Table 1. *Molecular weights of digestion products from rabbit γ-globulins*

Rabbit γ-globulin was prepared by sodium sulphate fractionation of serum (Kekwick, 1940).

Source	Sample	Buffer	Refractive increment of protein solution	$S_{20,w}$	Molecular wt. ± S.D.
Rabbit γ-globulin (batch 1)	Whole globulin	Phosphate (pH 6·8; *I* 0·2)	0·00149	6·50 s	187 600 ± 1400
Rabbit γ-globulin (batch 2)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00147	3·59 s	49 600 ± 1800
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00138	3·55 s	52 600 ± 2600
	Fraction III	Acetate (pH 4·0; *I* 0·2)	0·00149	3·40 s	80 200 ± 9100
Rabbit γ-globulin (batch 3)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00132	—	50 400 ± 2000
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00118	—	55 700 ± 2000
	Fraction III	Acetate (pH 4·5; *I* 0·2)	0·00133	—	80 200 ± 1600

at the finite concentrations indicated should therefore not differ greatly from values which would be obtained by extrapolation to infinite dilution.

The molecular weights quoted in Table 1 were each derived by taking the mean value calculated from five photographic exposures made at 8- or 16-min. intervals. The standard deviations of individual values about the corresponding mean are given in the last column as a measure of internal consistency. Comparisons between means must in addition take into account the uncertainty residing in refractometer measurements, which amounted to about ±0·6%, but which could affect a molecular weight to the extent of about ±0·9%. Agreement between different measurements on corresponding fractions is considered to be reasonably good when allowance is made for these sources of variation. The only unsatisfactory set of measurements in Table 1 refers to fraction III at pH 4·0, where very erratic results were obtained, with some suggestion of a downward trend with increasing time. Moreover, there was a detectable asymmetry of the peak obtained in the ultracentrifuge at high speed, although the sedimentation coefficient of the maximum ordinate (3·40 s) was lower than for similar preparations at pH 4·5, which gave about 3·7 s. It was concluded that complications, which may include some aggregation and some molecular-shape change, make pH 4·0 unsuitable for investigation of fraction III.

The sum of the molecular weights of the three fractions is in good agreement with the molecular weight of whole γ-globulin, the figure for which corresponds fairly well with that most commonly accepted (170 000; see Porter, 1959).

REFERENCES

Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
Charlwood, P. A. (1952). *Biochem. J.* **51**, 113.
Charlwood, P. A. (1955). *Canad. J. Chem.* **33**, 1043.
Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Porter, R. R. (1959). In *The Plasma Proteins*. Ed. by Putnam, F. W. New York: Academic Press Inc.

The *N*-Acetylation and Estimation of Hexosamines

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(*Received* 5 *March* 1959)

N-Acetylglucosamine is usually determined by Morgan & Elson (1934) colour reaction, as modified by Aminoff, Morgan & Watkins (1952). A more recent modification by Reissig, Strominger & Leloir (1955) is greatly superior on grounds of sensitivity, convenience and reproducibility, and in addition is unaffected by small quantities of acid or alkali in the test sample. Glucosamine in relatively large amounts does not interfere in the Morgan–Elson reaction.

The standard procedure for the estimation of glucosamine calls for preliminary treatment with alkaline acetylacetone (Elson & Morgan, 1933; Rondle & Morgan, 1955); under the usual conditions *N*-acetylglucosamine interferes to some extent. Interference by other tissue constituents is greater than in the *N*-acetylglucosamine determination. Conversion of glucosamine into *N*-acetylglucosamine as a prelude to assay by the Morgan–Elson reaction offers certain advantages

(Smithies, 1953; Pontis, 1955; Roseman & Daffner, 1956), not least that *N*-acetylglucosamine can be distinguished from *N*-acetylgalactosamine by the intensity of the colour produced.

There is some confusion on the subject of *N*-acetylation of glucosamine (see Kent & Whitehouse, 1955). *N*-Acetylglucosamine was first prepared by treating the free base with acetic anhydride in methanol (Breuer, 1898), but because of the alleged instability of the base the usual method of preparation employs glucosamine hydrochloride suspended in methanol and a mixture of silver acetate and acetic anhydride (Zuckerkandl & Messiner-Klebermass, 1931; White, 1940).

