the deamination product yielded a derivative with fluorescence properties similar to those from acetaldehyde or DNA. This observation has been developed into a procedure for

the sensitive determination of 2-amino sugars, heparin etc. The relevance to the DNA methods, and to the accepted mechanisms of the reaction of aldehydes with the reagent, is discussed.

Experimental

Materials

NaNO₂, ammonium sulphamate and acetic acid were AnalaR grade. 3,5-Diaminobenzoic acid dihydrochloride was Gold Label grade from Aldrich Chemical Co., Gillingham, Dorset, U.K. *m*-Phenylenediamine dihydrochloride was from Hopkin and Williams, Chadwell Heath, Essex, U.K. α -D-Glucosamine hydrochloride and α -D-galactosamine hydrochloride were from Koch-Light Laboratories, Slough, Berks., U.K. They contained only one component in the naphthyl isothiocyanate system (Scott, 1970). They were analysed by Mr. M. Hart of the Microanalytical Laboratory, Department of Chemistry, University of Manchester, and respectively contained: C, 33.3 and 33.7; H, 6.7 and 6.7; N, 6.3 and 6.4; C₆H₁₄NO₅Cl requires C, 33.4; H, 6.49; N, 6.49%. Gulosamine hydrochloride was a gift from Dr. C. J. Morel of Ciba-Geigy, Basle, Switzerland, and *N*-acetylmannosamine was from Koch-Light. Heparin samples were from Evans Biological, Runcorn, Cheshire, U.K., or were made by the potassium acetate extraction and fractionation technique (Scott, 1971) from beef lung or small intestine. Dermatan sulphate was prepared from pig skin by the method of Scott (1960). Chondroitin 4-sulphate was from Serva, Heidelberg, Germany. Desulphated *N*-acetylated heparin and heparan sulphate were gifts from Dr. L. Roden, University of Alabama, Birmingham, AL, U.S.A. Other chemicals were of the best available commercial grade.

Methods

Hexosamines were determined by the Elson-Morgan reaction as modified by Kraan & Muir (1957). The qualitative identification of hexosamines was by the naphthyl isothiocyanate method of Scott (1970).

Standard conditions

Deamination. A hexosamine-containing aq. solution (1 vol.) was mixed with 1 vol. of 10% (v/v) acetic acid. Aq. 5% (w/v) NaNO₂ (1 vol.) was added and mixed. The two reagents can be mixed in bulk and added as one, provided they are used freshly. The solution (pH approx. 3.5) was left unstoppered for 10 min (method A, for free hexosamines) or 80 min (method B, for heparin or heparan sulphate). Ammonium sulphamate (1 vol.; 12.5%, w/v) was added, and after active effervescence had ceased, the

tubes were closed with silicone-greased glass stoppers and mixed with inversion. It is important that mixing be complete, to avoid colour production from unreacted HNO₂ and the reagent.

Fluorescence development. After 30 min, 2 vol. of 0.8% (w/v) 3,5-diaminobenzoic acid dihydrochloride was added, and the mixture was left at 37°C for 1 h. Conc. HCl (0.4 vol.) was then added. Fluorescence was estimated at an emission wavelength of 514 nm, the exciting wavelength being 422 nm. The excitation and emission spectra of the fluorescent product are both quite sharp, and approximately symmetrical, with half-peak band-widths of about 50 nm.

Controls. The NaNO₂, acetic acid and sulphamate solutions were mixed together and left at room temperature for 30 min. The 'reagent blank' consisted of this solution (3 vol.) plus 1 vol. of the undeaminated test solution (or water), followed by 0.4 vol. of conc. HCl, depending on whether the fluorescence from the reagents plus the undeaminated sample, or the reagents themselves, was to be measured.

