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# An Improved Rapid Method for the Determination of Glucosamine and Galactosamine on an Amino Acid Analyzer

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## Introduction

The employment of ion-exchange resin according to the Pearson's procedure[10] and the subsequent application of the Elson-Morgan method modified by Boas[1] for the quantitative estimation of hexosamines in glycoproteins are available, because the use of expensive instruments is not necessary. However, the combined procedure using the two methods mentioned above requires considerable time for an estimation, and moreover, is inadequate to determine small amounts of hexosamines in some proteins. Spackman, Stein and Moore[16] carried out a chromatographic analysis of amino acid mixtures and related compounds including hexosamines in physiological fluids with an automatic amino acid analyzer, but the resolution and determination of glucosamine and galactosamine could not be performed on a single column.

Plummer *et al.*[12,13] also determined the glucosamine amount in glycoproteins by the method of Spackman *et al.*[16]. Since then, some modified methods for the determination of hexosamines on a single column with an amino acid analyzer have been published. Peterson *et al.*[11] and Rosan[15] successfully performed the resolution and quantitation of hexosamines on a long column using the modified two-buffer system at a higher temperature. Fauconnet *et al.*[4] also developed a single column amino acid analysis method which resolves hexosamines and cysteine derivatives, employing three kinds of modified buffers. The eluant buffer system, moreover, was greatly improved by Cheng *et al.*[2]. However, these quantitative analyses necessitated at least 110 to 240 min, excepting the longer period of time required in the case of the procedures by Spackman *et al.*[16] and Plummer[12,13]. Fanger and Smyth[3] performed the hexosamine analysis using a short column for the basic amino acids after the removal of interfering materials with the aid of Dowex 2. Recently, Murayama *et al.*[9] and Plummer[14] could resolve and estimate the hexosamines on a short column, using buffers which they modified, independently.

We have been investigating the constitutions and functional properties of glycoproteins in avian egg whites. It is, therefore, very useful to develop a rapid and accurate method for the quantitative estimation of small amounts of hexosamines, because the amount in some glycoproteins is noted to be very small[5]. The present paper describes a more rapid method for the resolution and quantitation of glucosamine and galactosamine in glycoprotein hydrolysates than the others on a short column with an amino acid analyzer, and without such pre-treatment as in the procedure of Fanger *et al.*[3].

## Materials and Methods

Amino acid analyzer — A Yanagimoto LC-5S type automatic amino acid analyzer was used

for the experiments, and a short column (0.9×20 cm) packed to a height of 7.5 cm with Aminex A-4 resin for the basic amino acid analysis was employed. The flow rate for the buffers was adjusted to 100 ml/hr and that for the ninhydrin reagent to 50 ml/hr in all analyses, and the column temperature was maintained at 52°C.

Chemicals — Glucosamine hydrochloride and galactosamine hydrochloride were obtained from Nakarai Chemicals Co. Amino acid calibration mixture was obtained from Kokusai Reagent Co. Ltd., and tryphophan was from Ajinomoto Co. Ltd. Other chemicals for preparing the buffer and ninhydrin solutions were purchased from Nakarai Chemicals Co. Ltd.

Preparation of eluant buffers — Using sodium citrate·$2H_2O$, concentrated hydrochloric acid of high purity, 25% Brij 35, n-caprylic acid and deionized distilled water, the various buffers differing in pH values and $Na^+$ concentrations were prepared as follows.

Buffer A: pH 5.28, $Na^+$ 0.35 M, Buffer B: pH 4.25, $Na^+$ 0.2 M, 0.16 M, 0.14 M, Buffer C: $Na^+$ 0.15 M, pH 5.45, 5.70, 6.00.

All pH adjustments were made with concentrated hydrochloric acid. An example of the constitutions of the buffers is shown in Table 1.

Table 1. Constitution of the citrate buffer (pH 5.70, $Na^+$ 0.15 M) improved for the resolution of glucosamine and galactosamine

| | |
|---|---|
| $Na_3$-Citrate·$2H_2O$ | 14.7 g |
| Concentrated HCl | 2.0 ml |
| 25% Brij 35 soln. | 3.0 ml |
| n-Caprylic acid | 0.1 ml |
| Final vol. with water* | 1 lit. |

* Deionized distilled water
Adjustment of pH was done with hydrochloric acid.

