STUDIES ON CHITIN

II. REACTION of *N*-ACETYL-D-GLUCOSAMINE WITH α-AMINO ACIDS, PEPTIDES, AND PROTEINS

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Summary

The reaction of *N*-acetyl-D-glucosamine with α-amino acids, peptides, and proteins has been investigated. The course of the reaction has been followed by methods which involved the use of paper partition chromatography, polarimetric measurements, measurement of pH depression, and equilibrium dialysis. It is considered that products of the Schiff base (or azomethine) type are formed but the combination is unstable and is split even in the biological pH range. No reaction occurs under acidic conditions but under alkaline conditions the amount of reaction increases with the pH. At a given pH the percentage combination depends upon the equilibrium constant of the reaction and upon the dissociation constant of the α-amino nitrogen, as this determines the initial concentration of the reagent. Differences in the degrees of ionization are more important in determining the percentage combination than are the differences in the equilibrium constants. The reaction is of a similar type to that which occurs between glucose and α-amino acids or peptides. Tyrosine, as well as free α-amino groups, appears to play an important part in the binding of *N*-acetyl-D-glucosamine by insect cuticular proteins. 2-Acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester has been synthesized from *N*-acetyl-D-glucosamine and glycine ethyl ester and its properties have been investigatd.

I. INTRODUCTION

The arthropod cuticle is generally thought to contain a glycoprotein resulting from the combination of chitin and protein. The nature of this association is not clear and it is not known whether such an association is useful in the insect's physiology or whether it is the result of the mechanism of chitin synthesis. Although the nature of the bonding is unknown a number of conclusions can be drawn from published papers.

The data obtained from X-ray diffraction (Fraenkel and Rudall 1947) and electron microscope (Richards and Korda 1948) studies on chitin micelles have been interpreted as indicating the presence of a weak bonding between chitin and protein in the soft cuticles of insects. This idea of a weak bonding is supported by the fact that the major part of the protein in soft cuticles can be separated from the chitin by relatively gentle methods, e.g. extraction with water and buffer solutions (Hackman 1953*a*, 1953*b*; Trim 1941). On the other hand Trim (1941) has shown that the puparia of blowflies (*Sarcophaga falcu-*

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lata) can be dispersed in lithium thiocyanate at 170°C and reprecipitated without effecting a separation of the chitin and protein components. This suggests that in blowfly puparia there exists quite a strong bond between the chitin and protein. It can be concluded from these papers that, in insect cuticle, there is probably some chitin-protein bonding and that the bonding in hardened cuticle is much stronger than that in soft cuticles.

Although no work has been published on the reaction between amino acids or peptides and *N*-acetyl-D-glucosamine (the recurring chemical unit of chitin) reference has been made to the reaction of *N*-acetyl-D-glucosamine with "dry" casein (pH 6·3, 37°C, and 70 per cent. R.H.) (Lea and Rhodes 1952). The reaction between the protein and *N*-acetyl-D-glucosamine was very slow when compared with the reaction of other carbohydrates, e.g. glucose and "dry" casein under similar conditions. The authors suggested that the slowness of the reaction may be due to the acetamido group inhibiting reaction at the terminal carbonyl group. In contrast to this lack of information on the reaction between amino acids and *N*-acetyl-D-glucosamine are the extensive investigations which have been carried out on the mechanism of the reaction between amino acids and aldose sugars such as glucose.

II. Experimental

(*a*) *Reagents*

The *N*-acetyl-D-glucosamine was a commercial preparation which was crystallized from a mixture of water, ethanol, and ether. It had m.p. 205°C (decomp.) and $[\alpha]_D^{24.9}$ 40·4° (*c*, 8·98 in water).

The amino acids and peptides used were shown to be single identities by paper chromatography and before use they were dried to constant weight.

The protein used was the water soluble protein extracted from larval cuticles of *Diaphonia dorsalis* Don. (Coleoptera). For a description of the method of extraction see Hackman (1953*a*). The protein contained 13·9 per cent. N (micro-Kjeldahl).

(*b*) *Paper Partition Chromatography*

Lysine monohydrochloride (900 mg) and *N*-acetyl-D-glucosamine (2·21 g) were dissolved in a small quantity of water and the pH of the solution adjusted to approx. 10 with 7·5N aqueous sodium hydroxide. The total volume of the solution was 4·5 ml, which gave 1M lysine and 2M *N*-acetyl-D-glucosamine. The solution was kept at 37°C for 6 days, when the pH of the solution had dropped considerably and the formerly colourless solution had become reddish brown. The solution was diluted 75 times with water and subjected to paper partition chromatography using *n*-butanol-acetic acid-water (76-6-17 v/v) as the solvent and Whatman No. 1 filter paper at a temperature of 25°C. Besides spots corresponding to lysine and *N*-acetyl-D-glucosamine there appeared a spot between the origin and lysine which was detected with ninhydrin.

