STUDIES ON CHITIN

III. ADSORPTION OF PROTEINS TO CHITIN

By R. H. HACKMAN*

[*Manuscript received April* 26, 1955]

Summary

The effects of pH, salt concentration, and temperature on the adsorption of a water-soluble insect cuticular protein to chitin have been investigated. The adsorption is dependent upon pH, decreasing rapidly as the pH increases from the region of the isoelectric point of the protein. Increase in salt concentration decreases adsorption but the adsorption appears to be little influenced by changes in temperature. Tyrosine-rich protein fractions are preferentially adsorbed. The adsorption is partly irreversible and an increase to pH 9 is necessary before all the adsorbed protein can be removed. It is concluded that there is only a weak bonding between the chitin and the water-soluble cuticular protein.

I. INTRODUCTION

In an earlier paper (Hackman 1955) the reaction between *N*-acetyl-D-glucosamine (the recurring chemical unit of chitin) and amino acids, peptides, and proteins was investigated. It was shown that compounds of the Schiff base (or azomethine) type are formed but no reaction occurs under acidic conditions. The compounds are unstable and undergo hydrolysis even in the biological pH range. Tyrosine residues, as well as free α-amino groups, were shown to play an important part in the binding of *N*-acetyl-D-glucosamine by insect cuticular proteins.

This evidence supports the theory that in soft insect cuticles there is a weak bonding between chitin and protein. To obtain further evidence along these lines the adsorption of a water-soluble cuticular protein to chitin has been investigated. The information gained must also be useful in further studies on the proteins of insect cuticles.

II. EXPERIMENTAL

(*a*) *Reagents*

The protein used was the water-soluble protein extracted from larval cuticles of *Diaphonia dorsalis* Don. (Coleoptera) prepared according to the method described by Hackman (1953). The protein contained 13·9 per cent. N (micro-Kjeldahl). The protein preparation was subjected to electrophoresis on filter paper using an E.E.L. paper electrophoresis apparatus. A phosphate buffer

* Division of Entomology, C.S.I.R.O., Canberra, A.C.T.

(0·100M potassium dihydrogen phosphate + 0·0024M potassium hydroxide, pH 5·3, ionic strength 0·1) and a current of 0·4 mA/cm width of paper were used, and the duration of the experiment was 24 hr. The protein was separated into five components.

The chitin was prepared from lobster shell by the method described by Hackman (1954). To purify the chitin further it was ground in an Eppenbach colloid mill, model QV6. The majority of the particles were below 5 μ in their greatest dimension and almost all the particles were less than 10 μ. However, there was present an occasional larger particle of up to 30 μ in its greatest dimension. The finely ground material was again extracted with cold 2N aqueous hydrochloric acid and hot N aqueous sodium hydroxide. It was finally dialysed against running water until free of alkali. Since the chitin was found to cake on drying it was retained as a suspension. The suspension was quite stable for the duration of these experiments and 2·55 ml contained 50 mg chitin and 2·5 ml water.

Except for the experiment described in Section II (*e*), all experiments were conducted in a constant temperature room at 26·7 ± 0·2°C.

(*b*) *Effect of pH on Adsorption of Protein*

A protein solution was prepared for each pH value in an appropriate buffer solution so that 1 ml contained 2 mg protein. Protein solution (2·5 ml) and chitin suspension (2·55 ml) were mixed well by a gentle rocking motion so as to avoid frothing and then rotated gently for 60 min. The rate of rotation, which was constant for all experiments, was sufficient to keep the suspension well mixed without any frothing. Previous experiments had shown that under the varied experimental conditions described in this paper adsorption was complete within 60 min. The final solutions all had ionic strengths of 0·1 and the pH of the solutions as measured by the glass electrode were 5·05, 6·00, 7·00, 8·09, 8·95, and 9·97. They had the following composition: (i) 0·045M acetic acid + 0·1M sodium acetate, (ii) 0·07M sodium dihydrogen phosphate + 0·01M disodium hydrogen phosphate, (iii) 0·019M sodium dihydrogen phosphate + 0·027M disodium hydrogen phosphate, (iv) 0·0017M sodium dihydrogen phosphate + 0·0317M disodium hydrogen phosphate. (v) 0·09M glycine in 0·09M sodium chloride + 0·01M sodium hydroxide, and (vi) 0·06M glycine in 0·06M sodium chloride + 0·04M sodium hydroxide.

