STUDIES ON CHITIN

V. THE ACTION OF MINERAL ACIDS ON CHITIN

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Summary

Chitin undergoes rapid and extensive degradation at 20°C when dissolved in 10N hydrochloric acid, 21N sulphuric acid, or 85% phosphoric acid. Most of the degradation occurs in the first few minutes after the chitin is dissolved and the products formed are oligosaccharides (which are to some extent deacetylated) and *N*-acetyl-D-glucosamine. Glucosamine was not found except possibly in the smallest traces. "Colloidal" chitin, prepared by precipitation of chitin from solution in cold 21N sulphuric acid, has a very much shorter chain length than the chitin from which it was prepared. Chitin is more stable in dilute (2N) hydrochloric acid. At 25°C only a negligible amount of hydrolysis occurs in 24 hr but at 100°C significant amounts of glucosamine and oligosaccharides are formed. For complete hydrolysis of chitin with 5·7N hydrochloric acid it is necessary to heat at 100°C for several hours. The preparation of chitin as a substrate for the enzyme chitinase is discussed in the light of these findings.

I. Introduction

Chitin, which is considered to be a polymer of *N*-acetyl-D-glucosamine (i.e. 2-acetamido-2-deoxy-D-glucose), is an important structural substance of arthropods, of some other invertebrates, and of some fungi. For reviews on the occurrence of chitin in nature see Foster and Webber (1960), Richards (1951, 1958), and Rudall (1955). Chitin is always associated with protein with which it forms covalently linked complexes (Hackman 1960). Considerable interest is now being taken in the chitinase complex of enzymes (e.g. Berger and Reynolds 1958; Jeuniaux 1959; Waterhouse, Hackman, and McKellar 1961; and Waterhouse and McKellar 1961), and for work in this field to be meaningful it is necessary to have substrates of known composition. Oligosaccharides of *N*-acetyl-D-glucosamine can now be prepared by acetylation of the acidic degradation products of chitosan (deacetylated chitin) (Barker *et al.* 1958). These compounds are useful for investigating the occurrence and properties of the chitobiase type of enzyme system. To study enzymes which can be correctly classed as chitinases it is necessary to use an undegraded chitin as a substrate.

Because chitin always occurs associated with other substances, purification procedures usually require lengthy treatments with cold dilute acids (e.g. for removal of calcium carbonate) and hot dilute alkali (e.g. 1N aqueous sodium hydroxide at 100°C to remove proteins and other organic material). Some workers have considered it necessary to decolourize the product by oxidation with dilute potassium permanganate. As a final step in the purification many workers dissolve

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chitin in cold concentrated hydrochloric acid (or other mineral acid) and precipitate it by the addition of water. Such drastic treatments may well cause structural changes in chitin.

As a substrate for investigation of chitinase activity most workers have prepared a so called "colloidal" chitin (e.g. Jeuniaux 1958; Berger and Reynolds 1958) while others (Waterhouse, Hackman, and McKellar 1961; Waterhouse and McKellar 1961) have used a finely powdered native chitin (a chitin–protein complex). To prepare "colloidal" chitin "pure" chitin is dissolved in cold concentrated mineral acid (e.g. 10N hydrochloric acid or 21N sulphuric acid) and after a suitable period of time in solution the chitin is precipitated by the addition of water, aqueous ethanol, or aqueous acetone. The length of time the chitin remains in solution depends on the temperature. For the preparation of a reproducible and stable suspension the chitin may have to be in contact with acid for many hours at 4°C. Popowicz (1959) prepares "colloidal" chitin by first dissolving chitin in boiling concentrated nitric acid.

Treatment of chitin with concentrated mineral acids would be expected to cause degradation and, in fact, Clark and Smith (1936) concluded on the basis of X-ray diffraction studies that even at room temperature the glycosidic linkages of chitin are hydrolysed by concentrated hydrochloric acid within 75 min and that concurrently but more slowly the amide groups are also hydrolysed. However, the extent of the degradation is not known. Meyer and Wehrli (1937) report that chitin undergoes degradation when dissolved in mineral acids (hydrochloric, sulphuric, phosphoric, and nitric acids). Zechmeister and Toth (1931, 1932) dissolved chitin in hydrochloric acid and kept the solution at 20°C for 15 days. From the solution they isolated *N*-acetyl-D-glucosamine, chitobiose as the octa-acetate, and chitotriose as the undeca-acetate.