Preparation of ninhydrin reagent — The buffer for the reagent was firstly prepared as follows: sodium acetate·$3H_2O$ 136 g, potassium acetate 294.5 g, potassium citrate·$H_2O$ 4 g, and glacial acetic acid 100 ml were dissolved, in that order in deionized distilled water, and adjusted to 1 liter of final volume, pH 5.20. To 1 liter of this buffer solution was added 3 liters of methyl cellosolve. The solution was freed of the contained oxygen by the introduction of the compressed nitrogen, and ninhydrin 80 g; stannous chloride·$2H_2O$ 1.6 g were added.

Preparation of glycoproteins — Avian ovalbumins were prepared in purity from the respective egg whites by salting out with ammonium sulfate followed by the CM-cellulose column chromatography and Sephadex gel-filtration[5,6]. Quail's ovomucoid and ovomucoid-like globulin[7], a trypsin inhibitor, were also prepared by a method similar to that mentioned above (unpublished). Human *r*-globulin was purchased from Miles Laboratories Co. (Kankakee, Illinois, USA).

Hydrolysis of glycoproteins for the release of hexosamines — Ten ml of 4 N HCl was added to 164 mg of ovalbumin in a pyrex test tube and after careful shaking, the air was removed by suction. After introduction of nitrogen gas into the tube, the tube was sealed. The hydrolysis was performed for 6, 8 and 10 hr at 100°C, respectively. The hydrolyzate was concentrated to dryness in vacuo, water was added, and the solution was again dried. Citrate buffer of pH 2.2, $Na^+$ 0.2 N was added, and then the solution was diluted to a given volume. The aliquot part was used for the hexosamine

analysis. Other glycoproteins were treated similarly, but the hydrolysis was performed for 7 hr at 100°C.

## Results and Discussion

The elution pattern of glucosamine, galactosamine, tryptophan and a standard amino acid calibration mixture when using an eluant buffer (pH 5.28, $Na^+$ 0.35 M) for the basic amino acid analysis, is shown in Fig. 1. Glucosamine, galactosamine and tryptophan were closely eluted between phenylalanine and lysine, and especially the resolution of galactosamine from glucosamine was deficient. Subsequently, the proper condition of the buffers for better separation of the two hexosamines were examined. Using buffers having different concentrations of $Na^+$ ion (0.20 M, 0.16 M and 0.14 M) at a constant pH 4.25, chromatographic separation was attempted. The resolution profiles are shown in Fig. 2. The figure represents that the resolution of glucosamine and galactosamine is improved considerably by the decreasing of $Na^+$ in the citrate buffers employed, although the time consumed for the elution of both the compounds is greater than that in Fig. 1.

The effect of the pH of the buffers on the resolution of hexosamines was examined at a constant concentration of $Na^+$ (0.15 M). The concentration corresponds to 0.05 M sodium citrate, being

