STUDIES ON CHITIN

I. ENZYMIC DEGRADATION OF CHITIN AND CHITIN ESTERS

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Summary

A study has been made of the enzymic degradation of chitin and the sodium salt of chitin sulphuric acid by a chitinase prepared from the intestinal tract of the snail, *Helix aspersa*. It is shown that, in a citrate-phosphate buffer, the pH for optimum activity is 4.8 for both substrates. Chitin, prepared from both lobster cuticle and fly puparia, is broken down by snail chitinase to *N*-acetyl-D-glucosamine, although there is also a trace of D-glucosamine. The sodium salt of chitin sulphuric acid also breaks down to *N*-acetyl-D-glucosamine and D-glucosamine. In both cases *N*-acetyl-D-glucosamine was isolated. The chitinase is without action on chitin nitrate. Acid hydrolysis of both chitin nitrate and the sodium salt of chitin sulphuric acid leads to the formation of D-glucosamine, which has been isolated as 2-hydroxynaphthylidene glucosamine. All attempts to phosphorylate chitin were unsuccessful. The structure of chitin is discussed.

I. INTRODUCTION

The work described in this paper is part of an investigation into the metabolic sources of chitin. In arthropods there must be very active systems both for the synthesis and for the solubilization of chitin because the cuticle is largely solubilized, reabsorbed, and then formed anew at each moult. No precursors of chitin are known although there are suggestions in the literature that the chitin arises from the transformation of glycogen, protein, and cellulose (for references see Richards 1951). However, the information is not sufficiently definite to warrant speculation on possible metabolic sources.

Chitin is a component of arthropod cuticles, it occurs in a number of other groups of animals and in certain fungi (Richards 1951). It is a high-molecular-weight polymer of *N*-acetyl-D-glucosamine (more correctly 2-acetamido-2-deoxy-α-D-glucopyranose) residues, linked in the 1,4-β-glucosidic manner of cellulose. Chitin, as usually prepared for chemical studies, is a colourless amorphous solid, insoluble in water, dilute acids, dilute and concentrated alkalis, alcohol, and all organic solvents. It is soluble in concentrated mineral acids, generally with some degradation to lower saccharides. When chitin is treated with alkali hydroxides at high temperatures (150-160°C) the acetyl groups are detached and the polysaccharide chitosan is formed. In "purified" chitin there is usually a strong attraction between adjacent molecular chains, as shown by its resistance to dispersion, but the nature of the bond involved is not known.

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It has been known for some time that many bacteria produce enzymes which will digest chitin, and such bacteria have been isolated from fresh water, sea-water, salt beds, mud, soil, decomposing manure, and from the gut contents of many marine, freshwater, and terrestrial animals (for review see Richards 1951). A recent paper by Veldkamp (1952) describes the isolation of two strains of bacteria from manured garden soil which are capable of decomposing chitin to *N*-acetyl-D-glucosamine, D-glucosamine, and ammonia. The enzymic breakdown of chitin has been studied in some detail by Karrer and his colleagues (Karrer 1930; Karrer and Hoffmann 1929; Karrer and François 1929), using a chitin-splitting enzyme obtained from digestive juices of vineyard snails (*Helix pomatia*). The hydrolysis of chitin by this chitinase was found to proceed best at pH 5.2 and from fungal chitin as much as 80 per cent. of *N*-acetyl-D-glucosamine together with no more than a trace of D-glucosamine was obtained. Yonge (1932) has shown that the digestive juices from the snail *Helix aspersa* are capable of dispersing lobster cuticle. In recent publications Jeuniaux (1950*a*, 1950*b*, 1950*c*) has described the isolation of bacteria from the gut of a snail (*H. pomatia*) which is capable of solubilizing chitin. These bacteria produce an extracellular chitinase, no chitinolytic enzymes being found in the snail hepatopancreas.