In a similar manner a solution containing glycine (1M) and *N*-acetyl-D-glucosamine (2M) at an initial pH of 9·5 when kept at 37°C for 7 days gave, after dilution with water and paper partition chromatography, a spot reacting with ninhydrin between the origin and the spot given by glycine. As before the final pH of the solution was less than 9·5 and the solution had become dark in colour.

The reaction products detected above are not stable on dilution with water. If any appreciable time elapses between diluting the solution with water and applying the diluted solution to the paper no additional spot is detected.

(c) Synthesis of 2-Acetamido-2-deoxy-N'-D-glucosylglycine Ethyl Ester

Freshly prepared glycine ethyl ester (2·8 g) was added to a suspension of anhydrous *N*-acetyl-D-glucosamine (6·0 g) (molar ratio 1 : 1) in magnesium-dried ethanol (15 ml). The mixture was boiled under reflux on a steam-bath for 5 hr and protected from moisture by a calcium chloride tube. Unreacted *N*-acetyl-D-glucosamine (2·07 g) was separated from the cooled solution by filtration and washed with absolute ethanol. The combined filtrates were concentrated to *c.* 5 ml, acetone (15 ml) added, and kept in a refrigerator overnight. The crystals which separated were collected by filtration, pressed well on the Büchner funnel, washed with ethanol-acetone (1-3; 5 ml), and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 3·1 g, m.p. 114°C. Repeated crystallization from absolute ethanol raised the m.p. to 141°C. (Found: C, 46·8; H, 7·1; N, 9·1 %. Calc. for $C_{12}H_{22}O_7N_2$: C, 47·0; H, 7·2; N, 9·1%).

The stability of 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester was determined by measuring the change with time in the pH of 0·001M solutions in water and 0·005M solutions in 0·005N aqueous hydrochloric acid. For purposes of comparison the change with time in the pH of an equimolar (0·001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl ester was determined.

(d) Stability of N-acetyl-D-glucosamine at Different Temperatures and pH

Since *N*-acetyl-D-glucosamine is unstable under alkaline conditions the relative amounts of degradation in 0·2M aqueous solutions at pH 8·0, 9·0, and 9·55 (buffers as for polarimetric experiments—see(*e*)) at 25°C and pH 9·0 at 37°C were determined as follows.

N-acetyl-D-glucosamine solution (1 ml) was diluted with glacial acetic acid, dimethylaminobenzaldehyde solution (1 ml of a 2 per cent. solution in glacial acetic acid containing 5 per cent. 10N hydrochloric acid) added, and the volume made up to 10 ml with glacial acetic acid. After 45 min the optical density of the solution was measured at 560 mμ. Water (1 ml) was used for preparation of the blank solution. Measurements were made of the amount of colour developed in buffer solution of *N*-acetyl-D-glucosamine which had been kept for varying periods up to 24 hr.

(*e*) *Polarimetric Measurements*

Glycine and glycylglycine were chosen for study by the polarimetric method because they are optically inactive and so the complications due to the change in the rotatory power of the amino acid or peptide itself were eliminated. The reactions were carried out at a constant pH, i.e. in buffer solution.

The glycine or glycylglycine was dissolved in water and 1N aqueous sodium hydroxide added to bring the solution to the desired pH, and then the solution was made up to volume with buffer (for pH 9·0 and 9·5 a carbonate-bicarbonate buffer (Delory and King 1945) and for pH 8·0 a veronal buffer (Michaelis 1930)). Final concentrations of glycine and glycylglycine were 0·4M. The *N*-acetyl-D-glucosamine (0·4M) was made up in buffer. Equal volumes of the *N*-acetyl-D-glucosamine and glycine or glycylglycine were mixed and for the purposes of control an equal volume of each was mixed with an equal volume of buffer. The reaction was allowed to proceed at the various pH values and various temperatures until equilibrium was reached, the progress of the reaction being followed by the decrease in optical rotation of the solution. In most experiments the molar ratio of the reactants was 1 : 1 but in one experiment this was altered to 1 mole glycine to 0·424 mole *N*-acetyl-D-glucosamine. As a check on the method, the amount of unreacted *N*-acetyl-D-glucosamine was determined iodometrically using the method for glucose as published by Dykins and Englis (1931). The method of Morgan and Elson (1934) could not be used to estimate the *N*-acetyl-D-glucosamine because under the hot alkaline conditions used further reaction would take place between the *N*-acetyl-D-glucosamine and the amino acid or peptide.

(*f*) *Measurement of pH Depression*

The amino acid or peptide was dissolved in water, the solution brought to pH 9·0 by the addition of 1N aqueous potassium hydroxide, water added to bring the solution to the desired concentration, and the pH again checked. In this manner 0·2M solutions of glycine, L-tyrosine, and L-lysine and 0·1M solutions of glycylglycine and L-leucylglycine were prepared. The *N*-acetyl-D-glucosamine (2M) was dissolved in water. Equal volumes of the *N*-acetyl-D-glucosamine and amino acid or peptide solutions were mixed. The progress of the reaction was followed by measuring the difference between the pH of the carbohydrate-amino acid or peptide solution and the pH of the amino acid or peptide solution after it had been diluted once with water. The pH of a 2M aqueous *N*-acetyl-D-glucosamine solution remained constant. The experiments with amino acids were conducted at 18°C, those with peptides at 22°C. All measurements were made on a Cambridge pH meter.