Selective *N*-acetylation of galactosaminic acid can be accomplished by adding acetic anhydride to a solution in excess of aqueous alkali (Karrer & Mayer, 1937), and this reaction has been extended to glucosaminic acid (Findlay, Levvy & Marsh, 1958). It therefore appeared probable that acetic anhydride could be employed to *N*-acetylate glucosamine in aqueous solution in the absence of excess of alkali, there being no question of zwitterion formation. [In this connexion one should a mention the use of keten for the selective acetylation of the amino group in glucosamine derivatives in aqueous solution (Neuberger & Pitt-Rivers, 1939; Weissmann & Meyer, 1954).]

Methods for the *N*-acetylation of glucosamine hydrochloride in aqueous solution with acetic anhydride have already been described: a mildly alkaline pH was maintained throughout, on the preparatory scale by adding a large quantity of a basic ion-exchange resin (Roseman & Ludowieg, 1954), and on the analytical scale with excess of sodium bicarbonate (Roseman & Daffner, 1956). It is not clear whether alkali is added to prevent *O*-acetylation, or to act as an 'acid acceptor' in hastening the reaction.

Glucosamine can be quantitatively converted into *N*-acetylglucosamine in a few minutes, simply by adding acetic anhydride to aqueous solutions of the amino sugar, which are more stable than is generally believed. When the hydrochloride is employed, sodium hydroxide is added on the preparatory scale, but this is unnecessary on the analytical scale, since the solution is made sufficiently alkaline during chromogen formation. The reaction mixture can be taken directly for analysis by the method of Reissig *et al.* (1955), and this procedure can be applied to galactosamine also.

EXPERIMENTAL AND RESULTS

Estimation of N-*acetylglucosamine*

It is not clear from the description of Reissig *et al.* (1955) whether the molarity of their 0·8 M-potassium tetraborate reagent refers to BO_3^{2-} or $B_4O_7^{2-}$, but from our own experiments it would appear to refer to the latter. They obtained optimum colour development when the final $B_4O_7^{2-}$ concentration during chromogen formation was 0·133 M, but as shown in Fig. 1 we required 0·29 M-$B_4O_7^{2-}$ at this stage. Stock solutions of potassium tetraborate were made 0·7 M, this being the limit of solubility at room temperature. The salt was prepared as described by Reissig *et al.* (1955), and the pH of the stock solution was 9·2. A commercial specimen of potassium tetraborate gave a considerably higher pH (about 9·8) and, even after pH adjustment with HCl, led to lower readings in the colour reaction.

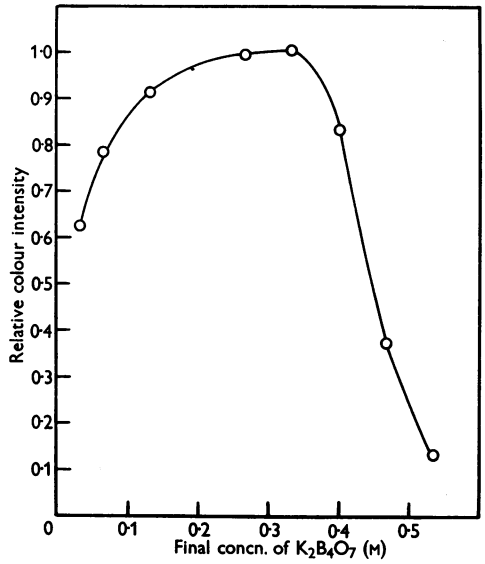

Fig. 1. Intensity of colour produced by *N*-acetylglucosamine (0·181 μmole) with varying concentrations of tetraborate during chromogen formation; the remainder of the procedure was as described in the text.

