STUDIES ON CHITIN

VI. THE NATURE OF α- AND β-CHITINS

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Summary

Enzymic digestion, acidic hydrolysis, deuterium exchange, infrared absorption spectra, and differential thermal analysis of chitins show that not only do the structures of α- and β-chitins differ but that differences occur within the α-chitins and within the β-chitins. The crystalline structure within an α-chitin varies and about 30% of the chitin is in a highly crystalline form. β-Chitins prepared from cuttlefish shell and squid pen contain significant amounts of free amino groups, on average one residue in five and two residues in five respectively being unacetylated. The free amino groups are in the less highly organized parts of the structure. The structures of the chitins and their associations with proteins are discussed.

I. INTRODUCTION

Although all chitins are considered to be polymers of 2-acetamido-2-deoxy-D-glucopyranose (i.e. *N*-acetyl-D-glucosamine) linked in a 1,4-β-glycosidic manner three different forms of chitin are recognized. These are α-, β-, and γ-chitin. Differences are concerned with the arrangement of the chains and the presence of bound molecules of water. Rudall (1963) has reviewed the latest ideas on the structure of chitins. β-Chitin is considered to have a more open type of chain structure than α-chitin and so its structure is such that it is more readily penetrated by chemical reagents. α-Chitin would appear to be the most stable form because both β- and γ-chitin are converted to α-chitin by the action of cold concentrated mineral acids and α-chitin is the form found when chitin is precipitated from solution. The conversion of α-chitin to β- or γ-chitin has not been reported. It is probable that there are some glucosamine residues in the chitin chains, i.e. all the residues are not *N*-acetylated.

X-ray diffraction is the only method by which these three forms of chitin have been identified. In this paper a study has been made of the action of hydrolytic agents on different forms of chitin. Deuterium exchange, infrared absorption spectra, and differential thermal analysis have also been investigated. The structures of α- and β-chitins are discussed.

II. EXPERIMENTAL AND RESULTS

(*a*) *Materials*

(i) *Chitins.*—Samples of chitin were prepared from the powdered shell of the crab *Scylla serrata* (Forskal), of the marine crayfish *Jasus verreauxi* H. M. Edw., of the freshwater crayfish *Cherax albidus* Clark, and of cuttlefish by the method described by Hackman (1962). A modification of this method (Hackman 1960) was used to

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prepare chitin from empty puparia of the blowfly *Lucilia cuprina* (Wied.), from the larval skins of the beetle *Agrianome spinicollis* McL., from the pen of the squids *Loligo australis* Gray and *Sepioteuthis australis* Quoy & Gaimard, and from the beak of the squid *L. australis*. The chitin from the pens of the squids was β-chitin and that from cuttlefish, although resembling in some respects γ-chitin, was probably a β-chitin (Rudall, personal communication). All the other chitins, i.e. those from arthropods, were α-chitins.

Chitosan hydrochloride was prepared from *Jasus* chitin by the method described by Barker *et al.* (1958). A sample of this hydrochloride was converted to the free base by precipitation from an aqueous solution with aqueous NaOH. The precipitate was collected, washed with water till free of alkali, and then with ethanol and ether and dried.

When incinerated all the chitins, except those from the squids, gave small amounts of ash. The quantities of chitin given in the experiments described below are on an ash-free basis. Each sample of chitin was sieved and two fractions collected —that which passed through a 150-mesh but not a 200-mesh sieve (fraction 1) and that which passed through a 200-mesh sieve (fraction 2). On an ash-free basis the nitrogen contents (Kjeldahl) of the following chitins were: *S. serrata*, 6·9%; *J. verreauxi*, 6·9%; *Lucilia cuprina*, 7·0%; *Loligo australis* pen, 7·4%; cuttlefish, 7·2%.

(ii) *Enzyme.*—A chitinase preparation was obtained from puff-balls (*Lycoperdon perlatum*) as described by Hackman and Goldberg (1964*a*).

(*b*) *Enzymic Hydrolysis*

Chitin (50 mg, fraction 1) was hydrolysed for 5 or 24 hr at 35° C with *Lycoperdon* chitinase (5 mg) in 10 ml (total volume) of acetate buffer (final concn. 0·08M, pH 4·4). The digest was centrifuged and the supernatant analysed for *N*-acetyl-D-glucosamine by the method described by Tracey (1955) except that the amount of borate used was increased to 0·5 ml. Citrate buffer (final concn. 0·05M, pH 4·5) could be substituted for the acetate buffer and gave identical results with no decrease in colour yield. With these experimental conditions the pH optimum for the reaction with each sample of chitin was found to be between 4·4 and 4·5. The results are given in Table 1. Also included in the table is the result obtained with fraction 2 of a sample of cuttlefish chitin.