(*g*) *Iodination of Insect Cuticular Protein*

Larval cuticular protein (200 mg) was dissolved in aqueous ammonia (sp.gr. 0·88; 2 ml) and the solution cooled in an ice-bath. An aqueous solution of iodine in potassium iodide (2·8N; 0·2 ml) was added gradually to the vigorously stirred protein solution maintained at 0°C. After 1 hr water was

added, the solution neutralized with glacial acetic acid, and the precipitate collected by centrifugation. The precipitate was supended in a small volume of water and dialysed against running water for 3 days and against distilled water for 2 days. The precipitate and solution was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 165 mg. (Found: N, 12·0 % (micro-Kjeldahl)).

(*h*) *Acetylation of Insect Cuticular Protein*

Larval cuticular protein (200 mg) was suspended in half-saturated aqueous sodium acetate (4 ml). Acetic anhydride (0·3 ml) was added during 1 hr to the vigorously stirred protein suspension maintained at 0°C. The suspension was diluted with water and dialysed against running water for 3 days and against distilled water for 2 days. The precipitate and solution was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide and potassium hydroxide. Yield: 154 mg. (Found: N, 13·7 % (micro-Kjeldahl)).

(*i*) *Interaction of* N-*Acetyl-*D-*glucosamine with Insect Cuticular Protein*

Cellophane bags were prepared from commercial sausage casing (24/32) and filled with 10 ml of protein solution (0·19 per cent. in carbonate-bicarbonate buffer (vide supra) pH 8·77 at 37°C). Three drops of toluene were added as a preservative. The bag was immersed in 20 ml of a solution of *N*-acetyl-D-glucosamine (concentration range 0·233 to 1·953 mM) in the same buffer contained in a tube 1·125 in. internal diameter. Five drops of toluene were added as a preservative. The tube was closed with a rubber stopper and kept at 37°C for 3 days, by which time equilibrium had been attained. The external solution was analysed for *N*-acetyl-D-glucosamine by a slightly modified form of the method of Morgan and Elson (1934). To develop maximum colour 0·3 ml 0·5N aqueous sodium carbonate solution was added for each ml of buffer solution and the colour intensity was read after 100 min instead of 45 min. For each concentration of *N*-acetyl-D-glucosamine a control tube was prepared which differed from the test tube in that the cellophane bag contained 10 ml buffer solution and not the protein solution. Analysis of the internal and external solutions of the control tube for *N*-acetyl-D-glucosamine ensured that equilibrium had been reached. From the difference in concentration of *N*-acetyl-D-glucosamine in the external solutions of the control and test-tubes the amount of carbohydrate taken up by the protein could be calculated. By this method of using control and test-tubes it was possible to correct automatically for any adsorption of *N*-acetyl-D-glucosamine by the cellophane bag. No adsorption was detected. All experiments were conducted in duplicate.

This same experiment was repeated using the iodinated and acetylated proteins, using an equivalent protein concentration.

III. Results and Discussion

From the filter paper partition chromatographic study of the reaction between amino acids and *N*-acetyl-D-glucosamine it is clear that an unstable condensation product is formed with glycine and lysine. The results obtained,

other than the instability of the compounds formed, are similar to those obtained by Gottschalk and Partridge (1950) for the reaction between glucose and lysine, and suggest that a similar type of reaction has occurred, namely, that initially compounds of the Schiff base (or isomeric *N*-glycoside) type are formed. The decrease in the pH of the solution indicates that reaction has occurred at the basic group of the amino acid. It is to be expected that a quantitative measure of the extent of the reaction can be obtained from the magnitude of the decrease in pH.

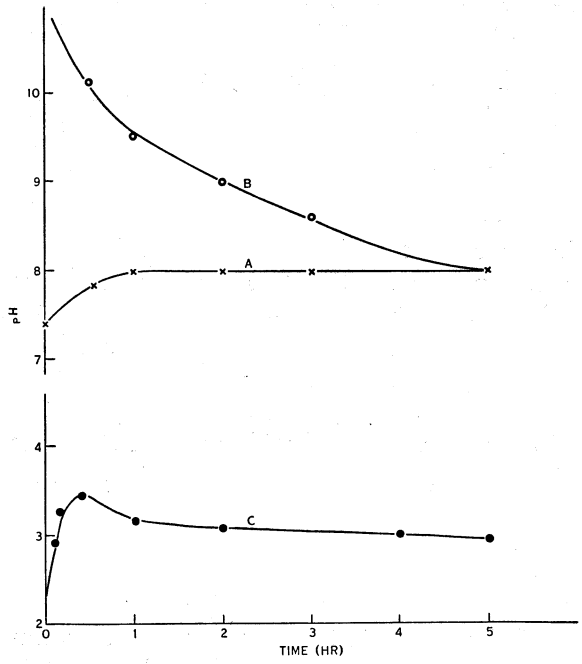


Fig. 1.—Stability (change in pH) at 23°C of 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester in water (0·001M; curve *A*) and to aqueous hydrochloric acid (0·005M in 0·005N acid; curve *C*). Curve *B* records the change in pH of a dilute aqueous solution of an equimolar (0·001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl ester.