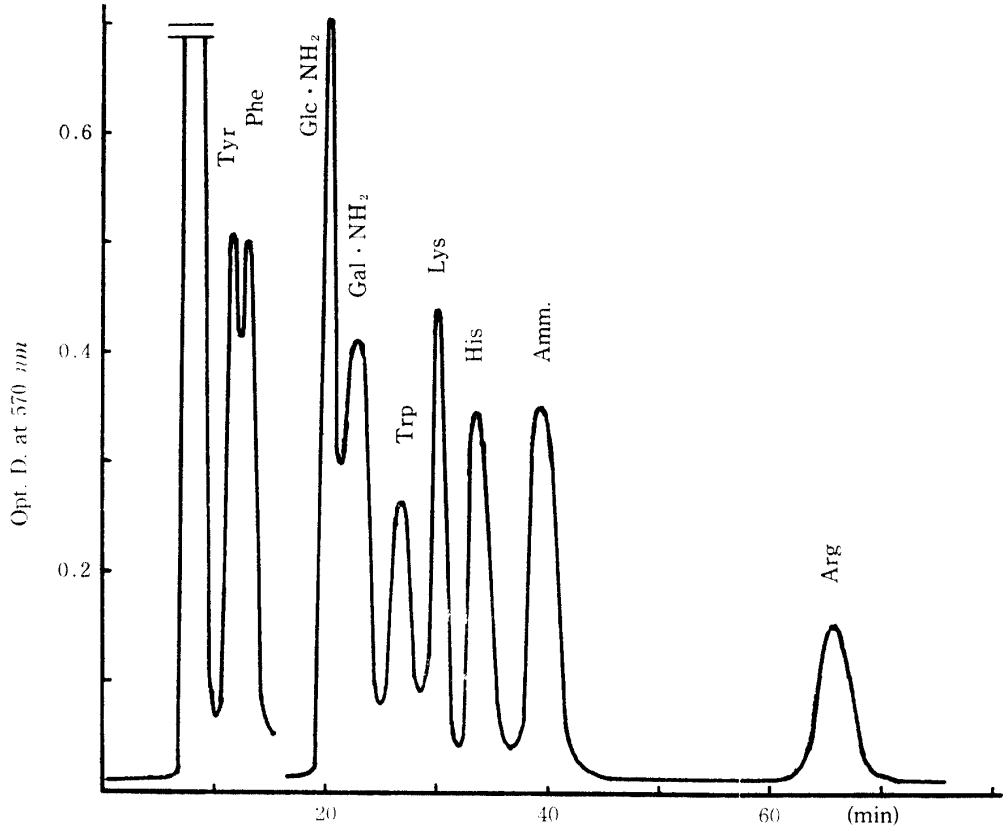


Fig. 1. Resolution of hexosamines from standard amino acids containing tryptophan on an amino acid analyzer.

Column: 0.9 × 7.5 cm length packed with Aminex A-4.
Glc·$NH_2$: glucosamine, Gal·$NH_2$: galactosamine.
Quantity applied: Glc·$NH_2$, Gal·$NH_2$ (0.1 $\mu$M each), standard amino acid plus tryptophan (0.08 $\mu$M), Eluant buffer: Citrate pH 5.28, $Na^+$ 0.35 M (general buffer for basic amino A.).

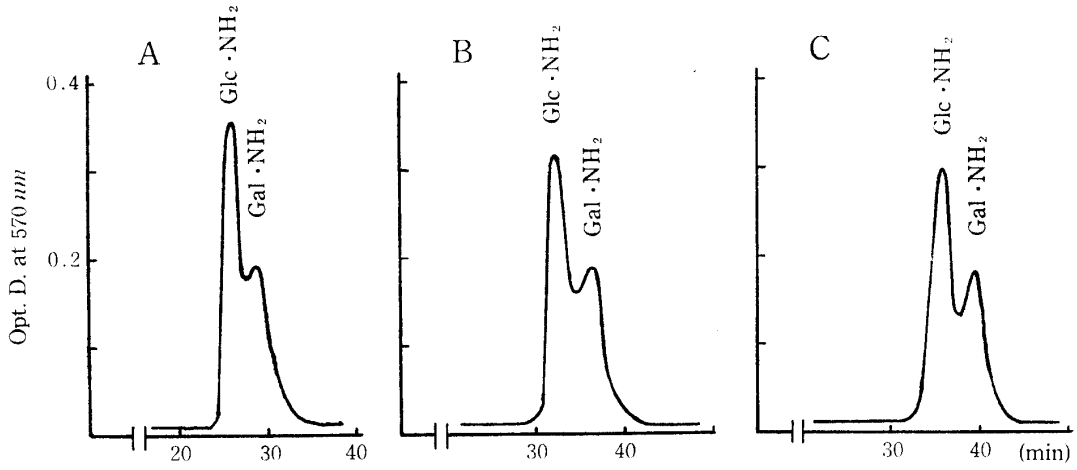


Fig. 2. Elution profiles of glucosamine and galactosamine at various sodium molarities in the citrate buffer, pH 4.25.

Quantity applied: Glc·$NH_2$, Gal·$NH_2$ (0.1 $\mu$M each).
$Na^+$ concentration: (A) 0.2 M, (B) 0.16 M, (C) 0.14 M.