Results

The fluorescent product

Both acetaldehyde and the deamination product from glucosamine gave yellow-green colours with the reagent in a few minutes at room temperature. The spectra showed peak absorption in the region 395–415 nm. The molecular absorbance of the yellow colour was about 1200, assuming that all the aldehyde reacted, and that one aldehyde molecule reacted per molecule of coloured product. The excitation spectrum of the fluorescent product and the absorption spectrum of the yellow-green colour were very similar.

In 0.6M-HCl, the long-wavelength absorption of the reagent at about 315 nm almost disappeared, whereas the yellow-green absorption was almost unchanged. The 'reagent blank' fluorescence was lower in hydrochloric acid, as would be expected from the shift to shorter wavelengths of the absorption spectrum of the reagent. All estimations were therefore performed in 0.6M-HCl.

The development of fluorescence from the deamination product practically ceased on the addition of conc. HCl. The lower the pH of the solution in which fluorescence developed, the lower the fluorescence finally observed. At pH 0.5, the fluorescence was only 5–10% of that observed at pH 2.8, which was the pH of the deamination solution plus the reagent. At pH 3.6 (acetate buffer) the yield of fluorescence declined slightly, and at pH 4.5 was appreciably lower.

At 40°C the final value of fluorescence was achieved 45–60 min after mixing reagent and deamination

product. At room temperature (22°C) fluorescence formation was slower, but the final values were slightly higher, after about 100–120 min. The fluorescent product was stable for at least 24 h at room temperature, and for days at –15°C, in 0.6M HCl.

The deamination products

The deamination products of D-glucosamine and D-galactosamine were labile in the solution in which they were formed. At 22°C the maximum yields of fluorescence were obtained at about 10 min, declining to less than one-third after 80 min (Fig. 1). At 0°C the deamination products were stable for at least 60 min, with a yield at 30 min similar to that at 22°C after 10 min (Figs. 1 and 2). The deamination product from heparin behaves quite differently. It was stable for at least 4 h at 22°C. The formation of the product was much slower than from 2-amino sugars, with a plateau at room temperature (22°C) after 70–100 min, or at 37°C after 35–45 min from the start of deamination (Fig. 3), the final value being the same in each case.

Fig. 1. Fluorescence yields from 5 μ g of glucosamine hydrochloride (▲ and △) or galactosamine hydrochloride (○) as a function of time in the HNO_2 reagent during the deamination stage

The glucosamine plot is for deamination at 0°C (▲) and at 22°C (△). Other manipulations were under standard conditions (see the text). The total volume of fluorescent solution was 1.6 ml. The units are the readings from the Aminco-Bowman spectrofluorimeter on the 0.1 scale.

Fig. 2. Fluorescence yields from 5 μ g of galactosamine hydrochloride with deamination for various times at 22°C (●) or 0°C (○)

Conditions were as described in the legend to Fig. 1.

Fig. 3. Fluorescence yields from 20 μ g of bovine mucosal heparin as a function of time in the deamination stage at 22°C (▲) or 37°C (○)

Conditions were as described in the legend to Fig. 1.

Sensitivity

There was a straight-line relationship between the fluorescence yield and the concentrations of 2-amino-2-deoxyhexose, heparin or heparan sulphate under the standard conditions. A 100% increase over the 'reagent blank' was given by 0.5 μ g of D-

glucosamine, or by about 7.5 μg of heparin per ml of fluorescent solution, an Aminco-Bowman fluorimeter being used on the 0.03 scale with a combination of 4mm and 5mm slits ('arrangement no. 5') for maximum sensitivity. Less than 0.1 μg of hexosamine per ml of final solution was easily measurable under the standard conditions. Heparin at 1 $\mu\text{g}/\text{ml}$ was easily measured by using standard cuvettes. By using commercially available microcuvettes, at least one-tenth these quantities were easily measurable (i.e. about 0.01 μg of total hexosamine).

Duplicates differed by less than 2%.

Fluorescence yields from individual 2-aminohexoses

At room temperature and at 0°C the fluorescence yield from D-galactosamine was about 25% lower than that of D-glucosamine, on a molar basis.