All attempts to isolate condensation products from mixtures of amino acids and *N*-acetyl-D-glucosamine in aqueous or in anhydrous alcoholic solutions were unsuccessful. However, 2-acetamido-2-deoxy-*N'*-D-glucosylglycine ethyl ester was prepared from glycine ethyl ester and *N*-acetyl-D-glucosamine in anhydrous ethanol. The compound had zero optical activity when dissolved in anhydrous ethanol. As is shown in Figure 1, a dilute aqueous solution (0·001M) of this *N*-glycoside undergoes hydrolysis at room temperature (23°C), the pH rising from 7·40 to 8·00 in 2 hr (curve *A*). The pH of a dilute aqueous solution of an equimolar (0·001M) mixture of *N*-acetyl-D-glucosamine and glycine ethyl

ester gradually falls from 10·34 to 8·00 during 5 hr (curve *B*). These two pH-time curves finally merge (Fig. 1) and these results are those which would have been predicted on the assumption that the *N*-glycoside hydrolyses partially to *N*-acetyl-D-glucosamine and glycine ethyl ester. Towards mineral acid the *N*-glycoside is very unstable at 23°C, as is shown by curve *C* in Figure 1. Not

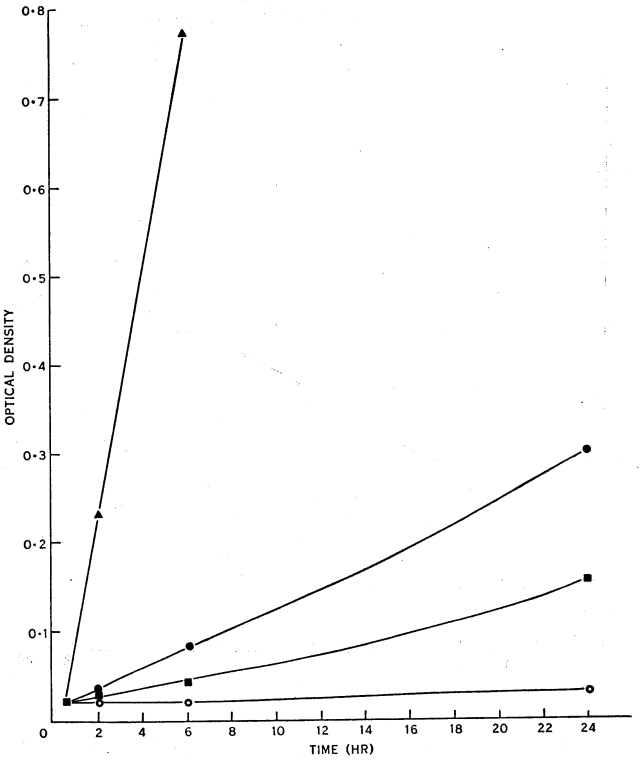


Fig. 2.—Stability of *N*-acetyl-D-glucosamine (0·2M) at different temperatures and pH. The optical density of the colour formed with Ehrlich's reagent has been plotted against time.
O—O, pH 8·0, 25°C.
■—■, pH 9·0, 25°C.
●—●, pH 9·5, 25°C.
▲—▲, pH 9·0, 37°C.

only is the *N*-glycosidic link hydrolysed but the ester grouping also undergoes hydrolysis. This hydrolytic behaviour shows that only a small amount of the *N*-glycoside could be formed by the interaction of *N*-acetyl-D-glucosamine and glycine ethyl ester in neutral aqueous solutions while no complex formation would be expected under acidic conditions. This conclusion is in general agreement with what is known about the interaction of amino acids and aldose sugars such as glucose.

In view of the failure to isolate condensation products from mixtures of amino acids and *N*-acetyl-D-glucosamine the course of the reaction in aqueous

solution was investigated further by means of polarimetric measurements and measurements of pH depression. Two types of reaction conditions were chosen, one at constant pH, the other in which the pH was not kept constant. As is shown in Figure 2, *N*-acetyl-D-glucosamine is not stable under alkaline conditions. With increase in pH and increase in time the amount of colour formed with Ehrlich's reagent increases. Since the nature of the degradation is unknown (cf. Aminoff, Morgan, and Watkins 1952) it is not possible to determine

