reaction extends to disaccharides and lower polysaccharides containing the 1:4 glycosidic linkage, irrespective of their stereochemical relationships. In agreement with this, sucrose (1:2) and melibiose (1:6) fail to give the reaction, as do the trisaccharides raffinose and melezitose.

The method seems to offer scope for further application to mixtures of lactose with other sugars, either by direct measurement—when the contaminating sugar gives no colour (raffinose)—or by measurement at two selected wavelengths, when interfering colours are produced (galactose). Estimations in the presence of maltose or lower dextrins may be made after preliminary fermentation with brewer's yeast, though the slow and variable fermentation rates of the latter, presumably to be associated with differences in the degree of polymerization of the dextrins capable of giving a red colour, may present special difficulties. When cellobiose is present an approximation to the correct lactose concentration can be made by first acting upon the solution with emulsin, which hydrolyses β-glycosides much more rapidly than lactose.

SUMMARY

1. A method is described for the semi-micro estimation of lactose; modifications are given for its application to solutions containing lactose in the presence of glucose and glycogen, and in tissue extracts, and more general recommendations made for its use when other contaminating sugars are present.

2. The method is most sensitive for lactose concentrations in the range 0·05–0·2 %.

We wish to express our thanks to Prof. D. C. Harrison for his interest in the progress of this work and to Dr Q. H. Gibson for valuable suggestions with regard to the graphical presentation.

REFERENCES

Archer, H. E. & Haram, B. J. (1948). *Lancet*, **1**, 558.
Fearon, W. R. (1942). *Analyst*, **67**, 130.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). *Nature, Lond.*, **160**, 86.
Grant, G. A. (1935). *Biochem. J.* **29**, 1905.
Harwood, J. A. (1946). *Bull. Inst. med. Lab. Tech.* **12**, 3.
Hawthorne, J. R. (1947). *Nature, Lond.*, **160**, 714.
Letonoff, T. V. (1934). *J. biol. Chem.* **106**, 693.
Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Scott, M. & West, E. S. (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 52.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Westall, R. G. (1948). *Biochem. J.* **42**, 249.

Displacement Chromatography on Synthetic Ion-exchange Resins

4. THE ISOLATION OF GLUCOSAMINE AND HISTIDINE FROM A PROTEIN HYDROLYSATE

By S. M. PARTRIDGE
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge

(*Received* 21 *April* 1949)

In Part 3 of this group of papers (Partridge, 1949) the fractionation of the hydrochloric acid hydrolysis product of commercial egg albumin was described. A displacement chromatogram, using a column packed with 'Zeo-Karb 215' showed the presence of seven discrete bands each of which contained simple mixtures of amino-acids. These were identified by carrying out partition chromatograms on filter paper by the method of Consden, Gordon & Martin (1944). Band VI contained histidine together with an unknown basic substance. This substance has now been identified as glucosamine and both glucosamine and histidine have been isolated from the amino-acid mixture in a pure condition. The amount of glucosamine in the hydrolysis product is very small and its origin is probably the ovomucoid contained in white of egg. Histidine is also present in small amount, the content of this amino-acid in crystalline ovalbumin being about 2·4 % (Chibnall, 1945).

The following is the order in which the more basic amino-acids appear in the effluent from a column of 'Zeo-Karb 215' when ammonia solution is used as the displacement developer: leucine, histidine, glucosamine, lysine, ammonia. The affinity of glucosamine for the resin is not much greater than that of histidine, and separation is only partial with columns of normal length. It has been pointed out (Partridge & Westall, 1949; Davies, 1949) that when the displacement developer is a free base, the order of displacement reflects the ability of the stronger bases to control the pH of the aqueous phase and thus to depress the cationic form of the weaker bases.

Davies (1949) shows that the controlling equilibrium in the case of the amino-acids may be represented by

$$A^+ \rightleftharpoons A^{\pm} + H^+. \quad (1)$$

In addition, there may be two further factors affecting the adsorption of an amino-acid by the resin: (*a*) van der Waals forces between the resin and the cation A^+ or the neutral molecule $A^{\pm}$, and (*b*) the affinity of the cation for the resin will be greater the higher its valency.