A standard curve was constructed by adding 0·5 ml. of 0·7 M-potassium tetraborate to 0·7 ml. of *N*-acetylglucosamine and following the procedure of Reissig *et al.* (1955): the results, which were perfectly reproducible from day to day, fell on the linear curve shown in Fig. 3 for glucosamine hydrochloride. Glucosamine in amounts up to 4·6 μmoles did not interfere in the colour reaction for *N*-acetylglucosamine.

Estimation of hexosamine

Standard procedure. The tube shown in Fig. 2 was convenient for carrying out the reaction. To 0·6 ml. of hexosamine solution (hydrochloride or free base) was added 0·1 ml. of 1·5% (v/v) acetic anhydride in acetone (prepared daily), followed by 0·5 ml. of 0·7 M-potassium tetraborate. A glass bulb was placed in the mouth of the tube, which was heated in a boiling-water bath for exactly 3 min. After cooling the tube, 6 ml. of the *p*-dimethylaminobenzaldehyde reagent of Reissig *et al.* (1955) was added. [AnalaR *p*-dimethylaminobenzaldehyde (British Drug Houses Ltd.) was entirely satisfactory.] The tube was closed with a glass stopper and the contents were mixed. Colour development was carried out for 20 min. at 37°, the glass stopper being momentarily released to equilibrate pressures. Colour

intensities were read almost immediately after cooling, with the Spekker photoelectric absorptiometer, Ilford no. 605 yellow–green filters (peak transmission at 545 mμ) and cells of 1 cm. length.

The total volume of the aqueous and acetone solutions was not appreciably altered after mixing, nor was there any change in volume after heating the mixture. Acetic anhydride in the quantity specified did not significantly alter the pH of the borate buffer. Pure *N*-acetylglucosamine was unaffected by the acetylation procedure.

Calibration curves for glucosamine hydrochloride and galactosamine hydrochloride are shown in Fig. 3. The specimen of galactosamine hydrochloride was presented by Dr P. G. Walker. It gave a colour intensity which was 35% of the value for glucosamine hydrochloride. This is in exact agreement with the figure obtained by Reissig *et al.* (1955) for *N*-acetylgalactosamine as compared with *N*-acetylglucosamine. Glucosamine itself gave readings identical with those for the hydrochloride.

The method is 2–3 times as sensitive as the acetylacetone method (Rondle & Morgan, 1955) for glucosamine; galactosamine gives the same intensity of colour as glucosamine in the latter procedure.

Stability of aqueous acetic anhydride. Fig. 4 shows the loss in efficacy of the standard acetic anhydride reagent in contact with water and glucosamine hydrochloride solutions. The acetone solution (0·1 ml.) was measured into 0·4 ml. of water and after varying periods 0·2 ml. of glucosamine hydrochloride solution (0·186 μmole) was added. After 1 or 15 min. the borate buffer was added and colour development was proceeded with in the usual way. It is evident that the interval before addition of borate should not exceed 15 min. There was a steep fall in the efficiency of the *N*-acetylation when the period of contact of glucosamine or glucosamine hydrochloride with acetic anhydride exceeded 20 min. before addition of borate.

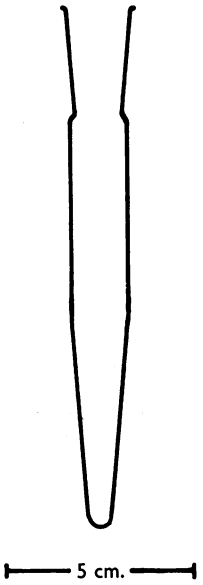

Fig. 2. Reaction tube for complete estimation, with B14 socket.

Stability of glucosamine base in aqueous solution. Very dilute solutions of glucosamine (0·93 mM, natural pH 8·5) were stable for several days at room temperature, as tested by the colour reaction, and could be maintained at 100° for 3 min. without decomposition (Table 1). More concentrated solutions (0·46 M, natural pH 9·5) showed no loss after 3 hr. and only 6% after 24 hr., but were rapidly destroyed at 100°. The more dilute solution, on being brought to pH 9·5 with NaOH, assumed the stability characteristics of the more concentrated solution.