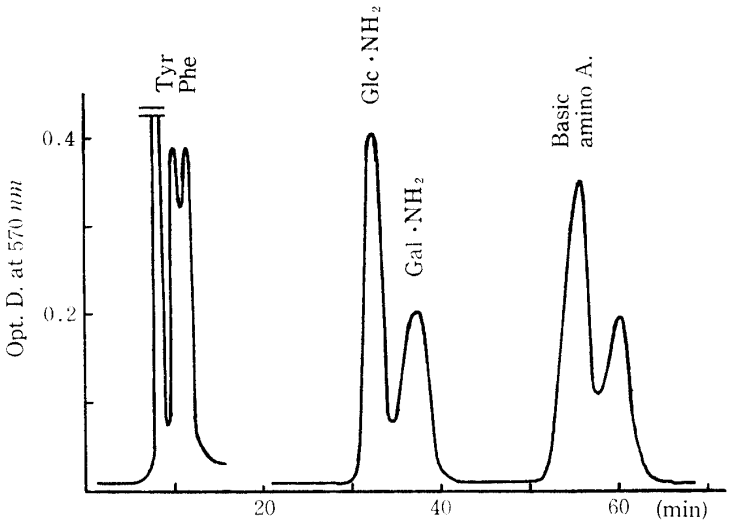


Fig. 3. Elution profile of hexosamines and standard amino acids, when 0.05 M citrate buffer, pH 5.45 was used.

Quantity applied: Glc·$NH_2$, Gal·$NH_2$ and amino acids (0.1 $\mu$M each).

favorable for preparing the buffer, hereafter. The results obtained are shown in Fig. 3, Fig. 4 and Fig. 5. Fig. 3 represents the profile eluted at pH 5.45; Fig. 4 and Fig. 5 represent those at pH 5.70 and 6.00, respectively. The resolution of glucosamine and galactosamine at pH 5.45 was better than that at pH 4.25, and moreover, the resolution at pH 5.70 was ascertained to be superior to that at pH 5.45, whereas the separation of basic amino acids was worse. Glucosamine and galactosamine, as seen in Fig. 4, were separately eluted between partially overlapped peaks of tyrosine and phenylalanine and the overlapped peaks of basic amino acids. The eluting profile was similar to those by other researchers[11,15,4], although it was different from that obtained by Spackman *et al.*[16].

At pH 6.0, a sharp glucosamine resolution was observed, but galactosamine and the basic

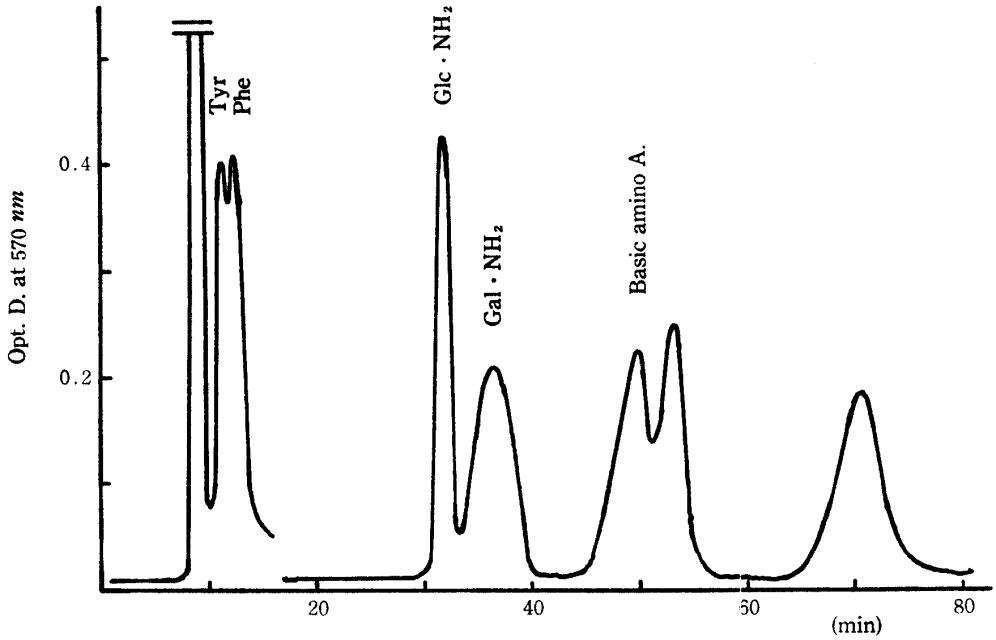


Fig. 4. Elution profile of hexosamines and standard amino acids, when 0.05 M citrate buffer, pH 5.70 was used.
Quantity applied: Glc·$NH_2$, Gal·$NH_2$ (0.08 $\mu$M) and amino acids (0.1 $\mu$M).