At 22°C, D-glucosamine yielded 38% of the fluorescence of D-glucosamine. D-Mannosamine, prepared by incompletely hydrolysing 2-acetamido-2-deoxy-D-mannose in 1.0M-HCl at 100°C for 70min gave 30% of the fluorescence produced by 2-acetamido-2-deoxy-D-glucose hydrolysed under exactly similar conditions. If all the D-mannosamine had been deacetylated, the fluorescence produced was about 20% of that of D-glucosamine. This is likely to be a better approximation, since 2-acetamido-2-deoxy-D-mannose is deacetylated about three times as rapidly as 2-acetamido-2-deoxy-D-glucose (Shively & Conrad, 1970). The 3,5-diaminobenzoic acid method indicated that 60% of the D-glucosamine had been liberated, compared with a prediction of 50%, on the basis of the data of Moggridge & Neuberger (1938). The excitation and emission spectra were identical for all four amino sugar derivatives.

Interfering substances

Under the standard conditions, the fluorescence yield from D-glucosamine at 25 $\mu\text{g}/\text{ml}$ without deamination was nil. The fluorescence yield from D-glucose, 2-acetamido-2-deoxy-D-glucose, D-ribose, D-glucuronolactone, D-mannose, N-acetylneuraminic acid, D-fructose and L-ascorbic acid (all without 'deamination') after 1h with the reagent was less than 2% (on a weight basis) of that from D-glucosamine under the same conditions, but with deamination. The fluorescence yield from DNA with or without deamination was less than 2% of that of deaminated D-glucosamine. Dermatan sulphate, chondroitin 4-sulphate and desulphated N-acetylated heparin gave no measurable fluorescence at 100–250 $\mu\text{g}/\text{ml}$, with or without 'deamination' for 80min at 22°C.

NaCl at 0.75M caused a small (10%) decrease in the fluorescence yield from D-glucosamine, under the standard conditions.

Acetic acid concentrations in the deamination solutions were varied from 3.3% (v/v) to 33% (v/v).

A small (10%) decrease in fluorescence at 8.3% (v/v) compared with 3.3% (v/v) acetic acid was observed, and a further 10% decrease from 8.3% (v/v) to 33% (v/v) acetic acid. The 'reagent blank' increased by about 220% from 3.3% (v/v) to 33% (v/v) acetic acid.

Removal of nitrous acid with ammonium sulphamate was complete in 15min at room temperature, as shown by the absence of crimson diazo product with 3,5-diaminobenzoic acid. The pH of the solution was about 3.0. The deamination product can be left in it at room temperature (22°C) for at least 45min without deterioration.

The suitability of 3,5-diaminobenzoic acid compared with m-phenylenediamine

Velluz *et al.* (1948) reported that *m*-diamines other than 3,5-diaminobenzoic acid react with aldehydes to give fluorescent products. *m*-Phenylenediamine was investigated, but was not preferred to 3,5-diaminobenzoic acid. We have found that the reaction proceeds less rapidly both at pH 1.5 and 3.6, and with less sensitivity. However, because the u.v. absorption of *m*-phenylenediamine was at shorter wavelengths, whereas the product absorbed similarly to that from 3,5-diaminobenzoic acid, the fluorescence of the reagent was considerably less (about 80% less, at the optimum settings). This compensated in part for the lower fluorescence produced.

Comparisons with Dische-Borenfreund method

By using method B, a commercial sample of heparin and heparan sulphate gave identical values, in terms of glucosamine equivalent, with those obtained by the Dische-Borenfreund indole method.

Acetaldehyde and glutaraldehyde at less than 50 $\mu\text{g}/\text{ml}$ both reacted with indole in the conditions of the Dische-Borenfreund method to give turbidities of an off-white colour that changed on heating to slightly soluble yellow materials. The spectrum of the acetaldehyde product, extracted into isopentyl alcohol (1 vol.) or dissolved by the addition of 1 vol. of ethanol, was identical with that of the Dische-Borenfreund product of D-glucosamine in similar conditions. Peak absorbances were at 492nm in ethanol/water or 496nm in isopentyl alcohol with a shoulder at 460–470nm of about 50% of the peak amplitude (cf. Lagunoff & Warren, 1962).