After the suspensions had been rotated for 60 min they were centrifuged. Aqueous sodium hydroxide (0·5 ml of an appropriate concentration) was added to 3·0 ml of the clear supernatants to make the sodium hydroxide concentration 0·75N. The solutions were mixed well and the optical densities read at 280 and 294·4 mμ in a Beckman photoelectric spectrophotometer, model DU. The amount of protein in 3·0 ml of these alkaline solutions was also estimated by the biuret method as given by Robinson and Hogden (1940). The optical densities of the coloured solutions were read at 550 mμ. The amount of protein present in each solution was determined by reference to calibration curves prepared by dissolving the protein in 0·75N sodium hydroxide and (1) measuring the optical density at 280 mμ, (2) measuring the optical density of the colour

formed in the biuret method. For every pH value solutions were prepared which contained protein but no chitin, and these served as controls. All experiments were performed at least in duplicate and the amount of protein adsorbed by the chitin was determined by difference.

(*c*) *Adsorption Isotherms*

The experiments were carried out in a similar manner to those described in Section II (*b*). Protein solution (2·5 ml) was added to various chitin suspensions (2·55 ml, 2 ml + 0·54 ml water, 1 ml + 1·52 ml water, 0·5 ml + 2·01 ml water, and 0·25 ml + 2·255 ml water). This gave suspensions containing 50, 40, 20, 10, and 5 mg chitin in 5 ml total volume. The solutions all had ionic strengths of 0·1 and two sets of experiments were performed, one at pH 6·00 and the other at pH 7·00 using the buffer solutions described above. The protein present in the supernatant after adsorption, was estimated by the biuret method.

(*d*) *Effect of Salt Concentration on Adsorption of Protein*

Three salt solutions were used. The protein (2 mg/ml) was dissolved in the salt solution and 2·5 ml of each solution were added to 2·55 ml chitin suspension. The final salt solutions had the following compositions: 0·019M sodium dihydrogen phosphate + 0·027M disodium hydrogen phosphate, ionic strength 0·1; the above phosphates + 0·05M sodium sulphate, ionic strength 0·25; the above phosphates + 0·15M sodium sulphate, ionic strength 0·55. The pH of all salt solutions was 7·00. The experiment was completed as described in Section II (*b*).

(*e*) *Effect of Temperature on Adsorption of Protein*

The experiments described in Section II (*b*) for pH 6·00 and 7·00 were repeated at 4°C, and the protein remaining in solution estimated by the biuret method.

(*f*) *Reversibility of the Adsorption*

(i) Cuticular protein (5·0 mg) was dissolved in a phosphate buffer of pH 7·00 (2·5 ml) and chitin suspension (2·55 ml) added. Ionic strength of the final solution was 0·1. The suspension was mixed well, rotated gently for 60 min, centrifuged, and the protein in the supernatant estimated by determining its optical density at 280 mμ. Phosphate buffer (3 ml, pH 7·00, ionic strength 0·1) was added to the chitin residue, the suspension mixed well, rotated for 60 min, centrifuged, and the protein in the supernatant estimated as before. Buffer was again added to the chitin residue and the extraction procedure repeated six times in all when no further protein was extracted.

(ii) The cuticular protein was adsorbed on to the chitin from a phosphate buffer of pH 7·00 as in Section (*f*) (i), but a glycine-sodium hydroxide buffer (pH 8·95, ionic strength 0·1) was added to the chitin residue instead of buffer of pH 7·00. Before adding the buffer solution the pH of the chitin residue was

adjusted to 9·00 by the addition of aqueous sodium hydroxide. A single extraction removed all of the protein from the chitin.

III. Results and Discussion

Earlier work (Hackman 1953) had shown that the water-soluble cuticular protein from *Diaphonia dorsalis* was not homogeneous but that it contained a number of components. Paper electrophoresis has shown that the protein can be separated into five components. It follows that the methods used to estimate the protein concentrations had to be such that they did not distinguish between the various protein fractions, or if they did then the nature of the discrimination would have to be known. Two methods were chosen: the development of the biuret colour which is almost independent of the nature of the protein, and measurement of the optical density of the protein solution at 280 mμ. The