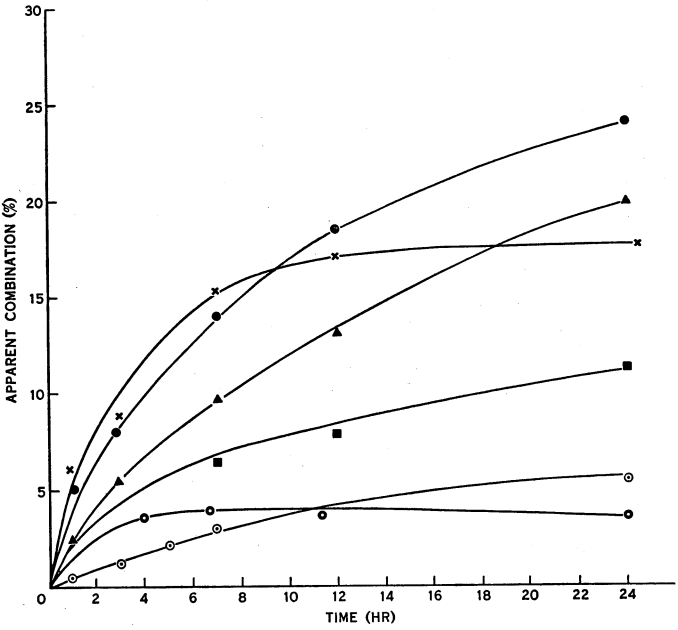


Fig. 3.—Apparent combination of *N*-acetyl-D-glucosamine (0·2M) with glycine and glycylglycine as determined by polarimetric measurements.

O —— O, Glycine 0·2M, pH 8·0, 25°C.
■ —— ■, Glycine 0·2M, pH 9·0, 25°C.
▲ —— ▲, Glycine 0·2M, pH 9·5, 25°C.
X —— X, Glycine 0·2M, pH 9·0, 37°C.
● —— ●, Glycine 0·472M, pH 9·0, 25°C.
⊙ —— ⊙, Glycylglycine 0·2M, pH 9·0, 25°C.

the absolute rate of decomposition but only the relative rate. Consequently the results are expressed in the form optical density *versus* time. It was not possible to prepare a true calibration curve because, on heating *N*-acetyl-D-glucosamine in aqueous sodium carbonate solution, the compound responsible for the formation of colour with Ehrlich's reagent is both formed and destroyed. Consequently the colour intensities as recorded in a calibration curve are probably lower than they would be if no destruction of the compound occured in hot alkaline solution. However, by use of such a calibration curve it is possible to calculate that after 24 hr at 25°C the amount of degradation at pH 8·0 is

less than 0·03 per cent., at pH 9·0 less than 0·15 per cent., at pH 9·5 less than 0·30 per cent., and at 37°C and pH 9·0 less than 2·28 per cent. In view of the instability of *N*-acetyl-D-glucosamine in alkaline solution experimental conditions were confined to the pH range 8-9·5.

The progress of the reaction between 0·2M *N*-acetyl-D-glucosamine and 0·2M glycine or glycylglycine at constant pH was followed by determining the change with time in the optical activity of the solution. The results are given in Figure 3. Calculation of the percentage combination and equilibrium constants were made on the assumption that the combination is in an equimole-

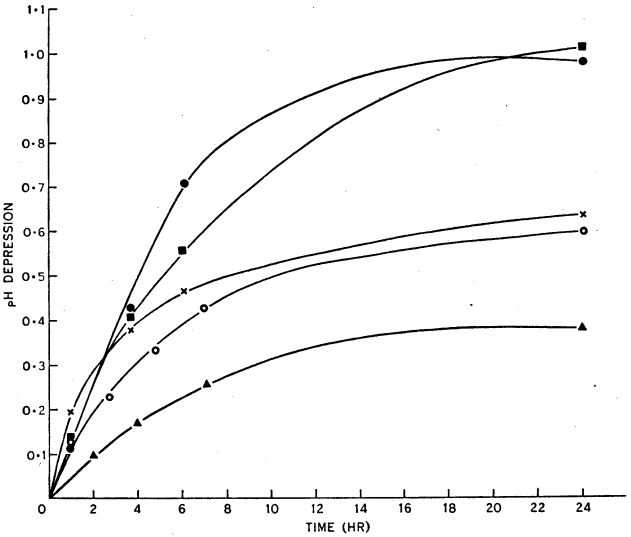


Fig. 4.—Increase of pH depression with time in the interaction of *N*-acetyl-D-glucosamine (1M) with amino acids (0·1M) and peptides (0·05M).

