The Determination of Hexosamines According to Elson and Morgan

GUNNAR BLIX

The Institute of Medical Chemistry, University of Upsala, Sweden

The method for the quantitative determination of hexosamines elaborated by Elson and Morgan [1] is based on the fact that those substances when treated with acetylacetone in alkaline solution are converted into a pyrrol derivative, which gives a coloured condensation product with p-dimethylaminobenzaldehyde. Although the colour reaction is not specific the method has proved to be of considerable value in biological work.

Elson and Morgan showed that under certain conditions various sugars and amino acids do not interfere with the colour reaction. Employing the method with protein hydrolysates Boyer and Fürth [2], however, obtained low values. On the other hand Nilsson [3], Sörensen [4] and Meyer et al.[5] successfully applied the method to material which contained protein. The explanation of this discrepancy is apparently to be found in the greater amounts of protein split products present in the reaction mixtures of Boyer and Fürth as compared with those of the other investigators. Following the directions given by Nilsson we have frequently observed, however, that lower values were obtained when a greater quantity of material was analysed than when a smaller amount was taken. For example, when 1 ml of blood serum was hydrolysed and one and two ml respectively of the hydrolysate were analysed, the percentage value found in the latter case was generally about 10 % lower than that obtained in the former. In both instances the hexosamine quantities measured were well within the range of the standard curve where a linear relation exists between hexosamine concentration and extinction coefficient. This experience prompted us to make a more detailed study of the method. In the first instance it was considered essential to establish whether or not protein split products influenced the results obtained with the procedure employed. Other details of the procedure were also considered.
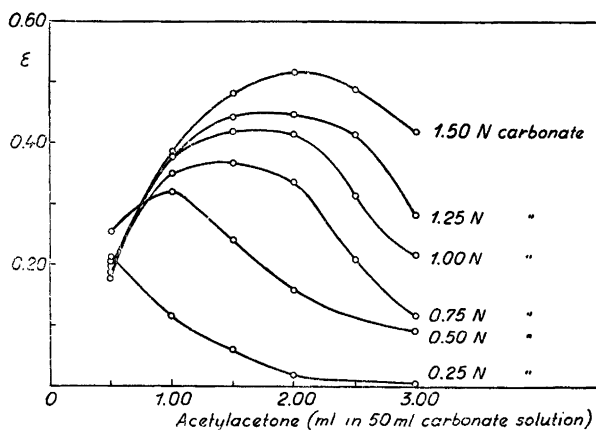
Fig. 1. Variation of extinction value with varying composition of the acetylacetone reagent.

EXPERIMENTAL

Working with solutions of pure glucosamine hydrochloride the following procedure was employed: To 2 ml glucosamine solution were added 2 ml of acetylacetone solution and the mixture heated at + 96° C in a closed Thunberg tube for 20 min. After cooling to room temperature 20 ml 96 % ethyl alcohol and 2 ml of Ehrlich's reagent were added. After standig for 45—60 min the light absorption was measured in a Zeiss' step photometer. Fig. 1 shows the results of a series of experiments in which the composition of the acetylacetone solution was varied. In all these experiments the Ehrlich reagent contained 1.6 g p-dimethylaminobenzaldehyde in a mixture of 30 ml concentrated HCl and 30 ml 96 % alcohol. The glucosamine solution used throughout these experiments contained 0.30 mg of the hydrochloride per ml.