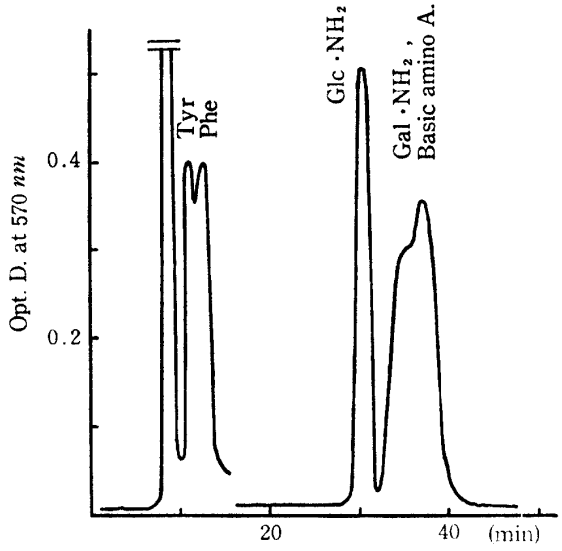


Fig. 5. Elution profile of hexosamines and standard amino acids when 0.05 M citrate buffer, pH 6.00 was used. Quantity applied: the same as Fig. 4.

amino acids were eluted overlapping, owing to the faster elution of the basic amino acids. The galactosamine-like material behind glucosamine on the chromatogram of partial hydrolyzates of rabbit IgG by Fanger *et al.*[3] was presumed to be tryptophan, because tryptophan was eluted behind hexosamines at pH 5.28 (Fig. 1), and a fair amount was contained in IgG[8]. To examine the eluting position of tryptophan at pH 5.70, $Na^+$ 0.15 M, we chromatographed the standard amino acid mixture solution containing the additional glucosamine, galactosamine and tryptophan on a short column, at pH 5.70. The result is shown in Fig. 6. Tryptophan was eluted ahead

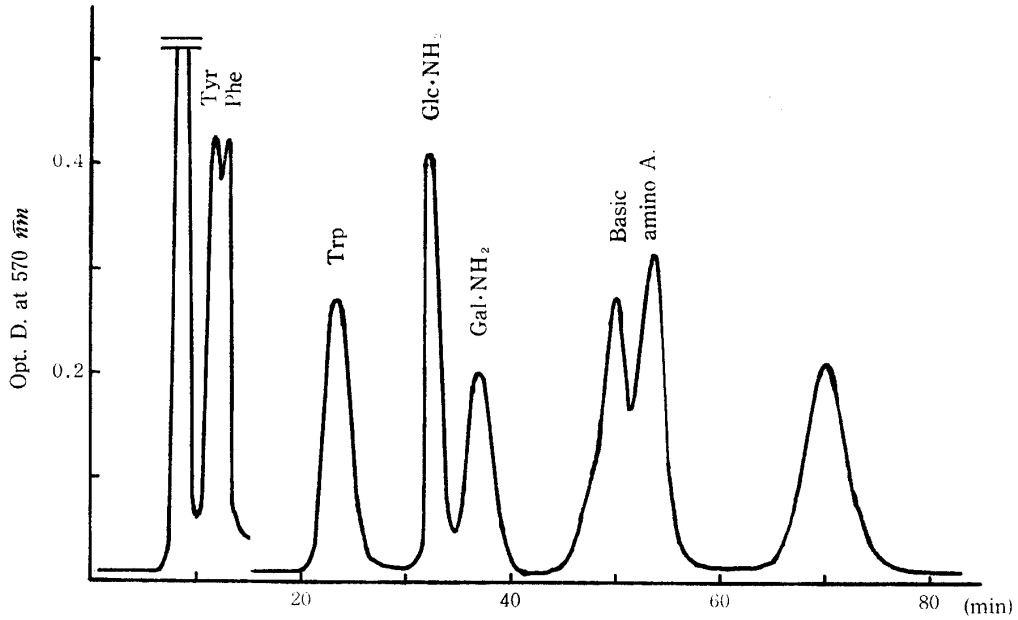


Fig. 6. Resolution of glucosamine, galactosamine and tryptophan from standard amino acids when 0.05 M citrate buffer, pH 5.70 was used, as eluant.