■——■, Glycine, initial pH 9·0 and 18°C.
●——●, L-Tyrosine, initial pH 9·0 and 18°C.
×——×, L-Lysine, initial pH 9·0 and 18°C.
O——O, L-Leucylglycine, initial pH 8·68 and 22°C.
▲——▲, Glycylglycine, initial pH 9·0 and 22°C.

cular ratio and that the compound formed is of negligible rotation so that the decrease in rotation is equal to the extent of combination. This assumption may be open to criticism but the following percentage combinations obtained by iodometric titration support the assumption as a working approximation. Percentage combination found for glycine (after 24 hr at 25°C) by iodometric titration: pH 8·0, 3·1 per cent.; pH 9·0, 9·7 per cent; and pH 9·5, 17·0 per cent. As was to be expected, an increase in temperature increased the rate of the reaction whilst altering the ratio of *N*-acetyl-D-glucosamine to glycine shifted the equilibrium in accordance with the law of mass action. The equilibrium constant for the system containing *N*-acetyl-D-glucosamine and glycine at pH 8·0 was found to be $0{\cdot}14 \times 10^{-2}$; at pH 9·0, $2{\cdot}7 \times 10^{-2}$; and at pH 9·5,

$4{\cdot}9 \times 10^{-2}$; and for glycylglycine at pH 9·0, $0{\cdot}92 \times 10^{-2}$. From Figure 3 it is clear that equilibrium was reached in 24 hr and that the rate of reaction increases considerably as the pH increases from 8 to 9·5. A similar conclusion has been reached by workers investigating the reaction of aldoses, e.g. glucose or xylose with amino acids.

The polarimetric method could not be used successfully to investigate the nature of the reaction with amino acids other than glycine because of the complications introduced by the optical activity of the amino acids. The method involving measurement of pH depression (cf. Frankel and Katchalsky 1941), however, is quite applicable. The theoretical considerations of Frankel and Katchalsky contain a number of assumptions, but the formulae derived by them do represent the experimental conditions with some degree of accuracy and are quite adequate for the present study. The equilibrium constants and the percentage combination have been calculated from the formulae

$$\text{antilog } \Delta \text{pH} = 1 + LG,$$

$$P = \frac{K}{K + H_0} \cdot \frac{LG}{1 + LG} \cdot 100,$$

where Δ pH = pH depression at equilibrium,
L = equilibrium constant,
G = molar concentration of *N*-acetyl-D-glucosamine,
P = percentage combination,
K = apparent dissociation constant of the amino acid or peptide, and
H_0 = initial hydrogen ion concentration of reaction mixture.

Figure 4 records the course of pH depressions with time in the interaction of *N*-acetyl-D-glucosamine with glycine, L-tyrosine, L-lysine, and glycylglycine for an initial pH value of 9·0 and with L-leucylglycine for an initial pH value of 8·68. Figure 5 gives the increase in percentage combination with time. The equilibrium constants (L) for these reactions at the pH values given above are: glycine 9·7, L-tyrosine 9·0, L-lysine 3·4, glycylglycine 1·4, and L-leucylglycine 3·1. The apparent dissociation constants given by Cohn and Edsall (1943) were used to calculate the percentage combination. Equilibrium was reached in 24 hr except for L-lysine, where there was a continued very small increase. This could be caused by reaction at the ϵ-amino group. Micheel and Klemer (1951) have reported that only the α-amino group of lysine reacts with 1-fluoro-D-glucose; the ϵ-amino group does not react. It can be seen that the percentage combination for the peptides is greater than that for the amino acids. Also the percentage combination for tyrosine and lysine are similar and both are more than twice that of glycine. This is due to the differences in the magnitudes of the dissociation constants of the amino acids and peptides, as well as to differences in the equilibrium constants of the reactions because the dissociation constant determines the initial amount of the reagent. The results show clearly that the differences in the degrees of ionization are more important in determining the percentage combination than are the differences in the equilibrium constants. It the past it has generally been considered that, in

contrast with many other amino acids, tyrosine manifests a strong affinity towards and forms compounds with polysaccharides in the biological pH range (Geidroyc, Cichocka, and Mystkowski 1935). This conclusion thus receives further support.

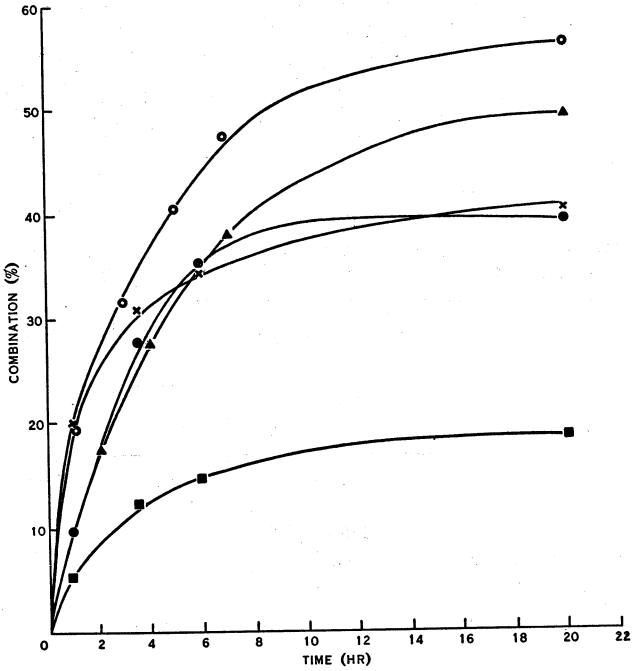
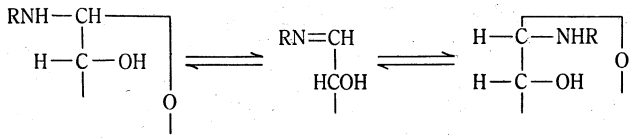


Fig. 5.—Percentage combination of *N*-acetyl-D-glucosamine (1M) with amino acids (0·1M) and with peptides (0·05M) as determined by measurement of pH depression.