Fig. 5 shows the rapid decay of a dilute solution of glucosamine hydrochloride in the presence of the borate buffer at room temperature before adding the acetic anhydride reagent: *N*-acetylglucosamine was completely stable under these conditions for 5 hr.

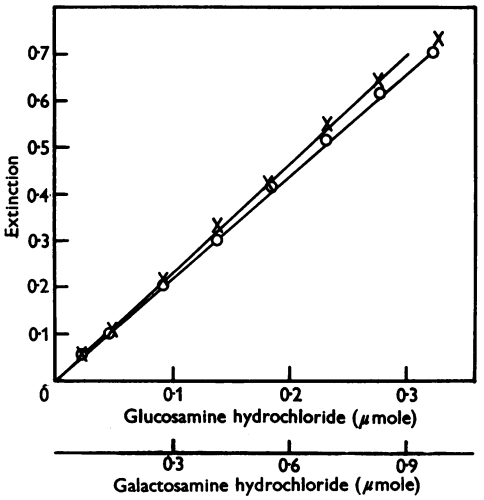

Fig. 3. Calibration curves for glucosamine hydrochloride (○) and galactosamine hydrochloride (×).

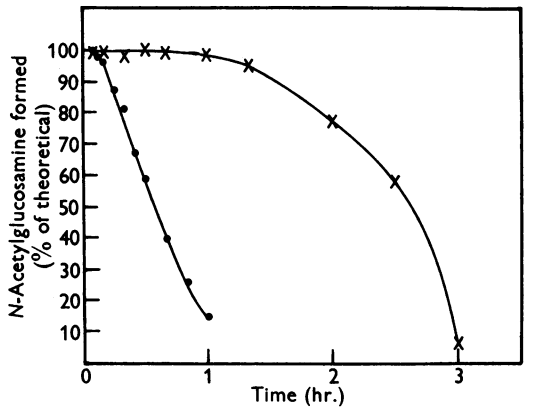

Fig. 4. Decay of acetic anhydride in aqueous solution as measured by the formation of *N*-acetylglucosamine; glucosamine hydrochloride was added after the intervals shown, and estimation proceeded with as described in the text. Borate buffer was added 1 min. (×) and 15 min. (●) after the glucosamine hydrochloride.

Table 1. *Stability of glucosamine base in aqueous solution*

NaOH was used for pH adjustment.

Concn. of glucosamine	pH	Temp.	Time	*N*-Acetylglucosamine (% of theoretical)
0·93 mM	8·5	Room temp.	4 days	98
		100°	3 min.	97
	9·5	Room temp.	3 hr.	98
			1 day	91
		100°	3 min.	52
	10·5	Room temp.	3 min.	98
0·46 M	9·5	Room temp.	3 hr.	100
			1 day	94
		100°	3 min.	68
	10·5	Room temp.	3 min.	98

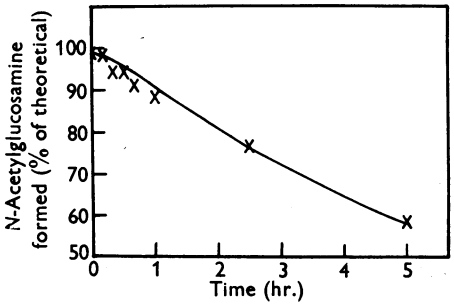

Fig. 5. Decay of glucosamine hydrochloride (0·186 μmole in 1·1 ml.) in the presence of the borate buffer. The acetic anhydride reagent was added after the intervals shown, and the tubes were then heated for chromogen development in the usual way.

Effect of varying the acetic anhydride concentration in the presence and absence of alkali. Fig. 6 shows the effect of varying the amount of acetic anhydride added during the standard assay procedure. The use of free glucosamine instead of the hydrochloride gave identical results. Addition of NaOH in low concentration did not diminish the amount of acetic anhydride required for complete development of colour (Fig. 7). Although esterification was incomplete, there was, however, greater acetylation of the amino group with low concentrations of acetic anhydride when alkali was added. Excess of NaOH relative to the acetic anhydride prevented the acetylation.