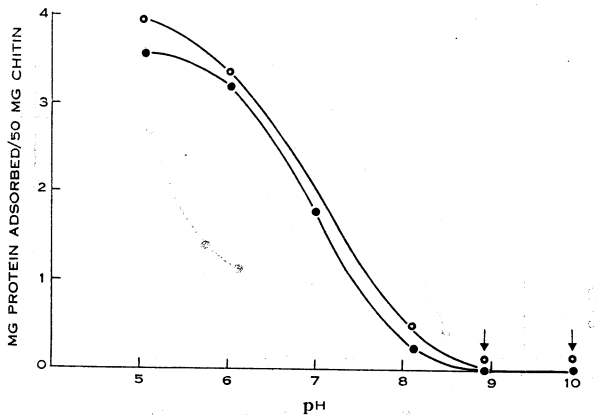

Fig. 1.—Effect of pH on the amount of protein adsorbed onto chitin from an 0·1 per cent. solution of the protein. ● Estimated by the biuret method. ○ Estimated by optical density at 280 mμ.

latter method is dependent upon tyrosine content and such measurements might indicate the adsorption of tyrosine-rich or tyrosine-poor fractions. Reproducibility of the biuret method was found to be excellent and a difference of 0·02 mg protein could be readily and consistently estimated. No alteration in the optical density of the protein solution at 280 mμ, in 0·75N sodium hydroxide was observed over a period of approximately one hour (by which time all measurements in any one experiment had been completed). However, changes which occurred within a few minutes would not have been detected. Even though the methods for analysis of the protein were reproducible with a high degree of accuracy, controls were included in all experiments to ensure that no unforeseen errors occurred.

The effect of pH on the amount of protein adsorbed on to chitin is shown in Figure 1. The graphs show clearly that a tyrosine-rich fraction is adsorbed.

Calculation of the tyrosine content of the protein solutions from the optical densities at 280 and 294·4 mμ (see Goodwin and Morton 1946) again indicates that a tyrosine-rich fraction is adsorbed. As the protein at a concentration of 1 mg/ml is not soluble in buffer solution of ionic strength 0·1 at pH 3 or 4, measurements could not be made in the region of the isoelectric point (pH 3-4) of the protein. The absence of adsorption at pH 9 or 10 is ascribed to the method of preparing the protein. The cuticular protein was prepared by extracting insect cuticles (which contain chitin) with a buffer of pH 9; consequently

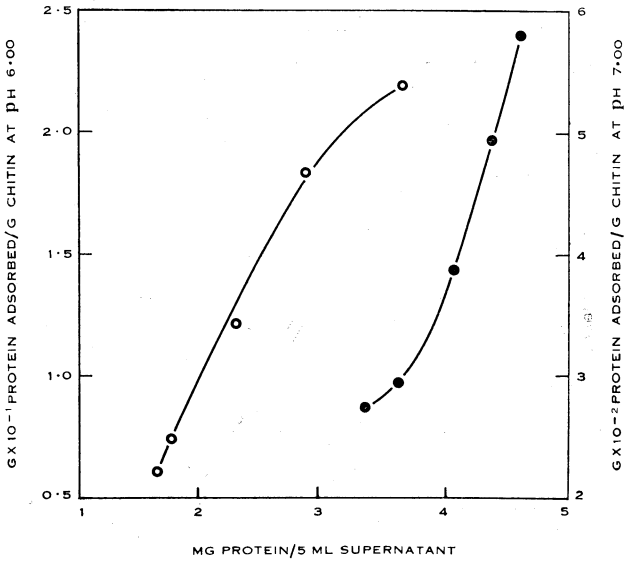

Fig. 2.—Adsorption isotherms obtained by the constant protein method. Protein concentration 0·1 per cent. ● Sodium phosphate buffer (pH 7·00, ionic strength 0·1). ○ Sodium phosphate buffer (pH 6·00, ionic strength 0·1).

no adsorption would be expected at pH 9 or at pH values higher than 9. The effect of pH on the amount of the cuticular protein adsorbed on to chitin is similar to that generally observed for the adsorption of proteins on to solids, i.e. the amount adsorbed decreases as the pH increases from the region of the isoelectric point of the protein.