Discussion

At the time of the introduction of 3,5-diaminobenzoic acid as a reagent for α -unsubstituted aldehydes, it was suggested (Velluz *et al.*, 1948) that the reaction was a Doebner-Miller synthesis involving two molecules of aldehyde with one of reagent. It has been noted (Velluz *et al.*, 1948), and re-emphasized (Kissane & Robins, 1962), that the reaction proceeds in mild conditions compared with the Doebner-

Miller synthesis. Even so, the conditions of Velluz *et al.* (1948) and Kissane & Robins (1962) are considerably more violent than those used in the present paper. The ease with which the fluorescent product is formed tends to rule out Doebner–Miller syntheses. The questions of how the product is formed, and of what nature it is composed, remain to be answered. The first stage must involve reaction of the aldehyde with one of the amino groups of the reagent. Such reactions are usually rapid and have an optimum pH in the less acid range.

Unfortunately, the acceptance of the reaction as a Doebner–Miller synthesis has deflected attention from its potentially wide use for many types of aldehyde, and has also discouraged investigations of milder conditions, in which, as shown in the present paper, the reaction proceeds with greater speed and sensitivity. Provided the aldehyde can be formed, there is nothing to be gained in using strongly acid conditions or higher temperatures. Apart from the enhanced sensitivity and convenience, the mild conditions of the proposed assay result in increased specificity. None of the common reducing sugars react, nor do chondroitin sulphates or desulphated *N*-acetylated heparin.

The fluorescence assays of DNA (Kissane & Robins, 1962) and sialic acids (Hess & Rolde, 1964) include strong acid and heating at temperatures of 60°C or 100°C for considerable periods. In the less drastic conditions of the proposed assay, neither DNA nor sialic acid react to any appreciable extent. Lagunoff & Warren (1962) found that DNA gave one-third the colour equivalent of 2-amino sugar in the Dische–Borenfreund method, and this must be due to the conditions of the indole reaction, which lead to breakdown of DNA.

The reaction of 3,5-diaminobenzoic acid with the deamination products from amino sugars suggests that they have considerable aldehyde character. Presumably other sugars with this property would also react. Compounds that produce aldehydes under the weakly acidic conditions of the deamination reaction or fluorescence-development stages (e.g. plasmalogens) could give fluorescence. To make allowances for such materials, it appears adequate to use a control solution containing the undeaminated sample mixed with a 'deamination' solution containing nitrite, acetic acid and sulphamate, as described in the Experimental section.

Notwithstanding the accepted assumption that the Dische–Borenfreund method involves reaction between indole and a furfuraldehyde (e.g. Dische, 1955*a*; Exley, 1957) it is at least as likely that the reaction takes place directly with the aldehyde–deamination product. The very close similarity between the reactions of acetaldehyde or the deamination product from glucosamine with indole suggests that no further stage is needed beyond the initial formation of

the 2,5-anhydro sugar. Similarly, the indole–DNA product is likely to be formed directly from aldehydes produced by degradation of DNA. The warm acid conditions of the DNA method are similar to those of the Feulgen reaction, from which Schiff-positive products are obtained. Seen in this light, the Dische–Borenfreund method and other similar indole reactions cannot be regarded as 'specific' for sugar-derived materials, but are general for aldehydes. The specificity of the Dische–Borenfreund method probably resides in the deamination-reaction-to-aldehydes, as does the assay proposed in the present paper. So far, only 2-aminocyclohexanols (McCasland, 1951), 2-aminohexoses and 2-aminopentoses are known to undergo this conversion.