Sulphuric acid esters of chitin have been prepared and their use as synthetic anticoagulants studied by a number of workers (Bergström 1935, 1936; Karrer, Koenig, and Usteri 1943; Astrup, Galsmar, and Volkert 1944; Piper 1946). Several different nitrate derivatives of chitin have been made and their structures investigated (von Fürth and Scholl 1907; Schorigin and Hait 1934; Clark and Smith 1936; Meyer and Wehrli 1937).

II. Materials and Methods

(*a*) *Preparation of Chitin*

Lobster shell was cleaned by washing and scraping under running water and dried in an oven at 100°C. Clean and dried lobster shell (220 g) was digested with 2N aqueous hydrochloric acid (2 l.) for 5 hr at room temperature, washed well, dried at 100°C (wt. 91.3 g), and ground to a fine powder in a hammer mill. The finely ground material was extracted for 48 hr with cold 2N aqueous hydrochloric acid (500 ml), the contents of the flask being vigorously agitated at frequent intervals. The material was collected by centrifugation, washed with water, and extracted with 1N aqueous sodium hydroxide solution (500 ml) for 12 hr at 100°C, the contents of the flask again being shaken at frequent intervals. The insoluble material was collected by centrifugation and again extracted with aqueous alkali for 12 hr at 100°C. The material was further extracted with alkali three times. The insoluble material was collected by centrifugation, washed with water (by centrifugation) until of neutral reaction, then with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide. Yield: 37.4 g (17 per cent.) of a cream-coloured powder (Found: N, 6.8% and ash nil. Calc. for $(C_8H_{13}O_5N)_x$: N, 6.9%).

In a similar manner chitin was prepared from the puparia of *Musca domestica* L. This sample of chitin, which was a pale brown powder, had N 6.5 per cent. and ash 5.8 per cent.

(*b*) *Preparation of the Sodium Salt of Chitin Sulphuric Acid*

Chlorosulphonic acid (9.0 ml) was added with cooling to pure dry pyridine (60 ml). To this mixture was added finely powdered chitin (lobster) (6 g) and the solution heated on a steam bath for 1 hr. The solution was cooled, ice water (200 ml) and 2N aqueous sodium hydroxide solution (120 ml) added, and the sodium salt of chitin sulphuric acid precipitated by the addition of ethanol (600 ml). The suspension was left in the refrigerator overnight, the precipitate collected by filtration, dissolved in water (200 ml), and dialysed against running water for 2 days. The solution was concentrated under reduced pressure and in an inert atmosphere to 100 ml, filtered through asbestos pulp, and the sodium salt precipitated by the addition of ethanol (100 ml). The salt was collected by centrifugation, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide. Yield: 7.7 g of a colourless solid. Tests for chloride and sulphate ions were negative (Found: S, 11.2%).

(*c*) *Preparation of Chitin Nitrate*

Finely powdered chitin (2.0 g) was mixed to a slurry with a small volume of fuming nitric acid (sp. gr. 1.5) while cooling the mixture in ice water. More fuming nitric acid was added (50 ml total vol.) and the mixture stirred until all the chitin was in solution (10 min). The solution was kept at room temperature for 2 hr and then poured slowly into ice water (1 l.). The precipitate was collected by centrifugation, washed with water until the washing had a neutral reaction, then with alcohol and ether, and finally dried to constant weight at 110°C. Yield: 2.0 g of a colourless powder (Found: C, 33.1; H, 4.2; N, 12.1%).

(*d*) *Phosphorylation of Chitin*

Attempts to phosphorylate chitin by the method used by Karrer, Koenig, and Usteri (1943) for phosphorylating starch were unsuccessful even when the reaction was carried out at 100°C. In every case the chitin was recovered in quantitative yield and it contained no phosphorus.