It is clear, therefore, that by altering the environment of the ions in the ambient phase it would be possible to exaggerate the effect of one of these mechanisms at the expense of the others and thus to produce conditions in which the order of displacement is altered. Thus, by using a dilute solution of a salt as displacement developer the pH of the ambient fluid would be lowered and equation (1) would lie entirely to the left (in so far as univalent cations are concerned). This has been made use of to secure the separation of glucosamine from histidine. When a solution of a salt flows through a column containing an adsorbed mixture of amino-acids, the cations of the salt are adsorbed and the anions are released together with the cations of the displaced amino-acids. The high acidity thus set up results in the mechanism depending upon equation (1) becoming inoperative and the mutual displacement of the amino-acids is thus controlled by mechanisms (*a*) and (*b*) only. In this case, as would be expected, the bivalent cations are strongly adsorbed and the observed order of displacement where 0·1 M-sodium chloride is used as the displacement developer is as follows: glucosamine, Na^+, leucine, histidine, lysine. The affinities of Na^+ and the leucine cation are very similar, but with long columns the Na^+ band tends to overtake that due to leucine. Lysine and histidine are much more strongly adsorbed than Na^+, and the sodium band overruns these two amino-acids and shows little tendency to cause their migration down the column by elution.

METHODS

Comparison of the effects of sodium chloride and sodium hydroxide solutions as displacement developers

The behaviour of the two displacement developers was compared directly by setting up two small identical columns and analysing with each the same sample mixture of bases.

The columns each contained 2 g. of air-dried 'Zeo-Karb 215' (80–100 mesh/in.; height of column 6·1 cm.). Before use the columns were treated alternately several times with 2N-HCl and 2N-NaOH. In both cases the mixture of solutes run through the column (in its hydrogen form) was: histidine, 0·1 M, 10 ml.; glucosamine hydrochloride, 0·1 M, 10 ml.; glycine, 0·1 M, 10 ml.

Column (1) was developed by displacement with 0·1N-NaOH applied at a rate of 1 ml./min. The effluent was collected in 3 ml. fractions, 34 fractions being collected in all. A filter-paper chromatogram was set up using a drop of solution from each fraction and the results are shown diagrammatically in Fig. 1*a*.

Column (2) was developed by passing at first a solution of NaCl (0·05 M) at a rate of 2 ml./min. A portion of the effluent (45 ml.) was allowed to pass before commencing to collect fractions. After 34 fractions (each of 3 ml.) had been collected the reservoir containing NaCl solution was replaced by one containing 0·1 N–NaOH and the experiment was continued until a further 26 fractions had been taken. The composition of the effluent was analysed by paper chromatography as before and the results are shown in Fig. 1*b*.

The effluent solution at first contained HCl arising by removal of the cations from the NaCl solution, but when the column became saturated with cations the hydrogen-ion concentration of the effluent fell rapidly and hydrochlorides of glycine and glucosamine appeared. Between fractions 10 and 14 (Fig. 1*b*) glucosamine and glycine hydrochloride were replaced by NaCl and the free acidity of the effluent fell to less than 0·001 N. On replacing the reservoir by one containing NaOH the residual NaCl solution was rapidly displaced and salt-free water flowed from the column until the appearance of the histidine band.

In order to collect data for the design of a suitable experimental procedure for the isolation of the components of the histidine-glucosamine band obtained in the primary separation, the experiment described above was repeated several times with small variations in the procedure. The results of these experiments may be summarized as follows.

(*a*) *Replacement of glycine by leucine.* The experiment illustrated in Fig. 1*b* was repeated with leucine in place of glycine. The chromatogram showed a good separation between glucosamine and leucine, the leucine front almost coinciding with that due to sodium chloride. The leucine band had a very diffuse rear boundary, showing that, under the conditions of the experiment, the affinities of the leucine and sodium ions for the resin were similar. A small part of the leucine was not eluted from the column by the volume of sodium chloride solution applied, and this appeared as a sharp but narrow band in front of the histidine band when the histidine was displaced by sodium hydroxide solution.

(*b*) *Effect of increasing the concentration of sodium chloride.* The sodium chloride concentration was increased to 0·1 M and the chromatogram compared with that shown in Fig. 1*b*. The result showed that while the glucosamine band was relatively shorter, and the glucosamine content of the effluent correspondingly higher, the leading edge of the band was very diffuse. This effect was probably due to the tendency of glucosamine to be eluted by the higher concentration of hydrochloric acid present.