The digests from the chitins were examined by paper chromatography [Whatman No. 1 filter paper and the solvent system pentan-2-ol–pyridine–water (1 : 1 : 1 v/v)]. The positions of the sugars were detected with alkaline silver nitrate, benzidine, the hexosamine reagent of Salton (1959), and the chlorination technique (Powning and Irzykiewicz 1963). The digests from the *Loligo* and cuttlefish chitins contained an oligosaccharide (shown below to be a triose) as well as *N*-acetyl-D-glucosamine. The amount of the oligosaccharide, as estimated from the paper chromatograms, was a little larger than that of the *N*-acetyl-D-glucosamine present, the *Loligo* chitin producing somewhat more of the oligosaccharide than did the cuttlefish chitin. Trace amounts only of other oligosaccharides were present. No oligosaccharides were detected in the digests from *Lucilia*, *Scylla*, or *Agrianome* chitins. Glucosamine was

present in all digests but amounted to less than 3% of the amount of *N*-acetyl-D-glucosamine found. These results were confirmed by ionophoresis on Whatman No. 3 paper using an acetate buffer (0·1M, pH 5, potential gradient 20 V/cm for 1 hr) in the apparatus described by Hackman and Goldberg (1964*b*).

Cuttlefish chitin (50 mg, fraction 2) was hydrolysed enzymically as described above and gave 6·6, 10·3, and 17·0 mg *N*-acetyl-D-glucosamine after 12, 24, and 48 hr digestion, respectively. After 12 and 24 hr digestion there was also present the oligosaccharide mentioned above. After digestion for 48 hr the amount of oligosaccharide present was the same as that present after 24 hr digestion. A colloidal sample of cuttlefish chitin (for preparation see Hackman and Goldberg 1964*a*) when hydrolysed enzymically (10 mg chitin, 5 mg enzyme, 5 ml total volume acetate buffer at 35°C for 5 hr) gave 7·35 mg *N*-acetyl-D-glucosamine, 0·22 mg glucosamine, and more than 2 mg of the same oligosaccharide.

TABLE 1

ENZYMIC DIGESTION OF CHITINS

Substrate (particle size 150–200 mesh, 50 mg) and *Lycoperdon* enzyme (5 mg) in acetate buffer (0·08M, pH 4·4, 10 ml) at 35°C

Source of Chitin	*N*-Acetyl-D-Glucosamine (mg) Formed after Digestion for:	
	5 Hr	24 Hr
Lucilia cuprina	0·15	0·32
Scylla serrata	0·80	1·51
Agrianome spinicollis	0·68	2·46
Loligo australis	3·70	14·00
Cuttlefish	2·28	7·83
Cuttlefish (pass 200 mesh)	3·45	10·30

The position of the oligosaccharide (from *Loligo* and cuttlefish chitins) on the paper chromatograms corresponded to that of a triose. It was not detected with ninhydrin but was detected with alkaline silver nitrate, benzidine, and the chlorination technique—it is therefore an amino sugar but not chitotriose. When subjected to ionophoresis on paper at pH 5 it migrated towards the cathode but with a migration rate less than either chitotriose or glucosamine. The oligosaccharide was eluted from paper chromatograms and selectively *N*-acetylated (Roseman and Ludowieg 1954), on a microgram scale, after which it was degraded to *N*-acetyl-D-glucosamine by *Lycoperdon* chitinase [as were all the oligosaccharides of *N*-acetyl-D-glucosamine prepared by the method of Barker *et al.* (1958)]. On paper chromatograms the acetylated oligosaccharide had an R_F value identical with that of tri-*N*-acetylchitotriose.

The same deacetylated triose was given by three different samples of cuttlefish chitin prepared on separate occasions over a period of three years. The yields of the triose were the same even though the cuttlefish shells were collected at different times.

A sample of cuttlefish chitin prepared by the use of EDTA (for preparation see Hackman 1960) was hydrolysed enzymically. This preparation contained about 50% chitin, the remainder being protein, and to maintain approximately the same ratio of chitin to enzyme 100 mg of the preparation was used. The preparation, which passed through a 200-mesh sieve, gave 13·9 mg *N*-acetyl-D-glucosamine after digestion for 24 hr. No oligosaccharides were present in the digest. Powdered pen of *Loligo australis* was also digested enzymically. Since the pen consisted of approximately one-third chitin and two-thirds protein, 150-mg samples were used. Very little digestion occurred in 24 hr and although *N*-acetyl-D-glucosamine was shown to be present in the digest no oligosaccharides were detected.