(*e*) *Preparation of Snail Enzyme*

Snails (*Helix aspersa* Müller) were collected at Canberra and starved for several days so that the gut was emptied of food. The snails were covered completely with water in a closed dish so that they were killed by lack of air. By use of this method the snails came out of their shells and extended themselves completely. The shells were removed and the intestinal tracts carefully removed without loss of intestinal contents. The collected intestines were ground in a mortar with toluene and sand, washed with a little water, and filtered through asbestos pulp. The brown filtrate was dialysed against running water for 3 days and diluted to such a volume that each 2 ml represented the

enzyme obtained from one snail. A few drops of toluene were added as a preservative. The preparation contained, besides a chitinase, an enzyme which attacked the cellophane membrane during dialysis. To prevent rupture of the membrane with consequent loss of material the solution was transferred to a new membrane each day.

(*f*) *Filter Paper Partition Chromatography*

One-dimensional filter paper partition chromatography (capillary ascent method of Williams and Kirby (1948)) was used for the detection of the products resulting from the action of the above chitinase preparation on chitin, the sodium salt of chitin sulphuric acid and chitin nitrate. The following two systems were used as solvents: phenol 74 per cent. and water 26 per cent. (w/w), and *n*-butanol 77 per cent., acetic acid 6 per cent., and water 17 per cent. (v/v). Whatman No. 1 filter paper was used, the chromatogram chambers were kept in a room maintained at 25 ± 1°C and the chromatograms were run overnight.

Finely powdered chitin or chitin derivative (20 mg), water (2 ml), McIlvaine (1921) citrate-phosphate buffer (pH 4.8) (2 ml), snail enzyme preparation (1 ml), and toluene (3 drops) were shaken continuously for 3 days at 25 ± 0.5°C. The mixture was centrifuged and 5 μl and multiples of 5 μl of the clear liquid were used for each chromatogram. Two controls were run for each preparation, the above mixture without enzyme and the above mixture without chitin or chitin derivative. For purposes of comparison pure D-glucosamine and *N*-acetyl-D-glucosamine were included in each chromatogram. Ninhydrin, aniline hydrogen phthalate (Partridge 1949), and the hexosamine reagent of Partridge (1948) were used as detecting agents. For *N*-acetyl-D-glucosamine better colour development was obtained if the paper was sprayed with alkali (0.1N alcoholic sodium hydroxide) before treatment with the *p*-dimethylaminobenzaldehyde reagent.

(*g*) *Enzymic Degradations*

(i) *Chitin.*—Since it was not found possible to obtain a reproducible product by the reprecipitation of chitin from cold concentrated hydrochloric acid according to the method of Karrer and Hoffman (1929), finely powdered chitin was used in all the work described in this paper. Lack of reproducibility was shown by differing rates of enzyme action.

To determine the optimum pH for the production of *N*-acetyl-D-glucosamine chitin (20 mg), water (2 ml), McIlvaine citrate-phosphate buffer (2 ml, pH range 4-7), enzyme preparation (1 ml), and toluene (3 drops) were shaken continuously for 3 days in a sealed tube at 25 ± 0.5°C. The mixture was centrifuged and 1-ml samples were taken for the estimation of *N*-acetyl-D-glucosamine and D-glucosamine. The solution from each tube was analysed in duplicate and each pH was done in duplicate. *N*-acetyl-D-glucosamine and D-glucosamine were estimated by a modification of the methods of Morgan (Elson and Morgan 1933; Morgan and Elson 1934; Aminoff, Morgan, and Watkins 1952). To develop maximum colour in the above buffered solutions it was found necessary

to add 0.5 ml 0.5N aqueous sodium carbonate solution for the estimation of *N*-acetyl-D-glucosamine and 1.5 ml for the estimation of D-glucosamine. It was observed that the time and temperature of heating and the rate of cooling influenced the intensity of colour developed and although these conditions were standardized a control containing 0.1 mg *N*-acetyl-D-glucosamine was included so that a correction could be made for any variation in colour development. For *N*-acetyl-D-glucosamine the colour intensity was read at 560 mμ and for D-glucosamine at 530 mμ. Standard curves were prepared by the use of known amounts of *N*-acetyl-D-glucosamine and D-glucosamine in buffered solutions. The presence of D-glucosamine had no effect on the estimation of *N*-acetyl-D-glucosamine but *N*-acetyl-D-glucosamine interfered in the estimation of D-glucosamine. To correct for this effect a standard curve was prepared, estimating *N*-acetyl-D-glucosamine by the D-glucosamine method. *N*-acetyl-D-glucosamine and D-glucosamine were found to be stable in the presence of the chitinase preparation at least for the duration of these experiments.