In this paper a study has been made of the action of concentrated and dilute mineral acids on chitin at room temperature and at 100°C. Particular attention has been paid to the rate of degradation of chitin when dissolved in acids and the type of products which are formed.

II. Experimental and Results

(*a*) *Preparation of Chitin*

Clean dry shell of the marine crayfish *Jasus verreauxi* H. M. Edw. was ground to a powder in a vibrating ball mill. The powdered shell was decalcified by extraction with cold 2N hydrochloric acid and the residue repeatedly extracted with 1N aqueous sodium hydroxide at 100°C. The insoluble residue was collected and washed with water till of neutral reaction and then washed with ethanol and ether and dried. That fraction which passed through a 200-mesh sieve was used in the experiments described below. (Found: N, 6·9% corrected for ash (5·3%). Calc. for $(C_8H_{13}O_5N)_x$: N, 6·9%.) Glucosamine and small amounts of aspartic acid and histidine were detected in hydrolysates (HCl) of this chitin. Full details of the method for the preparation of chitin are given by Hackman (1954, 1960).

For some of the hydrolyses described in Section II(*d*) below with 2N and 5·7N hydrochloric acid, chitin prepared from puparia of *Lucilia cuprina* (Wied.) was used—for details of the preparation see Hackman (1960).

(*b*) *Chitin Turbidity Measurements*

A Brice–Phoenix Universal light-scattering photometer, 1000 series, was used together with a Leitz xenon arc lamp and monochromator (wavelength used 5100 Å). All solvents were clarified by centrifugation. All measurements were made at 20°C in a constant-temperature room in which all solvents were kept and all solutions were prepared. The nature of the experiments described below prevented the solvents and solutions being clarified by filtration so they were examined visually for the presence of dust. No bright points of light were seen when the forward scattered light was viewed at a small angle to the incident beam. Dust contamination was therefore low. Preliminary experiments showed that a linear relation existed between turbidity and concentration for concentrations of chitin up to 20 mg per 10 ml 10N hydrochloric acid.

(i) *Hydrochloric Acid*.—Chitin (5 mg) in a stoppered test tube was wetted with 1N hydrochloric acid (1 ml) and 11N hydrochloric acid (9 ml) added. The chitin went into solution immediately and the test tube was inverted three times to ensure that a solution of uniform concentration was obtained. The solution was transferred carefully to a 1 cm rectangular cuvette and the cell cover placed in position. The light scattered at 90° was measured over a period of 24 hr together with the corresponding measurements of transmitted light at the 0° position. The light scattered by and transmitted by the solvent was also measured. The results are given in Figure 1. The turbidity is the solute turbidity obtained by subtracting the apparent turbidity of the solvent from the apparent turbidity of the solution.

(ii) *Sulphuric Acid*.—Chitin (5 mg) in a stoppered test tube was wetted with 10N sulphuric acid (2·67 ml) and 7·33 ml 25N sulphuric acid, cooled to 4°C, added. Cooled sulphuric acid was added to prevent the temperature of the solution rising above 20°C. The chitin went into solution immediately and the experiment was completed as in Section II(*b*)(i) above. Because the solution was somewhat viscous it was necessary to let it stand in the cuvette for a time until all striations had disappeared. The first readings of scattered and transmitted light were therefore taken after a time interval of 20 min had elapsed and additional measurements were taken during the following 24 hr. The results are given in Figure 1*B*. Measurements were made on many solutions prepared at different times and from these it became obvious that, even though measurements made during the first 20 min were likely to be unreliable (due to striations), the initial solute turbidity was high and comparable to that of solutions in hydrochloric acid.

(iii) *Phosphoric Acid*.—Chitin (5 mg) in a stoppered test tube was wetted with water (0·45 ml), 89% phosphoric acid (9·55 ml) added, and the solution mixed well. The chitin went into solution immediately and the solution was centrifuged for 5 min at 1500 *g* to eliminate air bubbles. The solution was transferred carefully to a 1 cm cuvette and allowed to stand until all striations had disappeared.