Effect of varying the pH *of the borate buffer.* Solutions of glucosamine hydrochloride were treated with acetic anhydride in the usual way, and then, after 1 min., with borate buffer of varying pH: 30 min. later the tubes were heated for chromogen formation. As shown in Table 2, replacing the standard borate buffer by one at pH 8 did not affect the result, but the use of a buffer at pH 10 led to much less development of colour. If the tubes were brought to pH 9·1 with NaOH or HCl immediately before heating, the same colour was seen in all. In other words, acetylation proceeded equally well at all three pH values, but chromogen formation was diminished at pH 10. Control experiments in which glucosamine hydrochloride was added last showed that no effective concentration of

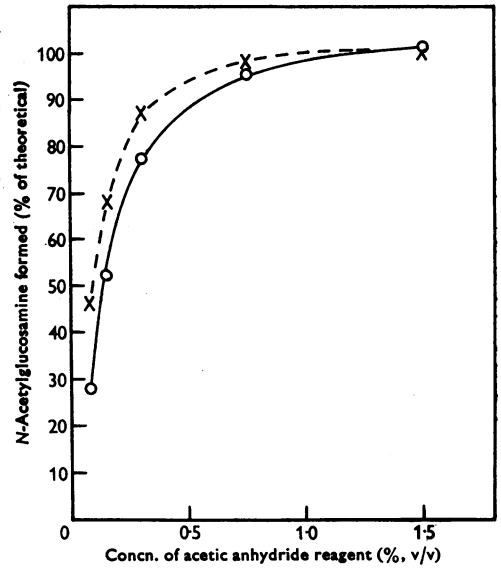

Fig. 6. Effect of varying the concentration of the acetic anhydride reagent in acetone; 0·1 ml. at the concentrations shown was added to glucosamine hydrochloride (0·186 μmole) in 0·6 ml. of water, and the estimation was continued as described in the text. ×, Borate added after 1 min.; ○, borate added after 15 min.

acetic anhydride remained after 30 min. contact with any of the borate buffers.

Similar experiments with *N*-acetylglucosamine (the addition of acetic anhydride being omitted) gave almost identical results. Reissig *et al.* (1955) found a sharp maximum in chromogen formation from *N*-acetylglucosamine in borate buffer at pH 9. Aminoff *et al.* (1952) found that borate buffers ranging from pH 9 to 11 were equally effective.

Preparation of N-*acetylglucosamine*

From glucosamine hydrochloride. At the amino sugar concentrations employed on the preparatory scale, it was possible to reduce the amount of acetic anhydride to only

a little more than the theoretical. Under these conditions, it was necessary to add NaOH to the hydrochloride. The effect of varying the amount of NaOH is shown in Fig. 8. For the purposes of isolation, 1–1·5 equiv. of NaOH was employed.

The preparation was carried out as follows. Glucosamine hydrochloride (10 g.) was dissolved at room temperature in water (60 ml.) containing 1 equiv. of NaOH. Acetic anhydride (7·5 ml.) was added, and after standing for 20 min. with occasional shaking the mixture was evaporated to dryness under reduced pressure. The white, crystalline residue was taken up in hot absolute methanol (400 ml.), the solution filtered, and the volume of filtrate reduced to 200 ml. Ether (600 ml.) was slowly added to precipitate a product (9·6 g.), m.p. 186–188°, containing Cl^- ion. It was dissolved in the minimum volume of hot water, 9 vol. of hot ethanol was added, and the hot mixture was filtered. The product that crystallized (6·8 g.), m.p. 193–195°, contained only a trace of Cl^- ion. Recrystallization from 75% (v/v) aqueous methanol yielded a chloride-free product (6·0 g.), m.p. and mixed m.p. with an authentic specimen 198–200° (corr.), $[\alpha]_D^{23}$ +73° → +37° in water (c, 1) (Found: $CH_3 \cdot CO^-$, 18·8. Calc. for $C_8H_{15}O_6N$: $CH_3 \cdot CO^-$, 19·4%). A second crop (0·6 g.), m.p. 196–198°, was obtained from the mother liquor. The pure product gave a single spot on a paper chromatogram (solvent, butanol–pyridine–water; spray, alkaline silver nitrate). Passage through acid ion-exchange resins or mixtures of acid and basic resins did not elevate the m.p. Recent literature gives m.p.'s ranging from 196° to 210° for *N*-acetylglucosamine; these may reflect differing proportions of the α- and β-anomers.