The original acetylacetone reagent described by Elson and Morgan is a solution of 1.00 ml acetylacetone in 50 ml 0.50 N Na_2CO_3. Using this strength for the carbonate solution the greatest light absorption was, as seen, obtained with 1.00 ml acetylacetone in 50 ml solution. This result agrees with those of earlier investigators. However, during the heating of the reaction mixture free acetic acid is probably formed, and if the amount of acetylacetone is augmented above that originally used, the concentration of the carbonate has also to be increased in order to get maximal extinction. If the strength of both these reagents are somewhat increased, the conditions for the formation of the coloured product become more favourable, *i. e.*, the extinction values rise. A solution of, say, 2.00 ml acetylacetone in 50 ml 1.25 or 1.50 N Na_2CO_3 is advantageous not only because a greater amount of the coloured
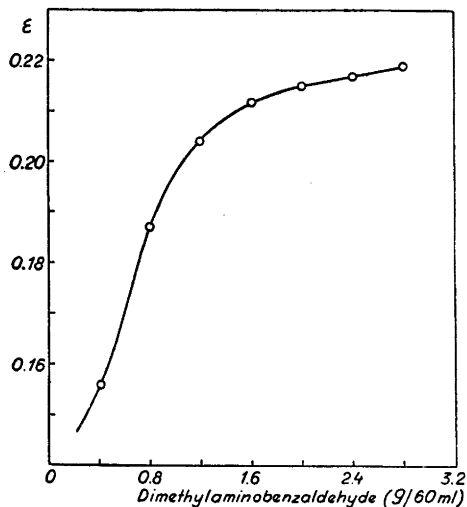
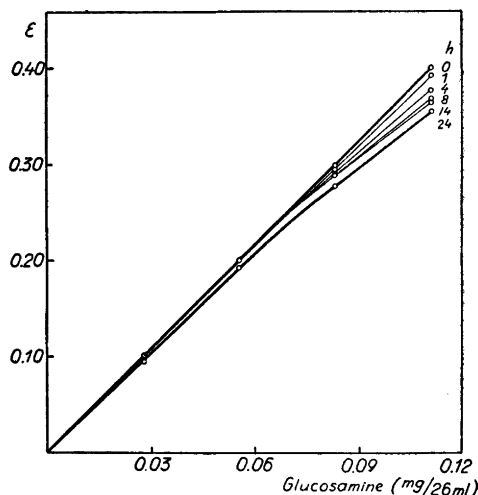Fig. 2. *Variation of extinction value with varying concentrations of p-dimethylaminobenzaldehyde.*

Fig. 3. *Influence on extinction value of treatment of the glucosamine with hydrochloric acid in the heat at varying glucosamine concentrations.*

product is formed but also because the extinction values are less influenced by small variations in the acetylacetone concentration than are those obtained with the original reagent. With increased concentration of the carbonate the reagent becomes less stable. Whereas the original reagent keeps at $+ 4°$ for at least 3 days, the reagents with 1.25 or 1.50 N Na_2CO_3 must be made fresh every day. Because of shortage of acetylacetone and of the increasing instability of the reagent with rising carbonate concentration we have in all subsequent determinations used a solution composed of 1.50 ml acetylacetone and 50 ml 1.25 N Na_2CO_3.

The results of a series of experiments in which the concentration of the p-dimethylaminobenzaldehyde was varied is given in Fig. 2. The reagent contained 1.50 ml of acetylacetone in 50 ml 1.25 Na_2CO_3.

It appears that the value obtained with the original Ehrlich reagent (0.8 g/60 ml) is situated on the edge of the steep slope of the curve. Because of this fact and because the extinction values are somewhat higher with the higher concentrations of the p-dimethylaminobenzaldehyde we have found it advisable to change over to an Ehrlich reagent containing 1.6 g of the aldehyde in 60 ml.

After a considerable number of experiments we concluded that the amounts of protein and other foreign substances present in the materials examined by

us (blood serum, joint fluids, cartilage and other tissues) did not cause any
error in the analyses, and that the difficulties mentioned earlier were due to
an action on the glucosamine of 2 N HCl at + 100° C. We had studied earlier
the stability of glucosamine under these conditions and found no change in
glucosamine concentration up to a heating period of 18 h, but a small decrease
was observed in 24 h. These experiments were, however, carried out with one
single and relatively low concentration of glucosamine. Fig. 3 shows the results
of a number of experiments, where glucosamine in varying concentrations was
heated for different lengthes of time with 2 N HCl.

In all these experiments 0.50—1.00 ml of solutions of pure glucosamine
hydrochloride were heated with 5.00 ml 2 N HCl in closed glass bulbs at
+ 100° C. After cooling, the solution was made up to 10 ml with distilled
water. Of this solution 5 ml were carefully titrated with 4 N NaOH using
methyl red as an indicator. The quantity of 4 N NaOH required for the
neutralisation was added to the remaining 5 ml of the experimental solution
and the mixture then filled up to 8 ml. Of the solution thus obtained 2 ml
were heated at + 96° C for 20 min in closed Thunberg tubes with 2 ml of the
acetylacetone reagent. After addition of alcohol and Ehrlich's reagent the
photometric reading was performed as above described.

When the treatment with acid was excluded the same procedure gave a
linear relation between extinction coefficient and glucosamine concentration
up to a concentration of about 0.20 mg in 26 ml solution (2 ml test solution +
2 ml acetylacetone solution + 2 ml Ehrlich reagent + 20 ml alcohol). On
heating with hydrochloric acid for 1—14 h a deviation from the linear relation
begins to appear when the concentration of the glucosamine exceeds 0.07—
0.08 mg/26 ml. The course of the deviating curves is not consistent with a
monomolecular decomposition reaction. On heating for as much as 24 h the
deviation of the curve appears already at lower concentrations and the lower
part of the curve seems to indicate a simple destruction. The same results
were obtained with solutions which in addition contained protein split pro-
ducts.