Measurements of the light transmitted and scattered by the solution and solvent were made for the time interval 30 min to 24 hr (from time of first preparing the solution). Solute turbidities were calculated and results are given in Figure 1*B*.

(iv) *"Colloidal" Chitin.*—"Colloidal" chitin was prepared as follows: Dry powdered chitin (5 g) was suspended in 10N sulphuric acid (120 ml) at −10°C and 25N

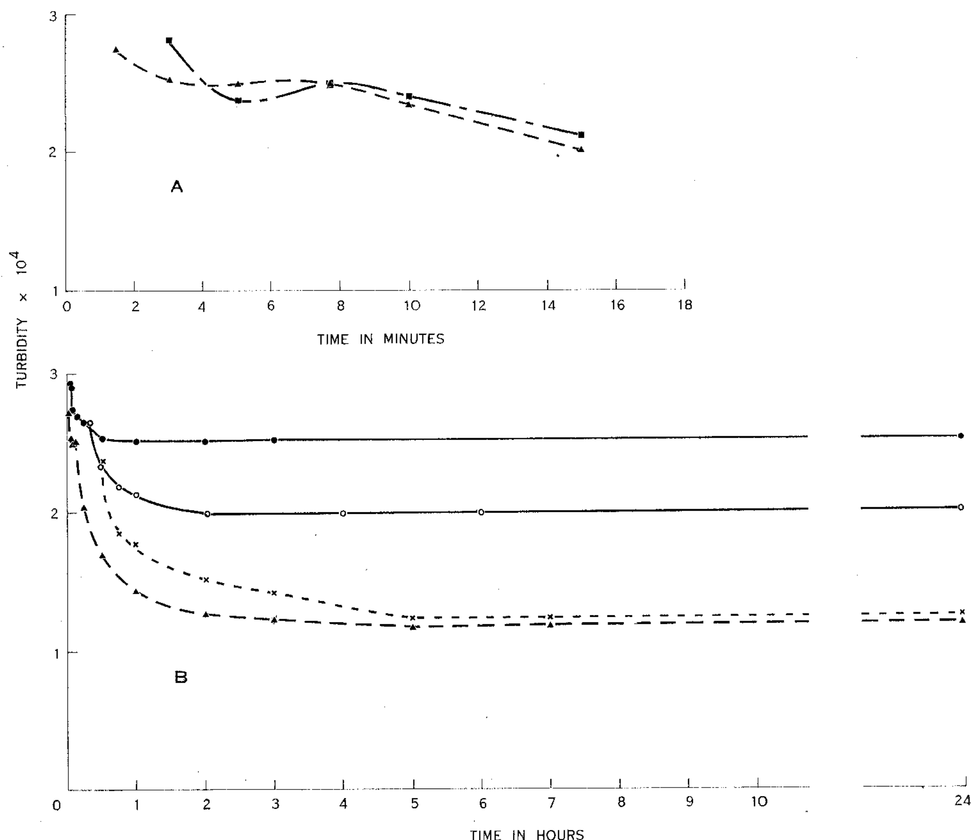

Fig. 1.—Change in turbidity with time for solutions of chitin (5 mg) in concentrated mineral acids (10 ml):

A ▲— — — —▲ 10N hydrochloric acid (curve 1).
■- — — — -■ 10N hydrochloric acid (curve 2).

B ▲— — — —▲ 10N hydrochloric acid.
x - - - - - - - - - x 21N sulphuric acid.
O————O 85% phosphoric acid.
●————● "Colloidal" chitin in 10N hydrochloric acid.

sulphuric acid (330 ml) was added slowly with continuous stirring (at −10°C) to give a final acid concentration of 21N. The temperature did not rise above 5°C and the solution was kept at 4°C for 18 hr. The solution was filtered through glass wool and added to water (5 l.) to precipitate finely dispersed chitin which was collected, washed with water, and dialysed against water until the aqueous suspension

was of neutral reaction. Unless the acidic solution was kept for at least 18 hr at 4°C the aqueous suspension of chitin was not stable.

To 1 ml of this suspension (containing 5 mg chitin) was added 11·11N hydrochloric acid (9 ml). The chitin went into solution immediately. Measurements of the light transmitted and scattered by the solution were made over 24 hr as in Section II(*b*)(i). Solute turbidities were calculated and the results are given in Figure 1*B*.