Adsorption isotherms of proteins may be prepared from data obtained by varying the protein concentration or by varying the amount of adsorbent, i.e. by working with a constant protein or constant adsorbent concentration. If the protein preparation is homogeneous then identical results should be obtained by both methods. The cuticular protein used is known to be non-homogeneous and it has been shown that a tyrosine-rich fraction is preferentially adsorbed. Results obtained by the constant adsorbent method indicated the presence of a

small fraction which was not adsorbed at all; consequently the constant protein concentration method was used to prepare the adsorption isotherms. The results are shown graphically in Figure 2. The slope of the adsorption isotherms indicates that satisfactory elution of the proteins would not be obtained with the same solvents. This fact is considered further when the irreversible nature of the adsorption is discussed.

The effect of salt concentration on the adsorption of the insect cuticular protein on chitin is given in Table 1. An increase in salt concentration brings about a decrease in the amount of protein adsorbed on to the chitin. The protein is not completely soluble at pH 7·00 in solutions of ionic strength greater than 0·55 (i.e. approx. 1/17 sat. sodium sulphate) so the results are of neces-

TABLE 1

EFFECT OF SALT CONCENTRATION ON THE AMOUNT OF PROTEIN ADSORBED ON TO CHITIN FROM 0·1 PER CENT. SOLUTION OF THE PROTEIN AT pH 7·00

Total Ionic Strength	Mg Protein Adsorbed/50 mg Chitin	
	Biuret Method	Optical Density Method (at 280 mμ)
0·10	1·70	2·10
0·25	0·92	1·26
0·55	0·61	0·90

sity limited. Calculation of the tyrosine content of the unadsorbed protein shows a decrease as the ionic strength increases from 0·1 to 0·25 after which it remains constant. Therefore increase in salt concentration at first favours the adsorption of tyrosine-rich protein fractions. In contrast with results produced by changes in salt concentration, temperature had little, if any, effect on the adsorption of protein on to chitin. Experiments carried out at 4°C gave the same results as those carried out at 26·7°C for both pH 6·00 and 7·00.

Adsorption of the insect cuticular protein by finely divided chitin is partly irreversible under the conditions studied. The chitin (50 mg) adsorbed 1·75 mg protein from 5 ml of a buffer solution of pH 7·00 and ionic strength 0·1 which initially contained 1 mg protein/ml. Subsequently by washing the chitin with buffer solution of the same pH and ionic strength, only 0·71 mg (i.e. 40 per cent.) of this adsorbed protein could be removed. It would appear that there is a layer of protein firmly held on to the chitin, and an outer, elutable layer held by secondary forces. However, all the protein adsorbed at pH 7·00 can be removed by extraction with a buffer of pH 8·95 and ionic strength 0·1. Lack of reversibility of adsorption is frequently encountered with proteins, and

in the case of this cuticular protein an increase in pH is necessary before the adsorbed protein can be removed.

In the work described above some of the factors affecting adsorption of an insect cuticular protein to chitin have been investigated. It is quite clear from the results that tyrosine-rich protein fractions are preferentially adsorbed and there is little doubt that the tyrosine residues in the protein play an important role in this adsorption. The adsorption is sensitive to both changes in pH and changes in salt concentration but is largely unaffected by changes in temperature. Since no adsorption occurs at pH 9 and since the protein may be extracted from the chitin at this pH it would appear that neither hydrogen bonds nor covalent bonds are involved in the adsorption (cf. Hackman 1955). This evidence indicates that there is only a weak bonding between the chitin and the water-soluble cuticular protein. The apparent absence of hydrogen bonds is of interest since both the chitin and the protein contain large numbers of groups which could form hydrogen bonds. The application of the results obtained in this paper to a study of insect cuticular proteins will be reported in later papers.

IV. Acknowledgment

The author wishes to acknowledge the helpful advice of Professor A. E. Alexander, N.S.W. University of Technology, Sydney.

V. References

Goodwin, T. W., and Morton, R. A. (1946).—The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **40**: 628-32.

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

Hackman, R. H. (1954).—Studies on chitin. I. Enzymic degradation of chitin and chitin esters. *Aust. J. Biol. Sci.* **7**: 168-78.

Hackman, R. H. (1955).—Studies on chitin. II. Reaction of *N*-acetyl-D-glucosamine with α-amino acids, peptides, and proteins. *Aust. J. Biol. Sci.* **8**: 83-96.

Robinson, H. W., and Hogden, C. C. (1940).—The biuret reaction in the determination of serum proteins. I and II. *J. Biol. Chem.* **135**: 707-25; 727-31.