Quantity applied: hexosamines (0.1 $\mu$M each), tryptophan (0.123 $\mu$M), standard amino acids (0.1 $\mu$M).

of glucosamine, and moreover, the well-resolved glucosamine and galactosamine were between tryptophan and the basic amino acids. The order of elution of tryptophan was the reverse of that in Fig. 1. The result shown in Fig. 6 was presumed to be satisfactory for the resolution of the two hexosamines from amino acid mixtures containing tryptophan, such as the partial hydrolyzates of proteins. Table 1 represents the constitution of the citrate buffer improved for the resolution of glucosamine and galactosamine. When the tryptophan, glucosamine and galactosamine mixture was chromatographed with this modified buffer as eluant, their retention times and dot numbers (corresponding to the peak width at half height) on the recording chart were ascertained to apparently be mutually different, as can be seen in Table 2. These differences are capable of being

Table 2. Retention time and width (at half height) of tryptophan, glucosamine and galactosamine peaks on the recording chart

| Material | Retention time (min) | Peak width at half height (dot number) |
|---|---|---|
| Tryptophan | 23.0 | 28.0±0.5 |
| Glucosamine | 31.5 | 20.9±0.7 |
| Galactosamine | 36.0 | 47.4±0.7 |

Chart speed: 9 inch/60 min, 15 dots required 1 min. Experiments were repeated 5 times, using the eluant buffer of pH 5.70, 0.05 M citrate, on a column of 0.9×7.5 cm.

utilized to identify tryptophan, glucosamine and galactosamine on the chromatogram of the partial hydrolyzate of glycoprotein. Subsequently, after the authentic glucosamine and galactosamine mixture was chromatographed, the recoveries of both the compounds were estimated. The results

are presented in Table 3. The recoveries of both the hexosamines were about 100% respectively, even when they were applied in such minute quantities as 0.020 $\mu$M of glucosamine and 0.019 $\mu$M of galactosamine.

Table 3. Recoveries of glucosamine and galactosamine when the mixture was chromatographed on a short column

| Exp. No. | Glucosamine | | | Galactosamine | | |
|---|---|---|---|---|---|---|
| | Applied amount ($\mu$M) | Found value ($\mu$M) | Recovery (%) | Applied amount ($\mu$M) | Found value ($\mu$M) | Recovery (%) |
| 1 | 0.100 | 0.101 | 101 | 0.093 | 0.092 | 99 |
| 2 | 0.100 | 0.100 | 100 | 0.037 | 0.036 | 98 |
| 3 | 0.100 | 0.100 | 100 | 0.019 | 0.019 | 100 |
| 4 | 0.040 | 0.039 | 98 | 0.093 | 0.091 | 98 |
| 5 | 0.020 | 0.020 | 100 | 0.093 | 0.091 | 98 |

Experiments were repeated 5 times. The chromatographic conditions are same as those below Table 2.

The hydrolyzates of quail's ovalbumin having a small amount of tryptophan[5] and the human $r$-globulin containing a fair amount of tryptophan[8] were separately chromatographed, using the modified buffer shown in Table 1. The elution profiles are represented in Fig. 7. Glucosamine could be observed to be a sharp peak in two elution diagrams, respectively, and galactosamine could not be observed. The peak eluted ahead of the glucosamine was identified as tryptophan from the retention time and the peak width at half height. The amount of tryptophan was ascertained to be smaller in the ovalbumin than in the $r$-globulin. The partial hydrolyzates of quail's ovalbumin hydrolyzed for 6, 8 and 10 hr were separately chromatographed. The elution diagrams of well-separated tryptophan and glucosamine are shown in Fig. 8. No variation in the emergence of glucosamine during the hydrolyzing time adopted was observed, whereas tryptophan was ascertained to be increasing in the 8 hr hydrolysis and to be decreasing in the 10 hr. Tryptophan is generally decomposed through acid hydrolysis for the amino acid determination, however, it was ascertained that tryptophan remains partially in mild acid hydrolysis for the hexosamine determination, and does not interfer with the resolution of hexosamines. From these results, it was presumed that the galactosamine-like material on the chromatogram for the hexosamine determination carried out by Fanger *et al.*[3] without pre-treatment was tryptophan. In such a case, the use of the buffer modified by the authors is assumed to give a satisfactory result without pre-treatment. Glucosamine amounts in some glycoproteins, which were determined by the Pearson's procedure followed with the Elson-Morgan method modified by Boas[1] and by the method with an amino acid analyzer, are represented in Table 4. Galactosamine was not detected in those glycoproteins. Excepting for the glucosamine content of the quail's ovalbumin, the contents of the other glycoproteins obtained by using an amino acid analyzer were ascertained to be approximately equal to those by the modified Elson-Morgan method[1].