(*c*) *Products Formed during Solution of Chitin in* 10N *Hydrochloric Acid*

(i) Chitin (50 mg) in a 250-ml round-bottomed flask was wetted with 1N hydrochloric acid (10 ml) and 11N hydrochloric acid (90 ml) added. The chitin went into solution immediately. The flask was stoppered and the contents well mixed and kept in a water-bath at 20°C for 3 hr. The solution was evaporated to dryness *in vacuo* at 20°C on a rotary evaporator and the time required for the evaporation was 2 hr. The residue was kept overnight in a desiccator *in vacuo* over solid potassium hydroxide. This experiment was repeated seven times, the total weight of chitin used being 340 mg (on a dry, ash-free basis). The residue in each flask was extracted with six lots of water (5 ml), the extracts were filtered through glass filter paper, and the insoluble residue from each flask was washed onto the filter at the same time. The combined extracts were evaporated to dryness *in vacuo* at 20°C. Yield: 193 mg of solid containing 14·7% moisture, 10·4% ash (550°C), and 5·0% N. Yield (on a dry, ash-free basis): 144 mg (42%) containing 6·7% N. The water-insoluble material was recovered from the filter but this could not be done quantitatively. Nitrogen content of water-insoluble fraction was 6·5%.

(ii) The water-soluble and water-insoluble fractions were hydrolysed by heating with 5·7N hydrochloric acid at 100°C for 24 hr and the hydrolysates examined by ionophoresis on paper (acetate buffer, pH 5) as described by Hackman (1960). In both fractions glucosamine, aspartic acid, and histidine were detected.

(iii) Solutions of the water-soluble fraction were examined by the techniques of ionophoresis and chromatography on paper. Acetate buffer (pH 5) was used for ionophoresis according to the method as described by Hackman (1960). Material reacting with ninhydrin remained at the origin and there was possibly a trace of glucosamine present. One-dimensional filter paper partition chromatography (capillary-ascent method of Williams and Kirby 1948) was used for investigation of the sugars present in the water-soluble fraction. Two solvent systems were used, viz: pentan-2-ol–pyridine–water (1 : 1 : 1 v/v) (Jeans, Wise, and Dimler 1951) and isopropanol–water (80 : 20 v/v) (Smith 1960) and the sugars were detected with benzidine (Smith 1960), ninhydrin, and alkaline silver nitrate (Trevelyan, Proctor, and Harrison 1950). *N*-acetyl-D-glucosamine and oligosaccharides were detected together with a trace only of glucosamine. A considerable amount of material remained at the origin and it reacted with ninhydrin. After acidic hydrolysis only glucosamine was detected.

(iv) The amount of *N*-acetyl-D-glucosamine present in the water-soluble fraction was estimated by the method described by Tracey (1955). 5·6% of the fraction (on a dry, ash-free basis) was *N*-acetyl-D-glucosamine.

(v) To confirm the presence of free amino groups in the water-soluble fraction it was subjected to the action of nitrous acid (cf. Bera, Foster, and Stacey 1956). *N*-acetyl-D-glucosamine was unaffected by the method used except that it was converted to glucosamine on hydrolysis.

Water-soluble fraction (40 mg) in 0·2N hydrochloric acid (1·5 ml) was kept at room temperature for 5 hr to reach mutational equilibrium. Aqueous sodium nitrate (5%, 0·5 ml) was added, the solution mixed well, and kept at room temperature for 1 hr. The solution was aerated under reduced pressure for 2 hr to remove excess nitrous acid; hydrochloric acid was removed by extraction with 5% methyldi-n-octylamine in chloroform and the excess amine removed by extraction with chloroform and finally ether. The aqueous solution was evaporated *in vacuo* at <35°C (rotary evaporator) and the residue dried overnight *in vacuo* over solid potassium hydroxide and phosphorus pentoxide. The residue was dissolved in 2N hydrochloric acid (10 ml), heated at 100°C for 5 hr, and cooled to room temperature. Hydrochloric acid was removed by extraction with methyldi-n-octylamine, the solution evaporated, and the residue dried as before. The residue was dissolved in water (1 ml) and the solution subjected to ionophoresis and chromatography on paper.