(ii) *Sodium Salt of Chitin Sulphuric Acid.*—The optimum pH for the production of *N*-acetyl-D-glucosamine was determined by the same method as was used for chitin. Since the sodium salt is soluble in water in the pH range 4-7 the solutions were not shaken but were kept at 37 ± 0.25°C and the reaction was allowed to proceed for 6 days. Excess chitin sulphuric acid was precipitated during the estimations of D-glucosamine and *N*-acetyl-D-glucosamine and to remove the precipitate the solutions were centrifuged after the addition of the colour reagent but before the colour commenced to develop.

(iii) *Chitin Nitrate.*—Repeated experiments showed that the chitinase preparation was without action on finely powdered chitin nitrate.

(*h*) *Isolation of* N-*acetyl-D-glucosamine from the Enzymic Degradation of Chitin*

Finely powdered chitin (1.0 g), snail enzyme preparation (50 ml), toluene (3 ml), and sufficient 0.1N aqueous hydrochloric acid to adjust the pH of the solution to 4.8 were thoroughly mixed and kept at 37 ± 0.25°C for 56 days. The solution was shaken vigorously twice each day. Ethanol (350 ml) was added and the precipitated protein and unchanged chitin removed by centrifugation. The clear solution was evaporated to dryness *in vacuo* at 50°C in an inert atmosphere and the residue boiled under reflux with absolute methanol for 90 min to dissolve the *N*-acetyl-D-glucosamine. The hot solution was filtered, concentrated to a small volume (*c.* 10 ml) at 40°C *in vacuo* in an inert atmosphere, and left in the refrigerator for the *N*-acetyl-D-glucosamine to crystallize. A further crop of crystals was obtained on concentrating the mother liquor. Total yield 0.48 g (44 per cent.) m.p. 195°C (decomp.). One crystallization from dry methanol raised the m.p. to 204-205°C (decomp.), undepressed in admixture with an authentic specimen of *N*-acetyl-D-glucosamine, m.p. 204°C (decomp.) (Found: C, 43.4; H, 6.9; N, 6.3%. Calc. for $C_8H_{15}O_6N$: C, 43.4; H, 6.8; N, 6.3%). Colorimetric analysis showed the presence of 4.5 mg D-glucosamine in the solution.

(*i*) *Isolation of* N-*acetyl*-D-*glucosamine from the Enzymic Degradation of the Sodium Salt of Chitin Sulphuric Acid*

The sodium salt of chitin sulphuric acid (1.0 g) was subjected to the same enzymic degradation as was chitin except that it was necessary to add alkali instead of acid to adjust the pH to 4.8. Yield of *N*-acetyl-D-glucosamine 0.33 g, m.p. 190°C (decomp.) raised to 204-205° (decomp.) on crystallization from methanol, undepressed in admixture with an authentic specimen of *N*-acetyl-D-glucosamine, m.p. 204°C (decomp.) (Found: C, 43.5; H, 6.9; N, 6.4%). Colorimetric analysis showed the presence of not more than 5 mg D-glucosamine in the solution.