■——■, Glycine, initial pH 9·0 and 18°C.
●——●, L-Tyrosine, initial pH 9·0 and 18°C.
X——X, L-Lysine, initial pH 9·0 and 18°C.
O——O, L-Leucylglycine, initial pH 8·68 and 22°C.
▲——▲, Glycylglycine, initial pH 9·0 and 22°C.

The non-enzymic condensations of amino acids with aldoses such as glucose have been extensively studied. It has been established that the initial reaction



II I III

is one in which the aldose reacts with the amino acid in equimolecular ratio, the reaction product being a Schiff base (i.e. an azomethine) (I) although it may be in the isomeric *N*-glycoside form (II or III). From the experimental

evidence presented in this paper it is concluded that the reaction between amino acids and *N*-acetyl-D-glucosamine is of the same type, i.e. a Schiff base is formed from equimolar quantities of each reactant. The reaction mechanism involves the addition of a nucleophilic reagent (the amino acid) to a carbonyl group (the *N*-acetyl-D-glucosamine) with the subsequent elimination of water from the addition product although rearrangement may take place to give an *N*-glycoside. In aqueous solutions such a reaction would be reversible. Under acidic conditions amino acids and peptides are almost completely ionized so it follows that almost no combination would occur, which is in agreement with all known facts.

TABLE 1

THE BINDING OF *N*-ACETYL-D-GLUCOSAMINE BY INSECT CUTICULAR PROTEINS AT 37°C AND pH 8·77 AS DETERMINED BY THE METHOD OF EQUILIBRIUM DIALYSIS

	Protein Tube		Control Tube	
	Outside Bag	Inside Bag	Outside Bag	Inside Bag
Volume of solution	20 ml	10 ml	20 ml	10 ml
Protein (mg)		19·0		
Concn. *N*-acetyl-D-glucosamine	1·953 mM		2·052 mM	2·052 mM
Log free concn. *N*-acetyl-D-glucosamine	−2·7093			
N-acetyl-D-glucosamine bound (mg)		0·66		
N-acetyl-D-glucosamine bound per g protein (mg)		34·7		
pH of solution	8·77	8·77	8·77	8·77

N-acetyl-D-glucosamine is definitely bound by the water-soluble protein isolated from larval cuticles of *D. dorsalis*. The affinity of this protein for *N*-acetyl-D-glucosamine at pH 8·77 and 37°C is shown in Figure 6 where *N*-acetyl-D-glucosamine bound per g protein has been plotted against the logarithm of the free concentration of *N*-acetyl-D-glucosamine. A typical set of results from which these graphs have been drawn is given in Table 1. With a concentration of free *N*-acetyl-D-glucosamine of 1·95mM the protein binds 3·3 per cent. of its own weight of *N*-acetyl-D-glucosamine. No binding of *N*-acetyl-D-glucosamine could be detected at pH 5·5 and 37°C, and the addition of zinc nitrate (cf. Klotz and Loh Ming 1954) at pH 5·5 did not cause any binding to take place. In order to obtain information on the types of groupings which may be involved in the binding which occurs at alkaline pH, the protein was both iodinated and acetylated. Acetylation would inactivate the free α-amino groups while iodination would affect the tyrosine residues. From Figure 6 it can be clearly seen that the iodinated protein shows a greater loss of affinity for *N*-acetyl-D-glucosamine than does the acetylated protein. Clearly the tyrosine groups as well as the free amino groups are involved in the interactions of the protein with *N*-acetyl-D-glucosamine. It has been shown that this cuticular

protein has a high tyrosine content and that the free α-amino groups are located on glutamic acid, glycine, serine, and alanine residues while the ϵ-amino group of lysine is also free (Hackman 1953*a*).

It can be concluded that α-amino acids, peptides, and proteins react with *N*-acetyl-D-glucosamine. No reaction occurs under acidic conditions but under alkaline conditions the amount of reaction increases as the pH increases. At a given pH value the amount of combination depends upon the equilibrium con-