Ionophoresis in borate buffer (pH 10) on Whatman No. 3 filter paper (Foster, Newton-Hearn, and Stacey 1956) and detection with alkaline silver nitrate revealed the presence of several components. One spot behaved in a manner identical with that of chitose prepared by the action of nitrous acid on glucosamine hydrochloride. Another spot proved to be glucosamine (positive ninhydrin reaction). Paper chromatography with isopropanol–water (80 : 20 v/v) as solvent and detection with ninhydrin, benzidine, and alkaline silver nitrate revealed the presence of a number of components, two of which were identified as glucosamine (an intense spot) and chitose (a weak spot) by comparison with the behaviour of authentic samples under the same conditions.

(*d*) *Acidic Hydrolysis of Chitin*

(i) Chitin (10 mg) and 5·7N hydrochloric acid (10 ml) were heated together at 100°C for 5 or 24 hr. The solution was filtered through glass filter paper, the flask and filter washed well with water, and the filtrate and washings evaporated to dryness *in vacuo* (rotary evaporator). The residue was kept overnight *in vacuo* over phosphorus pentoxide and solid potassium hydroxide. For chromatography on paper the residue was dissolved in 0·5 ml water. For estimation of glucosamine (method of Belcher, Nutten, and Sambrook 1954) the residue was dissolved in 50 ml water. When the hydrolysis was carried out in an open flask consistently higher recoveries of glucosamine were obtained than when the hydrolysis was carried out in a sealed tube in an atmosphere of nitrogen. If during the evaporation the solution became yellow the yield of glucosamine was reduced.

The percentage of chitin (from *J. verreauxi* or from *L. cuprina*) recovered as glucosamine after 24 hr heating with hydrochloric acid was 72·2–86·4. After heating for 5 hr the value for *J. verreauxi* chitin was 73·0% and for *L. cuprina* chitin

56·4%. When glucosamine was heated with 5·7N hydrochloric acid for either 5 or 24 hr the amount of glucosamine recovered was 73·7–86·9%.

Whatman No. 1 filter paper and the following solvent systems were used for examination of the hydrolysate by chromatography: butan-1-ol–pyridine–water (6 : 4 : 3 v/v) (Heyworth, Perkins, and Walker 1961), using both untreated paper and paper given a preliminary treatment with 0·1M barium chloride; isopropanol–water (80 : 20 v/v); and pentan-2-ol–pyridine–water (1 : 1 : 1 v/v). Ninhydrin, benzidine, alkaline silver nitrate, and the hexosamine reagent (Partridge 1948) were used to detect sugars. The only sugar detected was glucosamine. *N*-acetyl-D-glucosamine and the amino sugars (other than glucosamine) given by Heyworth, Perkins, and Walker (1961) could not be detected. Application of the method of Stoffyn and Jeanloz (1954) (deamination with ninhydrin) resulted in the detection of arabinose only.

(ii) Chitin (10 mg) was wet with 1N hydrochloric acid (1 ml) and 11N hydrochloric acid (9 ml) added. The chitin went into solution and was kept at 45°C for 15 hr (see Irvine 1909) and the experiment completed as in Section II(*d*)(i). 60·3% of the chitin was recovered as glucosamine. When glucosamine was treated with hydrochloric acid under the same conditions as used for chitin 73% of the glucosamine was recovered.

(iii) Chitin (1 g) was treated with 2N hydrochloric acid (20 ml) at 25°C or at 100°C for 24 hr, and the experiments completed as in Section II(*d*)(i). At 25°C only traces of glucosamine could be detected. At 100°C and with chitin from *L. cuprina* puparia 4·8% of the chitin was recovered as glucosamine and a further 3·3% was present as soluble oligosaccharides (hydrolysis of the soluble material with 5·7N hydrochloric acid and estimation of the additional glucosamine formed). No *N*-acetyl-D-glucosamine could be detected by chromatography on paper but the oligosaccharides may have contained *N*-acetyl-D-glucosamine residues. The insoluble chitin residue had a nitrogen content of 6·5%. When chitin from *J. verreauxi* was treated similarly the insoluble residue had a nitrogen content of 7·3%. (All results have been calculated on a dry, ash-free basis.)