Although no certain explanation can be given of the early deviation of the
curves obtained with acid-treated glucosamine some further insight in the
process was gained in the experiments graphically represented in Fig. 4.

Solutions of pure glucosamine-HCl were treated with acetylacetone in the
ordinary way but for varying periods of time. In one series the glucosamine
was previously heated for 14 h with 2 N HCl, in the other series the glucosamine
did not undergo any pretreatment with HCl. As seen from the graph the values
in the two series do not differ with the lower concentrations of glucosamine
tried (0.04—0.08 mg/26 ml, curve I, II and III). With a somewhat greater

Fig. 4. Variation of extinction value with
varying acetylation time.
●— — —● Glucosamine treated with hydro-
chloric acid at 100° C for 14 h.
○———○ Untreated glucosamine.
   I = 0.042 mg glucosamine/26 ml,
  II = 0.053 mg/26 ml,
 III = 0.085 mg/26 ml,
 IV = 0.106 mg/26 ml,
  V = 0.133 mg/26 ml,
 VI = 0.170 mg/26 ml.
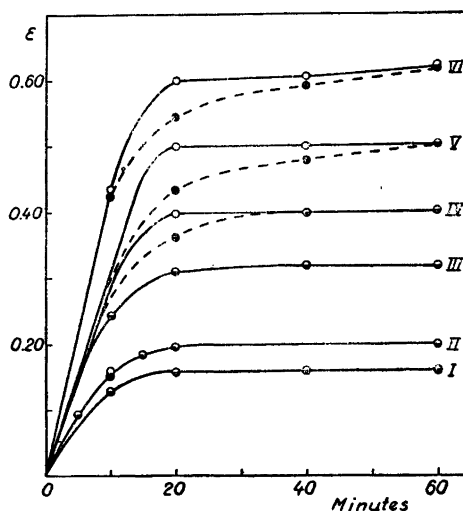
concentration of glucosamine (0.11 mg/26 ml, curve IV) the acetylation in the HCl-treated sample is not complete within 20 min, but is after 40 min. With still greater glucosamine concentration complete acetylation is attained only after about 60 min (0.13 mg/26 ml, curve V). With 0.17 mg/26 ml glucosamine the extinction value of the acid-treated sample has reached that of the non-treated in 60 min, but both were somewhat short of the expected values (E = 0.62 instead of 0.65). Evidently all or a part of the glucosamine in experiments IV—VI is changed in such a way that the acetylation proceeds slower than with the untreated substance. The non-appearence of this change with the lowest glucosamine concentration tried is difficult to explain. A relative change of the same order as in experiments IV—VI should lie outside the experimental error, at least in experiment III. Possibly the change might depend on a reaction of higher order, e. g. polymerization reaction.

Whatever may be the explanation of the HCl effect, due regard must be taken to its existence in the practical performance of the method. When carrying out the determination in the manner described in this paper an acetylation time of 20 min. can be used only with glucosamine amounts of 0.09 mg (in 26 ml) or less. Using an acetylation time of 60 min. it is possible to extend the range upwards to about 0.14 mg glucosamine.

For the convenience of the reader the details of the method as used by us are now given:

Reagents

p-Dimethylaminobenzaldehyde solution. — 1.6 g of the substance are dissolved in 30 ml concentrated HCl and 30 ml 96 % alcohol added. A pure sample of the reagent should be used. The solution has then a very pale yellow colour.

Acetylacetone solution. — 0.75 ml of pure acetylacetone is dissolved in 25 ml 1.25 N Na$_2$CO$_3$. This solution should be made fresh every day.

Procedure

Hydrolysis. — With the materials studied by us (various glucoproteins of animal origin, chondroitin sulfuric acid and hyaluronic acid) a complete liberation of hexosamine has always been attained by heating the material for 14 h in a closed glass bulb at + 100° C with about 2 N HCl. Frequently less time is required. When there has been no shortage of the material to be analysed we have used a standard procedure in which the analytical range is 0.25—0.70 mg in the sample taken for analysis. However, it is possible with slight changes to adapt the method for considerably smaller amounts of the hexosamine, down to 5—30 μg.