When a short column (0.9×20 cm) packed to a height of 10 cm with the Yanagimoto SCX-1001 resin instead of the Aminex A-4 resin was employed, the elution pattern of hexosamines and amino acid mixtures was the same as that in Fig. 6. The retention time of amino acids was affected by the change of pH in the eluant buffer, while that of the amino sugar was scarcely affected.

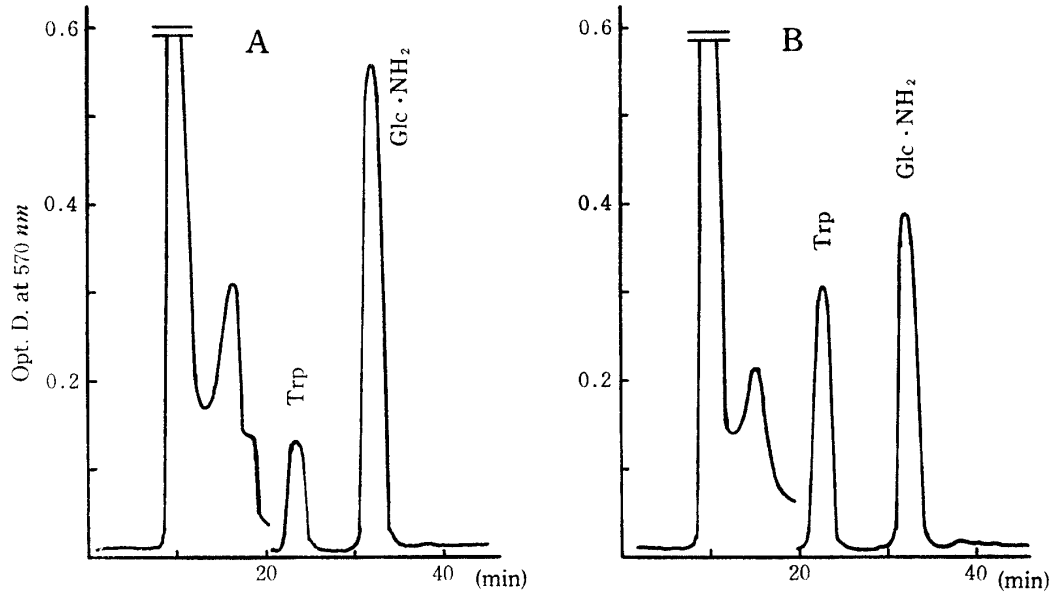


Fig. 7. Elution profiles of the hydrolyzates of quail's ovalbumin and human γ-globulin from a short column on the amino acid analyzer.

(A): Quail's ovalbumin hydrolyzed for 8 hr, at 100°C in 4 N HCl.
(B): Human γ-globulin hydrolyzed for 7 hr, at 100°C in 4 N HCl.
Eluant: 0.05 M citrate buffer, pH 5.70.