(*c*) *Effect of replacing the sodium hydroxide solution by ammonia* (0·1 N). The resulting chromatogram showed an improvement in the sharpness of the histidine band. Since the use of sodium hydroxide

leads to difficulties where larger columns are employed, dilute ammonia solution was adopted for all further experiments.

(*d*) *Effect of increasing the column length.* The experiment was carried out using glucosamine hydrochloride, leucine and histidine as the experimental mixture. The increase in column length was about 25 % and the chromatogram showed an improvement in the separation of glucosamine and leucine. It was considered that the extra column length allowed the sodium band to overtake that due to leucine, with the result that a narrow zone of sodium chloride was interpolated between the glucosamine and the mixed sodium chloride-leucine bands.

Primary fractionation of the protein hydrolysate

A sample of commercial egg albumin (moisture 13·8 %, ash 3·4 %; 40 g.) was hydrolysed with N-hydrochloric acid. It was then treated with charcoal and fractionated on a column of 'Zeo-Karb 215' exactly as described in Part 3 of this series (Partridge, 1949). Using the filter-paper chromatogram as a guide, three fractions of the effluent (each of 47 ml.) were combined together so as to comprise the whole of the histidine band. Further filter-paper chromatograms carried out on the mixed solution showed this to contain histidine and the 'unidentified substance', together with smaller quantities of leucine, lysine and cystine.

Identification of glucosamine

The unidentified substance was observed to have R_F values in a number of different solvents close to those given by glucosamine. Since the amino-sugar gives a good colour reaction with ninhydrin and is known to occur in the ovomucoid of white of egg, the identity of the two substances was probable. Like glucosamine, the unknown substance was shown to yield brown spots on chromatograms sprayed with ammoniacal silver nitrate and cherry-red spots with the Elson & Morgan (1933) reagents (cf. Partridge, 1948). Further chromatograms were carried out in phenol, collidine and butanol-acetic acid mixture in which the unknown substance was compared directly with authentic glucosamine, and these confirmed the identity.

The only naturally occurring ninhydrin-reacting substance known to have R_F values close to glucosamine in these three solvents is chondrosamine, but separation may be obtained between these two amino-sugars on filter-paper chromatograms by pro-

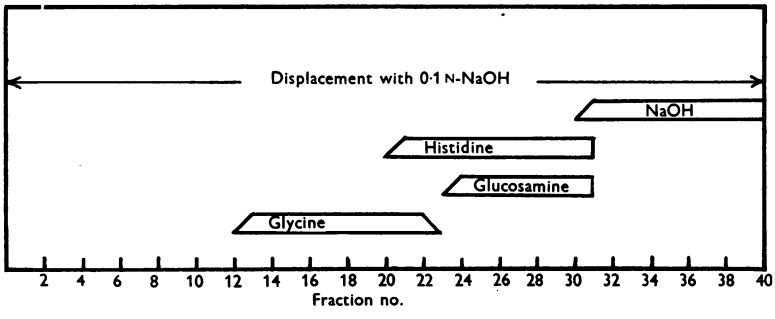

Fig. 1*a*.

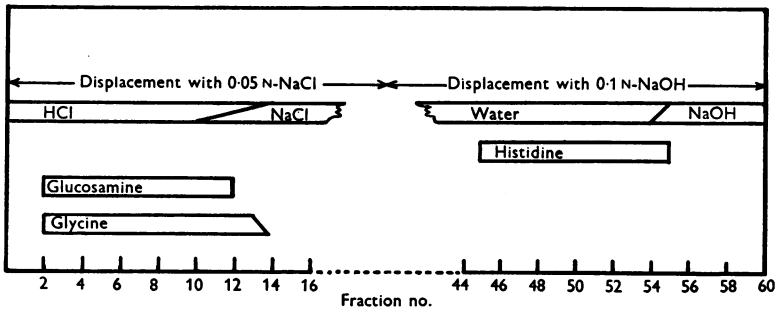

Fig. 1*b*.