III. Discussion

Measurement of the light scattered by a solution and by the solvent is used to calculate the solute turbidity and from this the molecular weight (weight average) of the solute. Thus turbidity measurements give an indication of molecular weight. Provided chitin is dissolved rapidly by concentrated mineral acids measurement of turbidity can be used to study the rate at which chitin is degraded. Dry powdered chitin does not dissolve rapidly in concentrated mineral acids because jelly-like aggregations are formed. Even vigorous stirring of the solution does not overcome the difficulty. However, chitin can be dissolved rapidly if it is first wetted with dilute acid or water and then concentrated acid added to give the desired final acid concentration. The solutions could not be clarified by filtration but dust contamination must have been low since visual examination failed to show the presence of dust particles.

The results reported in this paper clearly demonstrate that chitin undergoes rapid and extensive degradation at 20°C when dissolved in 10N hydrochloric acid. This conclusion is based on the turbidity measurements given in Figure 1 and the study of the products formed during solution of chitin in hydrochloric acid. The molecular weight is considerably reduced in the first few minutes. The rate of degradation then decreases and after a few hours an approximately stable condition is reached. The amount of degradation is greater than that indicated by the curves in Figure 1. The turbidity measurements show chitin to be a very large molecule and in order to calculate molecular weights the turbidity would have to be corrected for dissymmetry. This correction factor would decrease as the degradation proceeds and the molecule becomes smaller.

If the solution is evaporated to dryness when degradation appears to have ceased (after 3 hr) it is found that 42% of the chitin has been converted to water-soluble products. Glucosamine was not formed except possibly in the smallest of traces but 5·6% of the water-soluble material was *N*-acetyl-D-glucosamine. The remainder of the water-soluble material consisted of higher oligosaccharides which were to some extent deacetylated. That deacetylation had occurred was shown by paper ionophoretic and paper chromatographic studies (presence of components which reacted with ninhydrin) and by the presence of chitose in hydrolysates of the water-soluble fraction after it had been treated with nitrous acid. The nitrogen content of the water-soluble fraction was consistent with some deacetylation having taken place. In view of this deacetylation the absence of glucosamine is explained by the fact that the positive charge acquired by the amino groups electrostatically shields the neighbouring glycosidic substituent from attack by hydrions (cf. Foster, Horton, and Stacey 1957). The water-insoluble residue had a nitrogen content of 7·3% which, being higher than that of the original chitin, indicates that deacetylation has occurred. Hackman (1960) has shown that chitin, as it occurs naturally, is bound to protein and even after extensive purification amino acids (aspartic acid and histidine) can be detected in hydrolysates of chitin. These amino acids were detected in hydrolysates of both the water-soluble and water-insoluble fractions.

The rate of degradation of chitin by concentrated acids depends upon the agitation which the solution receives. When preparing solutions of chitin in acids variations in the amount of agitation occurred during the first 4 min after solution was effected, after this the solution remained undisturbed. The effect of this agitation is shown by the two curves in Figure 1*A* for solution in hydrochloric acid. Although the curve was not completely reproducible for degradation occurring in the 0–10 min time interval the increase in turbidity after 5 min always appeared, in some experiments more pronounced than in others. Curve 1 is typical, curve 2 is the least increase that was recorded. After an interval of 15 min the curves were reproducible. This increase in turbidity could be brought about by an increase in molecular weight. Reversion, the linking together of fragments of the chitin chain under the influence of a strong acid (cf. Foster and Horton 1958), would cause an increase in molecular weight. Since the chitin is being degraded the possibility of a change in shape should not be overlooked. At some stage in the degradation of chitin (or other very large molecule) fragments may be formed which would

immediately assume a shape different from that of the intact molecule. The fragments would take on the most stable configuration determined by their own physical and chemical properties. Depending on the nature of the change in shape the correction factor for dissymmetry would be decreased or increased without any change in the molecular weight. A decrease in the correction factor would cause an increase in turbidity.

The rapid degradation of chitin when in solution in acids is not confined to hydrochloric acid. Similar decreases in molecular weight occurred when chitin was dissolved in 21N sulphuric acid or in 85% phosphoric acid. Because of the viscous nature of these solutions it was not possible to obtain reproducible measurements of scattered light until some 20 or 30 min after dissolving the chitin in these acids. The curves obtained by plotting turbidity against time were very similar to those obtained with hydrochloric acid and showed that considerable degradation had occurred. Sulphuric acid could also bring about sulphation of the chitin chains.