To weighed amount of the substance or to ½—1 ml of the solution to be analysed are added 5 ml 2 N HCl and the mixture is heated for 14 h at 100° (in a boiling water bath or in an air thermostat). We have used 30 ml glass bulbs with ground stoppers held in position by a metal clamp. After hydrolysis the solution (if necessary filtrated) is made up to 10 ml. Of this quantity 5 ml are carefully titrated with 4 N NaOH using a drop of methyl red as indicator. The exact amount of NaOH required for neutralisation is added to the remaining 5 ml of the hydrolysate and this solution is made up to 8 ml. With the acetylacetone reagent here used an over-neutralization to pH 7—8 does not invalidate the determination. — As it has turned out that the methyl red does not interfere with the colour reaction with Ehrlich's reagent, the *total* hydrolysate may after addition of a drop of methyl red be directly neutralized and thereafter made up to 8 ml.

Acetylation. — Of the neutralized hydrolysate 2 ml are pipetted into a Thunberg tube (about 185 mm in length and holding 30—35 ml). 2 ml of the acetylacetone reagent are added. The tube is closed and put into a water bath of + 96° for 20 min.

The 2 ml of the glucosamine solution taken should not contain more than 0.09 and not less than 0.03 mg hexosamine. If the acetylation time is extended to 60 min. reliable results are obtained with quantities up to 0.14 mg.

Condensation with p-dimethylaminobenzaldehyde and photometric determination. — After cooling to room temperature with cold water, adding 20 ml 96 % alcohol, *thoroughly mixing* and adding 2 ml of Ehrlich's reagent the light absorption is measured in a Zeiss' step photometer using filter S 53 and a 10 mm cuvette. The reading is made at least 45 min after the addition of Ehrlich's reagent. The light absorption remains constant for 12 h but shows some decrease in 24 h. Of course a photoelectric apparatus, *e. g.* a Beckman photometer, may be used as well. Employing a Beckman photometer it is possible with the standard procedure given here to determine with reasonable accuracy as little as 15—20 μg in 2 ml of neutralized hydrolysate.

When it is possible to use very small amounts of the solution or of the substance to be analysed, *e. g.* 0.1 ml of joint fluid, the hydrolysis can be carried out with 3 ml 2 N HCl, suitably in a small Thunberg tube marked at 5 ml. After the hydrolysis the

solution is neutralized and made up to the mark. To 4 ml of this solution are added 4 ml of the acetylacetone reagent and the acetylation is performed as above. After cooling, 16 ml alcohol and 2 ml Ehrlich's reagent are added. The extinction values obtained are identical with those of the standard procedure for the same quantity of hexosamine. This modification has been introduced in this laboratory by Dr. L. Sundblad and proved to be as reliable as the »standard procedure». *

If 50 mm microcuvettes are used instead of the ordinary 10 mm cuvettes amounts of hexosamine as small as 5 μg in the hydrolysed and neutralized sample can be determined using the step photometer.

Within the analytical range an extinction coefficient of 0.100 corresponds to 0.0279 mg glucosamine in the part of the neutralized solution, which is acetylated. This ratio has been obtained as an average value from a series of analyses on two different, analytically pure specimens of glucosamine hydrochloride.

An idea of the accuracy of the method (standard procedure) can be gained from a series of 43 consequtive duplicate determinations of glucosamine in joint fluids (and a few pleural effusions). The average difference between the double analyses was 1.0 %. In one single case the difference was as high as 7.4 %, in no other instance did it exceed 3.2 %. The differences were calculated on the lowest value in each pair of analyses. Each single analysis was carried out separately commencing with an individual sample. The step in the analysis which most often limited the accuracy of the method was without doubt the photometric reading.

SUMMARY

Various factors influencing the formation of the coloured compound in the method of Elson and Morgan had been studied. As consequence of the experiences gained certain changes of the reagents and of the procedure have been proposed. The procedure adopted is given in details. With small variations in the performance the method may be used for the determination of hexosamine in quantities from about 5 μg up to 150 μg.

LITERATURE

1. Elson, L. A., and Morgan, W. T. J. *Biochem. J.* **27** (1933) 1824.
2. Boyer, R., and Fürth, O. *Biochem. Z.* **282** (1935) 242.
3. Nilsson, I. *Biochem. Z.* **285** (1936) 386.
4. Sörensen, K. M. *Compt. rend. trav. lab. Carlsberg* **22** (1938) 487.
5. Palmer, J. W., Smyth, E. M., and Meyer, K. *J. Biol. Chem.* **119** (1937) 491.

* With extinction values as low as 0.05—0.10 the blank should contain methyl red in the same concentration as the test.