Mechanisms of the reactions

The fluorescence yield is determined by the extent of conversion into an aldehyde. 2-Amino-2-deoxyhexoses with equatorial amino groups readily form 2,5-anhydro sugar aldehydes via attack by the ring oxygen atom on the carbonium ion at C-2 left by the breakdown of the diazonium group (Fig. 4). Glucosamine, galactosamine and gulosamine are in this class. The amino group of mannosamine is axial so that when the diazonium ion breaks down, the back side of C-2 is open to attack by solvent water (Fig. 4) with the formation of glucose. Since glucose does not react in our method, and the deamination product of mannosamine does to some extent, it appears that some aldehyde is formed, perhaps by the normal reaction route, or through the alternative chair conformation, as suggested by Shafizadeh (1958). Shively & Conrad (1970) also found low yields of a mixture of products from the deamination of mannosamine.

Extrapolation to zero time of the plots in Fig. 1 indicates that equimolar amounts of similar products are formable from glucosamine and galactosamine, but the greater rate of disappearance of the galactosamine product, coupled with its slower rate of formation (cf. Fig. 1 and 2) result in a smaller amount of fluorescent product in the final stage.

The logarithmic plots of Fig. 1 suggest first-order reactions leading to unreactive material, perhaps by intramolecular rearrangement. 1,4-Hemiacetal formation is one possibility, and in fact 2,5-anhydromannose is an unusual aldehyde in that it mutarotates. Although it reduces Fehling's solution at room temperature, it does not give a Schiff test (Grant, 1956). In the deamination product from heparin, position 4 of glucosamine is blocked by the glycosidic link, which would prevent hemiacetal formation. This could explain the stability of the heparin product, as shown by its unchanged reactivity toward 3,5-diaminobenzoic acid after 60 min (Fig. 3) at 37°C in HNO₂ solution. It does not account for the lability of the galactosamine product, since all the hydroxy groups (of 2,5-anhydrotalose) are *trans* to the alde-

Fig. 4. The pathways of reaction of HNO_2 -treated 2-amino-2-deoxyhexoses containing equatorial amino groups (e.g. I, D-galactosamine) or axial amino groups (e.g. III, D-mannosamine)

The intermediate stage (not shown) is the formation of a diazonium ion from the amino group, which then breaks down to a carbonium ion. Attack on the carbonium ion by the ring oxygen atom, or water, takes place as shown, with the formation of 2,5-anhydro sugars or hexoses (Shafizadeh, 1958). Structure (II) illustrates the *trans* arrangement of the aldehyde, compared with the hydroxy groups, in 2,5-anhydrotalose.

hyde (Fig. 4), which would hinder hemiacetal formation. Moreover, since the fluorescence yield declines only on standing in HNO_2 solution, and not in the HNO_2 -free solution after sulphamate addition, HNO_2 may enter into the reaction at some point. Being in great excess, this does not affect the kinetics, which therefore would be better described as pseudo-first-order.

The assay of amino sugars, heparin and heparan sulphate

The conditions for deamination and removal of HNO_2 established by Dische & Borenfreund (1950), and followed with small adjustments by Exley (1957), have been used as the point of departure.

Regulation of pH is important at several stages. At low pH, the reaction of 3,5-diaminobenzoic acid with aldehyde occurs less readily, but deamination requires an appreciable concentration of HNO_2 ($\text{p}K_{3.4}$) as does the removal of excess nitrite by ammonium sulphamate. At pH 4.2 the sulphamate/ HNO_2 reaction is slow, and this would probably be true of the reaction with the sulphamato group in heparin, by analogy. Without complicating the method by adjusting the pH at each stage, a pH of about 3.0 is a practical compromise.

The Dische-Borenfreund method requires almost 2.0M-acetic acid at the deamination stage, for reasons which are not clear, since it does not enter into the reaction, except to provide an acid pH. High

concentrations of acetic acid add to the 'reagent blank' and slightly diminish the fluorescence yield. In the proposed method the pH is controlled at a value of about 3.0, with a concentration of acetic acid 30% of that of the Dische-Borenfreund method.