From glucosamine. Glucosamine was prepared from the hydrochloride by the simple and elegant method of Breuer (1898). The product melted at 116° (corr.) and was evidently largely in the β-form (Westphal & Holzmann, 1942). It was quite stable if stored dry in the dark.

The base (2·5 g.) in water (17·5 ml.) was treated with acetic anhydride (2·25 ml.), and the product was precipitated from methanol with ether as described above (2·31 g., m.p. 193–195°) and crystallized from 75% aqueous methanol. Yield 2·13 g., m.p. 198–200° (corr.).

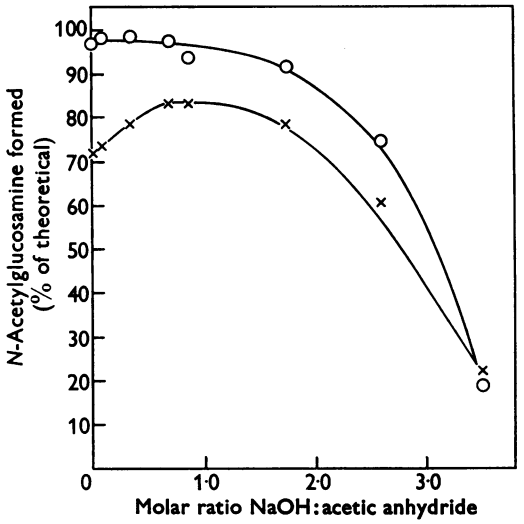

Fig. 7. Effect of adding varying proportions of NaOH during the acetylation of glucosamine hydrochloride (0·186 μmole). ○, Concentration of acetic anhydride reagent 0·75% (v/v) (8 μmoles employed); ×, concentration of acetic anhydride reagent 0·15% (v/v) (1·6 μmoles employed). Borate was added 1 min. after acetic anhydride.

Table 2. *Effect of varying the* pH *of the borate buffer during acetylation and chromogen formation*

Glucosamine hydrochloride (0·186 μmole) was treated with 1·5% (v/v) acetic anhydride solution and 0·7 M-$B_4O_7^{2-}$ solution in the usual way, but the borate solution was of varying pH. An interval of 30 min. was allowed to elapse before heating, and in the second experiment all the tubes were brought to the same pH (9·1) at this point.

Expt.	pH of borate buffer	Colour intensity (% of theoretical)
1	8·1	97
	9·2	97
	10·0	23
2	8·1	98
	9·2	99
	10·0	99

DISCUSSION

It would appear that fairly concentrated aqueous solutions of glucosamine are sufficiently alkaline for *N*-acetylation with acetic anhydride to proceed in

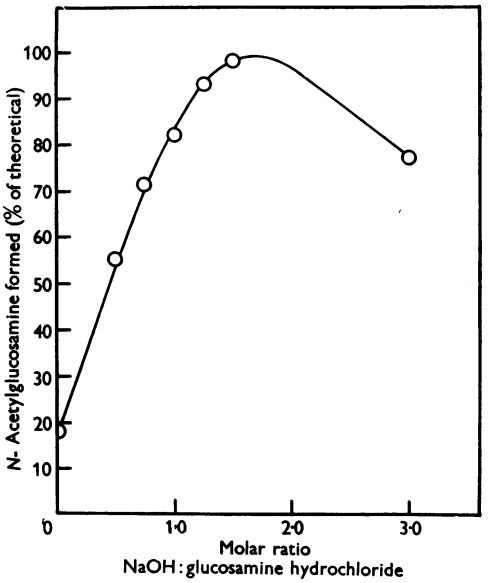

Fig. 8. Effect of adding varying proportions of NaOH during the acetylation of glucosamine hydrochloride on the preparatory scale; 100 mg. of glucosamine hydrochloride in 1·0 ml. of aqueous alkali was treated with 0·075 ml. of acetic anhydride (1·7 mol. prop.) for 15 min., and *N*-acetylglucosamine determined as described in the text.