"Colloidal" chitin, prepared by precipitation from solution in 21N sulphuric acid, was dissolved in 10N hydrochloric acid and the solute turbidity measured over a period of 24 hr. As can be seen by the shape of the curve given in Figure 1*B* the "colloidal" chitin did not undergo the extensive degradation reported above and, except for a small initial drop in molecular weight, the molecular weight appeared to remain unchanged. This result indicates that the initial solution in 21N sulphuric acid brought about the extensive degradation reported above and that the "colloidal" chitin has a very much shorter chain length than has chitin and it must have undergone some deacetylation. Methods for the preparation of "colloidal" chitin indicate quite clearly that stable suspensions are formed only after sufficient degradation of the chitin has taken place.

It is well known that 5·7N hydrochloric acid, at 100°C or at its boiling point, eventually hydrolyses all the glycosidic and amide linkages in chitin to give glucosamine. The yield of glucosamine depends upon such factors as the particle size and source of the chitin and the duration of the hydrolysis. Further losses may occur if the solution is evaporated to dryness. Chitin prepared from *L. cuprina* puparia (Hackman 1960) was more resistant to hydrolysis than the chitin prepared from *J. verreauxi*. In the experiments described in this paper 72–86% of chitin was recovered as glucosamine and consistently higher yields were obtained when the hydrolysis was carried out in an open flask than if a sealed tube was used. Under the identical conditions used for the hydrolysis of chitin recoveries of pure glucosamine hydrochloride were similar, indicating that the hydrolysis of the chitin was probably complete. Ogston and Stanier (1950) heated glucosamine hydrochloride with 4N hydrochloric acid in a sealed tube in boiling water for 8 hr and obtained a rather variable recovery of glucosamine (72–96%) while Haab and Anastassiadis (1961) reported that 4N hydrochloric acid at 100°C in sealed tubes caused no decomposition of glucosamine in 24 hr (see also Johansen, Marshall, and Neuberger 1960). Purchase and Braun (1955) also reported a 56–66% yield of glucosamine hydrochloride from chitin. For complete hydrolysis of chitin with 5·7N hydrochloric acid it was necessary to heat at 100°C for some hours. Glucosamine was the only sugar which could be detected in the hydrolysates. The method described by Irvine (1909), viz.

solution in concentrated hydrochloric acid at 45°C for 10 or 15 hr, gave much lower yields of glucosamine. The low yields are no doubt related to the method of preparation of the chitin. Irvine prepared his chitin by the "usual method" and precipitated it repeatedly (presumably from acid) until it was "perfectly white" and free of ash. From the experiments described in this paper it can be concluded that Irvine used an extensively degraded sample of chitin.

Chitin is more stable in dilute (2N) hydrochloric acid. At 25°C only a negligible amount of hydrolysis occurs in 24 hr. After 24 hr at 100°C, 4·8% of the chitin was recovered as glucosamine and a further 3·3% as soluble oligosaccharides. No *N*-acetyl-D-glucosamine was detected. The nitrogen content of the insoluble residue was 6·5% (corrected for ash) which is slightly lower than the nitrogen content of the chitin before acid treatment. When the more finely divided chitin from lobster shell was heated at 100°C for 24 hr with 2N hydrochloric acid the nitrogen content of the insoluble residue was higher than that of the original chitin, indicating that deacetylation had occurred. These results do not agree with Giles *et al.* (1958) who report that the nitrogen content of chitin fell from 7·1 to 3·7% on being immersed in aqueous 2N hydrochloric acid at 60°C for 24 hr. They concluded that not only is the amide bond hydrolysed but the amino groups are also lost during the acid treatment. In view of the results given above and of the action of 4N, 5·7N, 10N, and concentrated hydrochloric acid on chitin and glucosamine, loss of half the amino groups from chitin would not be expected with 2N hydrochloric acid. Again, Folkes, Grant, and Jones (1950) autoclaved glucosamine hydrochloride in a sealed tube at 15 lb pressure for 5 hr with 3N hydrochloric acid and obtained 20 and 21% loss of nitrogen as ammonia.