(*j*) *Acid Hydrolysis of the Sodium Salt of Chitin Sulphuric Acid*

Sodium salt (100 mg) was heated on a steam bath under reflux with 6N aqueous hydrochloric acid (5 ml) for 4 hr. The solution was evaporated to dryness *in vacuo* and the residue dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was extracted with hot water (0.5 ml), the solution treated with charcoal and filtered. The insoluble material was washed with two lots of water (2×0.25 ml). The pH of the solution was adjusted to 5 by the addition of solid sodium bicarbonate, and sodium acetate (200 mg) added. A solution of freshly synthesized (Duff and Bills 1934) 2-hydroxynaphthaldehyde (150 mg) in methanol (7.5 ml) was added and the mixture kept in the dark for 2 hr. The mixture was concentrated to 1 ml *in vacuo* at room temperature and in an inert atmosphere, a few drops of methanol added to the semisolid mass, and the slurry kept at 0°C overnight. The mixture was evaporated to dryness *in vacuo* at room temperature in an inert atmosphere and the residue thoroughly dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was extracted three times with 10-ml portions of dry chloroform-dry ether (3:1), freed of chloroform and ether, and extracted three times with 1-ml portions of water at 0°C, leaving the water in contact with the solid for some hours each time. Concentration of the aqueous extracts and extraction of the solid which separated with water as before yielded a small additional quantity of yellow solid. Total yield of 2-hydroxynaphthylidine glucosamine 31.3 mg, which after crystallization from methanol had m.p. 200°C (decomp.) undepressed in admixture with an authentic specimen of m.p. 202°C (Found: C, 61.0; H, 5.8; N, 4.2%. Calc. for $C_{17}H_{19}O_6N$: C, 61.2; H, 5.8; N, 4.2%).

Colorimetric analysis of the hydrolysate for D-glucosamine gave 18.1 mg; that isolated in the form of the Schiff base corresponds to 16.8 mg.

(*k*) *Acid Hydrolysis of Chitin Nitrate*

Chitin nitrate (100 mg) was hydrolysed and the Schiff base isolated by the method described for the sodium salt of chitin sulphuric acid. Yield of 2-hydroxynaphthylidene glucosamine 26.4 mg, m.p. 201°C undepressed in admixture with an authentic specimen of m.p. 202°C (Found: C, 61.1; H, 5.8; N,4.1%). Colorimetric analysis of the hydrolysate for D-glucosamine gave 15.2 mg; that isolated in the form of the Schiff base corresponds to 14.2 mg.

(*l*) *Stability of Chitin to Aqueous Hydrochloric Acid at* 100°C

Chitin (18.8 mg) and 2N aqueous hydrochloric acid (10 ml) were heated at 100°C for 7 hr. The mixture was cooled, neutralized, and its volume made up to 50 ml. The solution was centrifuged and 1-ml samples were analysed for D-glucosamine (Found: 1.10 mg glucosamine from 18.8 mg chitin).

(*m*) *Stability of Chitin to Aqueous Alkali at* 100°C

Chitin (2.0 g) and 1N aqueous sodium hydroxide solution (50 ml) were heated on a steam bath for 48 hr, the mixture being shaken at frequent intervals. Saturated aqueous solution of barium hydroxide (20 ml) was added to precipitate any carbonate, the solution kept warm and protected from carbon dioxide for several hours, cooled, and filtered. To test for the complete precipitation of carbonate an additional 1 ml saturated aqueous solution of barium hydroxide was added. Magnesium sulphate heptahydrate (35 g) and concentrated sulphuric acid (2.5 ml) were added and the solution distilled, using a still head incorporating a steam trap. Approximately 50 ml distillate were collected and titrated with 0.1N aqueous sodium hydroxide solution, using phenolphthalein as an indicator (cf. estimation of acetyl groups by Clark 1936, 1937). A control was included which contained no chitin. Difference between the amount of 0.1N alkali required by the control and the chitin solution was 1.25 ml.

III. Results and Discussion

The difficulty experienced in phosphorylating chitin is in marked contrast to the ease with which sulphuric acid and nitric acid residues can be introduced into the molecule. On the other hand, Karrer, Koenig, and Usteri (1943) were able to phosphorylate chitosan by less drastic methods than those which failed with chitin. These observations, together with the difficulty experienced in degrading chitin with alkali and the report by Schorigin, Makarowa-Semljanskaja, and Anurjewa (1935) that it is exceedingly difficult to methylate chitin by any of the known procedures, place chitin in a class distinct from most other polysaccharides.