Fig. 1. *a*, Composition of the effluent from a column of 'Zeo-Karb 215' on displacing a mixture of glycine, glucosamine, and histidine with 0·1 N-NaOH. *b*, Composition of the effluent obtained by displacing the amino-acid mixture first with 0·05 N-NaCl followed by 0·1 N-NaOH.

longed irrigation with collidine (cf. Aminoff & Morgan, 1948). A few trials were required in order to determine satisfactory conditions for the separation of the two amino-sugars, and the following procedure was finally adopted. A strip of Whatman no. 1 filter paper was cut to a point at one end and spots of glucosamine hydrochloride, chondrosamine hydrochloride, the unknown mixture and a mixture of the authentic amino-sugars were applied to the starting line. When the spots were dry, a drop of concentrated ammonia solution was put on each of the spots and allowed to dry off. The chromatogram was then irrigated for 42 hr. with collidine, allowing the solvent to drip off the pointed end of the paper. On drying and spraying with ammoniacal silver nitrate or the Elson & Morgan (1933) reagents a clear separation was obtained between the two amino-sugars, and chondrosamine was seen to be absent from the unknown mixture.

Isolation of glucosamine and histidine from band VI of the primary separation

The filtration tube was 13 mm. in diameter and contained 10 g. (dry wt.) of 'Zeo-Karb 215' (80–100 mesh/in.). The column was prepared by alternate treatment with 0·15N-ammonia and 2N-hydrochloric acid in order to compact the bed (height occupied by the resin, 18·0 cm.). The mixed solution obtained from band VI of the primary separation (140 ml.) was applied to the column at a rate of 1·5 ml./min. and the column was then washed with 10–20 ml. water. Since the primary fractionation was carried out by displacement with 0·15N-ammonia the concentration of bases in the mixed solution of band VI was about 0·09M (cf. Part 1 of this series; Partridge, 1948), and thus the experimental mixture was sufficient to saturate rather less than half the new column (Part 1, fig. 3).

A dilute solution of sodium chloride (0·05M, 200 ml.) was applied at a rate of 2 ml./min. and the effluent was collected in 20 ml. fractions. A further quantity of more concentrated sodium chloride solution (0·1M, 400 ml.) was then applied, after which the column was washed with water (5–10 ml.) and the reservoir changed for one containing 0·15N-ammonia. This solution (150 ml.) was applied at a rate of 1 ml./min. and the effluent collected in 10 ml. fractions.

Fig. 2 shows the filter-paper partition analysis of the effluent fractions. A good separation was obtained between glucosamine and leucine and between histidine and lysine, but the histidine fraction was contaminated by a little cystine. A series of very faint spots due to phenylalanine was noticed on the paper chromatogram. The bulk of this amino-acid was removed by the preliminary charcoal treatment of the protein hydrolysate and it had not been observed in previous chromatograms carried out on the experimental solution; however, its appearance in the histidine band was probably due to the concentration of small traces by the chromatographic procedure. Fractions 9–12 (glucosamine hydrochloride) and 43–46 (histidine) were set aside for concentration and crystallization.

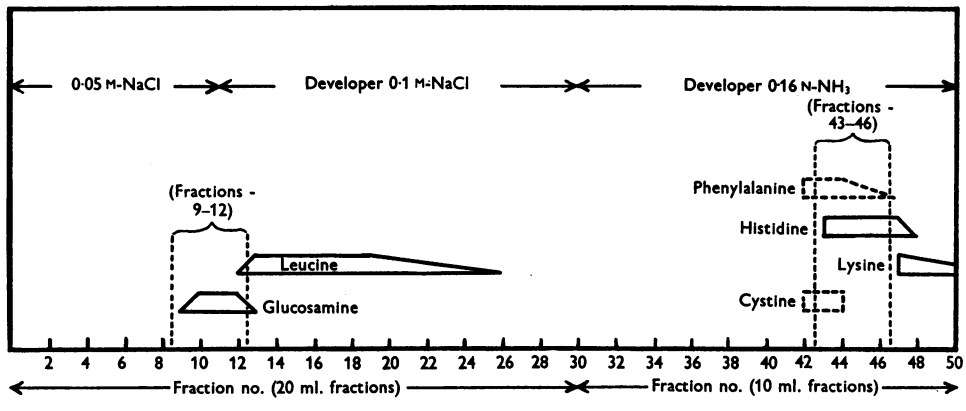

Fig. 2. Further fractionation of the histidine-glucosamine band obtained in the primary separation of the protein hydrolysate. The chromatogram shows the result of displacing the mixture from a column of 'Zeo-Karb 215' with an increasing concentration of NaCl followed by ammonia solution.