From the results reported in this paper it is concluded that although chitin is relatively stable in cold dilute acids it is less stable in hot dilute acids, while in cold concentrated acids extensive degradation occurs. It therefore follows that to prepare chitin in the least-degraded form it should be subjected only to the action of cold dilute acid for the removal of inorganic material and to the action of hot dilute alkali for the removal of protein and other extraneous organic matter. Even these treatments bring about some degradation (see Hackman 1954 and results given in this paper) and the use of ethylenediaminetetraacetic acid for the removal of inorganic matter is undoubtedly better (Foster and Hackman 1957; Hackman 1960). Precipitation from concentrated mineral acids cannot be recommended because of the very extensive degradation which occurs. To prepare stable suspensions of colloidal chitin the chitin must remain in solution in acid for some time. Extensive degradation must take place before the chitin can be precipitated as a stable suspension.

When "colloidal" chitin, prepared by precipitation of chitin from solution in acid, is used as a substrate in enzymic studies it should be remembered that a much degraded chitin is being used. Enzymes capable of digesting this substrate may not be able to attack "native chitin" (i.e. chitin as it occurs in nature) or chitin which has not been subjected to solution in acid. Crude chitinase preparations hydrolyse colloidal chitin to a greater extent than native chitin (Waterhouse, Hackman, and

McKellar 1961). However, these two substrates differed in particle size as well as in molecular weight and the relative importance of these two factors is not known.

Irvine (1909) has reported that in hydrochloric acid solution chitin initially has $[\alpha]_D^{20}$ $-14°$ which slowly changes to $+56°$ due to hydrolysis. His method for preparing chitin almost certainly brought about considerable shortening of the chains and therefore it is very likely that the true initial $[\alpha]_D$ for chitin differs considerably from the result he has given. A similar comment applies to any measurements made on chitin precipitated from solution in acid.

Chitin is said to be soluble in anhydrous formic acid (e.g. Schulze and Kunike 1923) but not all workers have succeeded in dissolving chitin in formic acid. This is well illustrated by Rudall (1955) who reports that purified chitin of the beak and skeletal pen of *Loligo* dissolves readily in strong formic acid while, under the same conditions, chitin from purified blowfly puparia does not dissolve. Chitin from the beak of *Loligo* and from the puparia of blowflies is described as α-chitin, that from the pen of *Loligo* as β-chitin. Rudall suggests that the difference in the behaviour of these two samples of α-chitin may be due to the more crystalline nature of the chitin in arthropod cuticles (i.e. the blowfly puparia). From published results it is not clear whether chitin undergoes degradation when in solution in formic acid.

Differences in the crystalline nature of chitin may account for the somewhat contradictory reports in the literature on the solubility of chitin in mineral acids. Meyer and Wehrli (1937) reported that chitin is soluble in both 45 and 85% phosphoric acid, degradation occurring in both solutions. Danilov and Plisko (1954) report that phosphoric acid (40–60%) does not dissolve chitin even after prolonged contact but 78·4–97·2% phosphoric acid gives complete solution with progressive hydrolysis of the chitin in time. Again Meyer and Wehrli (1937) report that chitin is soluble in 45 and 50% nitric acid but not completely soluble in 68% acid. Popowicz (1959) prepares "colloidal" chitin by first dissolving chitin in hot concentrated nitric acid. Such conflicting reports probably indicate that the chitins used have differed. All the chitins used were obtained from the shells of Crustacea and so would be classed as α-chitin.

Only α-chitin has been used in the work described in this paper so some reference should be made to the stability of β-chitin to acids. β-chitin is less crystalline than α-chitin and is readily soluble in formic acid. β-chitin is converted to α-chitin by cold 6N hydrochloric acid or by solution in formic acid (Rudall 1955 and personal communication) and by 45% fuming nitric acid (Lotmar and Picken 1950). β-chitin is stable in cold 3N hydrochloric acid and in hot dilute alkali (Rudall, *vide supra*). From these properties β-chitin would be expected to undergo similar degradation to α-chitin in solution in concentrated mineral acids because it would be transformed to α-chitin.

IV. Acknowledgment

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