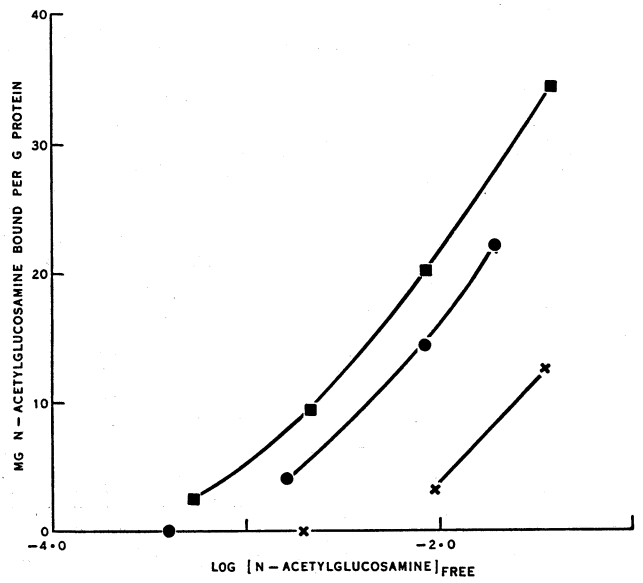


Fig. 6.—Binding of *N*-acetyl-D-glucosamine by proteins at 37°C and pH 8·77. Protein concentration 0·19 per cent.

■——■, Insect cuticular protein.
●——●, Acetylated insect cuticular protein.
×——×, Iodinated insect cuticular protein.

stant of the reaction and upon the dissociation constant of the α-amino nitrogen as this determines the initial concentration of the reagent. Differences in degrees of ionization are more important than differences in the equilibrium constants. Tyrosine, as well as free α-amino groups, appears to play an important part in the binding of *N*-acetyl-D-glucosamine by the cuticular protein. The compounds formed are of the Schiff base (or azomethine) type although they may be in the form of *N*-glycosides. They are unstable, being split even in the biological pH range. The reaction is therefore of a similar type to that which occurs between glucose and α-amino acids or peptides.

IV. References

Aminoff, D., Morgan, W. T. J., and Watkins, W. M. (1952).—Studies in immunochemistry. II. The action of dilute alkali on the *N*-acetylhexosamines and the specific blood-group mucoids. *Biochem. J.* **51**: 379-89.

COHN, E. J., and EDSALL, J. H. (1943).—"Proteins, Amino Acids and Peptides as Ions and Dipolar Ions." Ch. 4. (Reinhold Publ. Corp.: New York.)

DELORY, G. E., and KING, E. J. (1945).—A sodium carbonate-bicarbonate buffer for alkaline phosphatases. *Biochem. J.* **39**: 245.

DYKINS, F. A., and ENGLIS, D. T. (1931).—Determination of glucose in presence of fructose and glycine by iodometric method. *Industr. Engng. Chem.* (Anal. Ed.) **3**: 21-3.

FRAENKEL, G., and RUDALL, K. M. (1947).—The structure of insect cuticles. *Proc. Roy. Soc.* B **134**: 111-43.

FRANKEL, M., and KATCHALSKY, A. (1941).—Interaction of aldoses with α-amino acids or peptides. 4. The percentage combination. *Biochem. J.* **35**: 1028-33.

GIEDROYC, W., CICHOCKA, J., and MYSTKOWSKI, E. (1935).—Über chemische Gruppen der Proteine, die Affinitäten zu Polysacchariden besitzen. VII. Über die Natur der Verbindung Polysaccharid-Guanidin. *Biochem. Z.* **281**: 422-30.

GOTTSCHALK, A., and PARTRIDGE, S. M. (1950).—Interaction between simple sugars and amino acids. *Nature* **165**: 684-5.

HACKMAN, R. H. (1953*a*).—Chemistry of insect cuticle. 1. The water soluble proteins. *Biochem. J.* **54**: 362-7.

HACKMAN, R. H. (1953*b*).—Chemistry of insect cuticle. 2. The water insoluble proteins of the cuticle of *Aphodius howitti* Hope (Coleoptera). *Biochem. J.* **54**: 367-70.

KLOTZ, I. M., and LOH MING, W. C. (1954).—Mediation by metals of the binding of small molecules by proteins. *J. Amer. Chem. Soc.* **76**: 805-16.

LEA, C. H., and RHODES, D. N. (1952).—Studies of the reaction between proteins and reducing sugars in the "dry" state. V. The reaction of D-galactose, 2-deoxy-D-galactose, D-glucosamine and *N*-acetyl-D-glucosamine with casein. *Biochim. Biophys. Acta* **9**: 56-60.

MICHAELIS, L. (1930).—Diethylbarbiturate buffer. *J. Biol. Chem.* **87**: 33-5.

MICHEEL, F., and KLEMER, A. (1951).—*N*-glucoside von Aminosaüren. *Chem. Ber.* **84**: 212-15.

MORGAN, W. T. J., and ELSON, L. A. (1934).—A colorimetric method for the determination of *N*-acetylglucosamine and *N*-acetyl-chondrosamine. *Biochem. J.* **28**: 988-95.

RICHARDS, A. G., and KORDA, F. H. (1948).—Studies on arthropod cuticle. II. Electron microscope studies of extracted cuticle. *Biol. Bull.* **94**: 212-35.

TRIM, A. R. (1941).—Studies in the chemistry of the insect cuticle. I. Some general observations on certain arthropod cuticles with special reference to the characterization of the proteins. *Biochem. J.* **35**: 1088-98.