From the filter paper partition chromatographic study of the enzymic degradation of chitin by snail (*H. aspersa*) chitinase it is concluded that chitin breaks down to give *N*-acetyl-D-glucosamine together with a trace of D-glucosamine, these being the only degradation products detected. The appearance of D-glucosamine is, perhaps, significant and will be discussed later. Similarly the sodium salt of chitin sulphuric acid is degraded to *N*-acetyl-D-glucosamine and D-glucosamine and again these were the only degradation products detected. On the other hand the snail enzyme preparation appeared to be without action on chitin nitrate.

Graphs illustrating the amounts of *N*-acetyl-D-glucosamine found during the enzymic degradation of chitin and the sodium salt of chitin sulphuric acid at different pH's are given in Figure 1. From these, it can be seen that the chitinase preparation is most active in weakly acid solutions and the pH for optimum activity is 4.8 for both chitin and the sodium salt of chitin sulphuric acid in

McIlvaine's citrate-phosphate buffer, the former at 25°C and the latter at 37°C. During the short period used for these enzymic reactions no D-glucosamine could be detected by the Elson and Morgan (1933) method with chitin as a substrate. When the sodium salt of chitin sulphuric acid was used as the substrate about 0.02 mg D-glucosamine/20 mg substrate was detected, i.e. about 2 per cent. of the estimated amount of *N*-acetyl-D-glucosamine formed.

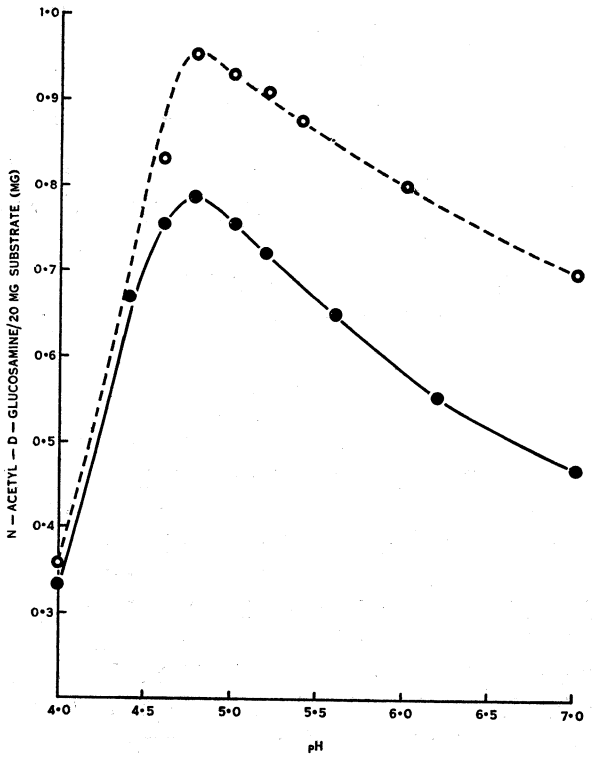

Fig. 1.—Effect of pH on the amount of *N*-acetyl-D-glucosamine formed during the enzymic degradation of chitin (●—●—●) and of the sodium salt of chitin sulphuric acid (O—O—O).

The appearance of D-glucosamine in the enzymic hydrolysate of the sodium salt of chitin sulphuric acid (in excess of that obtained from chitin) indicates that some deacetylation has occurred during the sulphation of chitin. The sulphur content of the sodium salt of chitin sulphuric acid corresponds to approximately 1.4 sulphate groups per *N*-acetyl-D-glucosamine residue. Since only D-glucosamine and *N*-acetyl-D-glucosamine were detected and estimated in the enzymic hydrolysate a sulphatase must be present in the snail enzyme preparation which attacked the *N*-acetyl-D-glucosamine sulphuric acid. The alternative explanation that in the sulphated chitin only about half the *N*-acetyl-D-glucosamine residues carry sulphuric acid groups is not attractive.