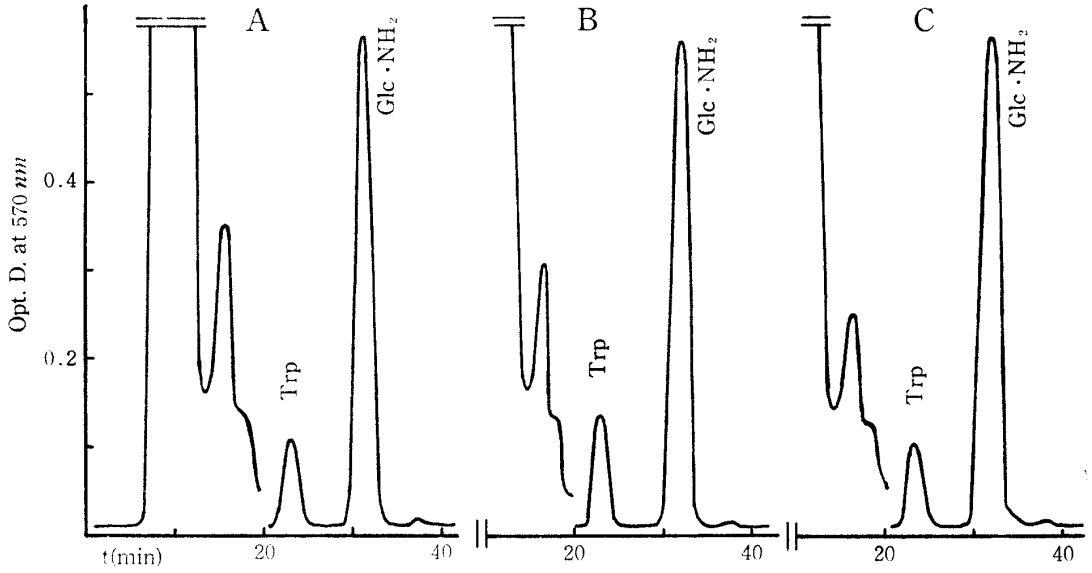


Fig. 8. Elution profiles of the hydrolyzates of quail's ovalbumin hydrolyzed for various time-units at 100°C.

Hydrolyzing time: (A) 6 hr, (B) 8 hr, (C) 10 hr.
Eluant: 0.05 M citrate buffer, pH 5.70.

Raising the pH value and decreasing the concentration of $Na^+$ appeared to be favorable for the resolution of hexosamines from amino acids. Murayama *et al.*[9] resolved the hexosamines successfully, using a citrate buffer having such a high concentration of $Na^+$ as, $Na^+$ 0.50 M (0.166 M citrate), which differed from the buffer used by the authors. It might be due to the superiority of their column (0.9×24 cm) length, compared with the column (0.9×7.5 cm) adopted by us.

Table 4. Glucosamine contents of several glycoproteins from avian egg whites

| Glycoprotein | Values by the modified Elson-Morgan method* | Values by the amino A. analyzer, using the modified buffer |
|---|---|---|
| Hen ovalbumin | 1.10 | 1.10 |
| Quail ovalbumin | 0.60 | 0.75 |
| Duck ovalbumin | 2.03 | 2.11 |
| Quail ovomucoid | 1.70 | 1.81 |
| Quail ovomucoid-like globulin | 2.90 | 3.08 |

* Elson-Morgan method modified by Boas[1]
Galactosamine was not detected in these glycoprotein hydrolyzates.

Peterson *et al.*[11] and Rosan *et al.*[15] consumed 240 min and 250 min for the resolution of hexosamines on a long column with an amino acid analyzer, and the columns employed were 0.9× 60 cm, respectively. Fauconnet *et al.*[4] and Cheng *et al.*[2] consumed an analytical time of 110 min, using the respective columns of 0.9×46 cm and 0.9×56 cm for the acidic and neutral amino acids. Murayama *et al.*[9] and Plummer[14] shortened the resolution time to about 60 min, using the respective short columns of 0.9×24 cm and 0.9×20 cm. The time for the resolution of hexosamines were further shortened by the authors: it was about 40 min. We recommend, therefore, the employment of the citrate buffer pH 5.70, $Na^+$ 0.15 M as shown in Table 1, for the resolution and quantitation of glucosamine and galactosamine in glycoproteins.

## Summary

This investigation was performed to simplify the determination of the glucosamine and galactosamine content of egg white glycoproteins. A rapid method for resolution and quantitation of hexosamines on a short column for basic amino acids with an amino acid analyzer have been developed, using the modified citrate buffer, pH 5.70, $Na^+$ 0.15 M and at 52°C of column temperature. The time consumed for the elution of galactosamine eluted behind glucosamine was about 40 min, and it was shorter than that hitherto published. Glucosamine contents in several avian ovalbumins and quail's ovomucoid estimated by this improved method were approximately equal to those obtained by the modified Elson-Morgan method.

This study was reported at the Meeting of the Nishi Nippon Division and the Kansai Division of the Agricultural Chemical Society of Japan, Naha, Okinawa, Jan. 10, 1974.

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