the absence of added alkali. With more dilute solutions and with solutions of the hydrochloride it is necessary to add alkali. For purposes of estimation by the colorimetric method of Reissig *et al.* (1955) for *N*-acetylglucosamine, the borate buffer employed for chromogen formation is sufficiently alkaline. If present in molar excess, relative to acetic anhydride, sodium hydroxide prevents acetylation by competition with the amino group.

Acetylation of the amino group in glucosamine with acetic anhydride in aqueous solution seems to be almost instantaneous. In their method for the estimation of hexosamine, based on this reaction, Roseman & Daffner (1956) employ sodium bicarbonate as the catalyst. After 10 min. at room temperature, excess of acetic anhydride is destroyed by heating to 100°. Sodium carbonate is then added for the determination of *N*-acetylglucosamine by the procedure of Aminoff *et al.* (1952).

SUMMARY

1. Glucosamine and galactosamine can be determined by the method of Reissig, *et al.* (1955) for the *N*-acetylated hexosamines by preliminary treatment in aqueous solution with acetic anhydride. The borate buffer employed for chromogen formation catalyses the *N*-acetylation of the hexosamines.

2. *N*-Acetylglucosamine can be prepared from glucosamine base by treatment of aqueous solutions with acetic anhydride. If glucosamine hydrochloride is employed alkali must also be added.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Breuer, R. (1898). *Ber. dtsch. chem. Ges.* **31**, 2193.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Findlay, J., Levvy, G. A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 467.
Karrer, P. & Mayer, J. (1937). *Helv. chim. acta*, **20**, 407.
Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Aminosugars*, p. 215. London: Butterworth.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Neuberger, A. & Pitt-Rivers, R. V. (1939). *J. chem. Soc.* p. 122.
Pontis, H. G. (1955). *J. biol. Chem.* **216**, 195.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Roseman, S. & Daffner, I. (1956). *Analyt. Chem.* **28**, 1743.
Roseman, S. & Ludowieg, J. (1954). *J. Amer. chem. Soc.* **76**, 301.
Smithies, W. R. (1953). *Biochem. J.* **53**, xxix.
Weissmann, B. & Meyer, K. (1954). *J. Amer. chem. Soc.* **76**, 1753.
Westphal, O. & Holzmann, H. (1942). *Ber. dtsch. chem. Ges.* **75**, 1274.
White, T. (1940). *J. chem. Soc.* p. 428.
Zuckerkandl, F. & Messiner-Klebermass, L. (1931). *Biochem. Z.* **236**, 19.

Studies on the Phosphoproteins of Brain: the Intracellular Localization in Brain of a Phosphoprotein Involved in the Metabolic Response of Cortical Slices to Electrical Stimulation

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(*Received* 30 *October* 1958)

The application of electrical pulses for 10 sec. to slices of cerebral cortex which had metabolized radioactive phosphate for a few minutes resulted in marked changes in the distribution of radioactivity in phosphocreatine, adenosine triphosphate and in the phosphoprotein fraction (Heald, 1956, 1957). Under these conditions, the specific radioactivity of phosphocreatine and the γ-phosphorus of adenosine triphosphate decreased (Heald, 1956), whereas that of the phosphoprotein phosphorus increased (Heald, 1957). Subsequently it was found that acid hydrolysis of the radioactive phosphoprotein fraction yielded only radioactive phosphorylserine (Heald, 1958).

Sequences illustrating ways in which such changes in phosphates may be induced by electrical pulses have been outlined by McIlwain & Gore (1953) and Heald (1956). Briefly, pulses are considered to alter structures involved in the transport of ions from one membrane surface to another, and the accompanying changes induced in the phosphates are considered to reflect the increased