Crystallization of glucosamine hydrochloride. The mixed fractions 9–12 (80 ml.) were evaporated to small bulk under reduced pressure and the syrup diluted with about 4 vol. of methanol. The solution was then warmed, and warm acetone added until crystallization commenced. On cooling, a heavy crop of crystals separated (small prisms). Yield, 0·157 g.; ash, 0·7 %; N, 6·40 % (calc. for $C_6H_{14}O_5NCl$, N, 6·53%); $[\alpha]_D^{20°}$ + 74 (1 dm.; *c*, 1·5 in water); yield as percentage of protein dry wt., 0·47. A chromatographic analysis of the product showed it to be free from amino-acids.

Crystallization of histidine. The mixed fractions 43–46 (40 ml.) were concentrated to about 10 ml. under reduced pressure and the solution stored at + 2° overnight. It was then filtered to remove a light precipitate which was probably cystine. A small amount of charcoal was added and the solution warmed. It was then refiltered and the evaporation continued until a few crystals appeared in the liquid. An equal volume of warm ethanol was then added and a dense feltwork of crystals appeared on cooling. Yield, 0·303 g.; ash, 0·3 %; N, 26·5 % (calc. for $C_6H_9O_2N_3$: N, 27·1 %); $[\alpha]_D^{20°}$ − 32·6 in water (*c*, 2·3; 1 dm.); yield as percentage of protein dry wt., 0·92. Chromatographic analysis showed the product to be free from other amino-acids.

DISCUSSION

The separation described above illustrates a point that has already been stressed in Part 3 of this series (Partridge, 1949), namely, that fractionation by displacement from an ion-exchange column has an advantage not possessed by the process of distillation from a fractionating column which it otherwise resembles: in the chromatographic technique it is often possible to alter the adsorption affinity of a solute by altering the environment in which the adsorption takes place; thus a primary separation may be carried out in order to divide the components into a series of small groups, and each group may then be separated further by a secondary fractionation carried out with an alteration in experimental conditions calculated to produce a differential alteration in adsorption affinity.

Two distinct methods of using ion-exchange resins in displacement chromatography have been described here, displacement with a free base and displacement with a salt; the substitution of one method by the other leads to a remarkable change in the order in which the components leave the column. The differential technique has been used in this case to separate a univalent base, glucosamine, from a complex ampholyte, histidine; but, although no further experiments on these lines have yet been attempted, it is clear that a similar technique could be applied to other separations in which the components are either different in charge structure or are well separated in a homologous series.

The isolation of glucosamine from a protein containing it in small amount has a special interest since many proteins are known to contain small quantities of hexosamines. Isolation by conventional procedures is a difficult matter, and although the hexosamine content of a protein may be estimated readily by colorimetric or other methods, none of these differentiates one hexosamine from another.

The yields of both glucosamine and histidine obtained in these experiments were low, probably not exceeding 40 % in either case, but a large part of the loss occurred during the final process of crystallization which was carried out with 150–300 mg. of material. Higher yields should be obtainable by increasing the scale.

SUMMARY

1. Glucosamine has been identified as a constituent of the hydrolysis product of commercial egg albumin.

2. A primary fractionation of the hydrolysate carried out by displacing the components of the mixture from a column of 'Zeo-Karb 215' by means of dilute ammonia solution yielded a mixed band containing glucosamine and histidine together with other amino-acids in small amount.

3. The components of the mixed band were again displaced from a smaller column of 'Zeo-Karb 215' using dilute sodium chloride solution as the displacement developer. Glucosamine was displaced and was recovered in a pure condition as the hydrochloride. Histidine remained in the column.

4. The histidine was displaced from the column by means of ammonia solution and was recovered as the free base in a substantially pure condition.

5. The principle underlying the separation is discussed briefly and possible applications to other systems are pointed out.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The author wishes to acknowledge the assistance of Mr D. F. Elsden.

REFERENCES

Aminoff, D. & Morgan, W. T. J. (1948). *Nature, Lond.*, **162**, 579.

Chibnall, A. C. (1945). *Second Procter Memorial Lecture.* Croydon: International Society of Leather Trades Chemists.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Davies, C. W. (1949). *Biochem. J.* **45**, 38.

Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27** 1824.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Partridge, S. M. (1949). *Biochem. J.* **44**, 521.

Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.