That *N*-acetyl-D-glucosamine is in fact the compound formed when snail chitinase reacts with chitin or with the sodium salt of chitin sulphuric acid is

shown by the filter paper partition chromatographic studies mentioned above and by isolating 44 per cent. of the theoretical yield of *N*-acetyl-D-glucosamine from chitin and 330 mg from 1 g of the sodium salt of chitin sulphuric acid. Neither of these figures is corrected for unreacted substrate. In both cases D-glucosamine was present in the hydrolysate and it was estimated by the Elson-Morgan method. The D-glucosamine obtained from chitin represented *c.* 1 per cent. of the *N*-acetyl-D-glucosamine isolated and that from the sodium salt of chitin sulphuric acid 1.5 per cent. of the *N*-acetyl-D-glucosamine isolated. Hydrolysis of chitin nitrate or of the sodium salt of chitin sulphuric acid by 6N aqueous hydrochloric acid at 100°C leads to the formation of D-glucosamine, as is shown by the isolation of 2-hydroxynaphthylidene glucosamine. The amount of Schiff base isolated agrees very well with the amount of D-glucosamine estimated to be present. However, comparison of these results with those obtained in the enzymic degradation of the sodium salt of chitin sulphuric acid indicates that considerable decomposition must occur in hot acid solution.

The structure of chitin is a problem which has received a great deal of attention and it is generally accepted that chitin is a polymer built up of *N*-acetyl-D-glucosamine residues. The question as to whether there are any non-acetylated amino groups in chitin is possibly of great biological importance but remains unanswered. Chemists investigating the structure of chitin have invariably used a "purified" chitin, i.e. cuticle which has been repeatedly extracted with hot dilute aqueous alkali or acid, or both. Such samples of chitin give a positive ninhydrin and a positive periodic acid-Schiff (PAS) reaction. This indicates that in the purification process the chitin has undergone some degree of deacetylation. That this is so for chitin treated with hot dilute aqueous hydrochloric acid (cf. Fraenkel and Rudall 1940) is shown by the appearance of D-glucosamine in the aqueous extract and after 7 hr at 100°C 6.6 per cent. conversion of chitin to D-glucosamine occurred. Not only are the amino groups deacetylated but the chitin is hydrolysed. The effect of alkali is more difficult to assess as free D-glucosamine is not stable. However, estimation of the volatile acids formed from purified chitin by the action of 1N aqueous alkali at 100°C for 48 hr indicates that methods of purification involving such alkali treatments remove 1.3 per cent. of the acetyl groups. Again, enzymic degradation of purified chitin produces a small amount of D-glucosamine (cf. Karrer and Hoffmann 1929; Veldkamp 1952) but since the enzymes used cannot be considered pure, this deacetylation could be enzymic. There is little doubt, therefore, that such purified chitin contains free amino groups. The accepted structure of chitin is one which contains no α-glycol groups and no free amino groups and therefore it is expected that chitin would give a negative PAS reaction and a negative ninhydrin reaction. Richards (1952) showed the presence of PAS-positive substances in the epicuticle of many insects and assumed the presence of polysaccharides. However, as mentioned by Brunet (1952), a more reasonable conclusion is that the dihydric phenols known to be present in the epicuticle give the positive PAS reaction. The presence or absence of a positive PAS reaction in various insect procuticles noticed by Richards is more difficult to explain.

The procuticle is considered to be made up of chitin and protein. A positive PAS reaction therefore means that either the chitin contains free amino groups or that some unidentified material reacting positively in the PAS reaction is present. Such unidentified material could be polyphenols, which are being transported through the procuticle, or protein-carbohydrate complexes, which are known to be present in insect cuticles (Hackman 1953; Trim 1941).

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