The findings that the amino sugar deamination products are converted into non-reactive forms in the HNO_2 reagent, and that the galactosamine product with a half-life only 50% of that of the glucosamine product (Fig. 1) is the most labile, require that the deamination stage be accurately timed, if performed at room temperature. Instead, deamination can be performed in an ice bath and reproducibility is much improved, at a small expense in time.

Conversely, the time for estimation of heparin can be 50% shortened by 'deaminating' at 37°C. Even at the elevated temperature, the stability of the product allows some latitude in timing, while not affecting reproducibility.

Both heparin and amino sugar deamination products are sufficiently stable after the sulphamate reaction not to require exact timing of the addition of 3,5-diaminobenzoic acid.

The different stabilities of the deamination products from glucosamine and galactosamine may have led to inaccurate estimates of hexosamine in the past. Dische (1955a) found only 55% of the expected content in chondroitin sulphate. Exley (1957), however, found close to theoretical values in samples analysed by his procedure. From Exley's description,

the deamination samples were probably not left long before proceeding to the colorimetric stage.

The different fluorescent yields of glucosamine and galactosamine require that the proportions of the two amino sugars be known to obtain accurate estimates of the total hexosamine, where both are present. The glycosaminoglycuronans have mainly one or the other, and it is sufficient to apply a correction factor, with glucosamine as a standard. Comparisons can be carried out easily and accurately. Acceptable values for hexosamine contents of glycoproteins should be obtainable in which the difference between the fluorescence yields of the respective amino sugars is a smaller factor than e.g. relative destruction rates during hydrolysis. As with most fluorescence assays, internal standards are desirable for highest accuracy.

Because the influence of high concentrations of NaCl is quite small, hydrolysates in HCl can be directly neutralized with aq. sodium acetate solution, as a rapid alternative to removing the acid. A slight excess of sodium acetate is allowable, if it does not appreciably change the pH of the reaction solution. Internal standards are recommended in this procedure.

Heparin

Less than the expected amount of fluorescence in terms of glucosamine develops from heparin in Method B. The only valid comparison is between the maximum amount of fluorescence produced from the free amino sugar with that from the combined sugar in heparin. By using these optimal values, samples of heparin gave about 70% of the value expected from the hexosamine content measured by the Elson-Morgan reaction after 4h hydrolysis in 3.5M-HCl. Lagunoff & Warren (1962) also found that heparin gave less, by about 20%, than the expected value for glucosamine content in the Dische-Borenfreund indole method. Assuming complete conversion of glucosamine and heparin into their respective deamination products, the reasons for the short-fall in fluorescence yield remain to be established. The presence of *N*-acetyl groups in heparin must contribute to the discrepancy.

Recommended procedures

In drawing together the reported findings into a practical method, the important variables are the

time and temperature of deamination. The temperature of fluorescence development is of secondary importance. The most complicated situation is that in which the fluorescence yield from D-glucosamine is compared with that from heparin. In methods A and B two different time schedules are involved. It is more convenient to have one. With reagents and manipulations as described under 'standard conditions' amino sugars are deaminated at 0°C in an ice bath for 40min. The sulphamate/HNO₂ step is performed at room temperature for 30min and fluorescence development at 37°C for 60min (method A1). Heparin or heparan sulphate are deaminated at 37°C in a water bath for 40min. The sulphamate/HNO₂ step and the fluorescence development are as for amino sugars (method B1). In this way timing is simplified and the heparin assay is quicker.

The methods are very reproducible, 50–100 times as sensitive as the Elson-Morgan assay and 10–50 times as sensitive as the Dische-Borenfreund method. They are considerably more specific than the latter. They are convenient, require little equipment, and allow hydrolysates of amino sugar-containing compounds to be assayed directly, without removal of hydrolysing acid.

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