N-acetyl-D-glucosamine was digested with *Lycoperdon* enzyme under the same conditions as were used for the digestions of the chitins. A paper chromatographic examination of the digest failed to show the presence of any glucosamine. This result indicates that under the experimental conditions used the enzyme is not capable of deacetylating *N*-acetyl-D-glucosamine.

TABLE 2

ACIDIC HYDROLYSIS OF CHITINS

Chitin (particle size 150–200 mesh, 50 mg) in 2N HCl (10 ml) at 100°C

Period of Digestion (hr)	Chitin Hydrolysed (%)		
	Lucilia cuprina	*Loligo australis*	*Scylla serrata*
1	13·8	32·9	15·9
5	18·5	49·9	
24	27·5	88·7	

(*c*) *Hydrolysis with Hydrochloric Acid*

Lucilia and *Loligo* chitins (50 mg, fraction 1) were heated at 100°C with 2N HCl (10 ml) in sealed tubes for 1, 5, and 24 hr. In addition *Scylla* chitin was hydrolysed for 1 hr. The hydrolysates were cooled and their refractive indices measured in a Brice–Phoenix differential refractometer with 2N HCl as the reference liquid and compared with that of *N*-acetyl-D-glucosamine which had been hydrolysed in the same manner. This method gave an estimate of the total amount of chitin hydrolysed and the results are given in Table 2. The refractive index of an 0·5% solution of glucosamine hydrochloride in 2N HCl was not altered after it had been heated in a sealed tube at 100°C for 24 hr.

(*d*) *Deuterium Exchange*

All manipulations with deuterium oxide were conducted in a dry box and at no time was the deuterium oxide exposed to atmospheric moisture. The samples of chitin were dried *in vacuo* over phosphorus pentoxide for 4 months. A sample of each chitin was heated at 110°C *in vacuo* for 24 hr—none of the samples lost weight. All apparatus was dried at 110°C and then kept in the dry box until required.

Deuterium oxide (99·8% D_2O, 5 ml) was added to chitin (fraction 1, 400 mg, except for cuttlefish chitin when 200 mg was used) in a tube which was then stoppered, its contents thoroughly mixed, and allowed to stand, with occasional shaking, for 4 hr at room temperature. The stoppered tube was centrifuged for 10 min at 2000 *g* and the supernatant decanted. The absorbancy of a sample of the supernatant was measured in stoppered cells of 1 cm light path at 1·66 μ (Lecomte, Ceccaldi, and Roth 1953). A sample of the supernatant was frozen, lyophilized, and the absorbancy of the distillate measured at 1·66 μ. The results showed that it was unnecessary to distil the deuterium oxide. This together with the fact that the infrared spectra for the supernatant and for deuterium oxide containing water were similar showed that the supernatant probably consisted only of deuterium oxide and water. A calibration curve was prepared by determining the absorbancy of deuterium oxide to which had been added known amounts of water. The curve was linear over the range

TABLE 3
DEUTERIUM EXCHANGE — CHITIN IN LIQUID D_2O

Source of Chitin	Hydrogen Exchanged (mg-atom) per Gram of Chitin
Lucilia cuprina	9·66
Scylla serrata	11·08
Agrianome spinicollis	11·41
Cuttlefish	16·56
Loligo australis	18·35

0–4% water. The results are given in Table 3 and are expressed as the number of mg-atoms of hydrogen exchanged per gram of chitin. Fractions 1 and 2 of *Loligo* chitin gave identical results and the amount of exchange of deuterium for hydrogen after 1 hr was 17·6, after 2 hr 18·34, after 4 hr 18·35, and after 7 hr 18·38 mg-atom hydrogen exchanged per gram chitin.

(*e*) *Differential Thermal Analysis*

The apparatus consisted of an electrically heated cylindrical furnace in which the rate of increase in temperature was automatically controlled at 10° C per minute. The temperature differential between the sample (polysaccharide) and the inert standard (calcined alumina) was measured by a two-headed Pt–Pt-10% Rh differential thermocouple while the furnace temperature (that of calcined alumina in the sample holder) was measured by a Pt–Pt-10% Rh thermocouple. The voltage from the differential thermocouple was amplified and the data were recorded automatically on a Leeds and Northrup type G potentiometric recorder. There was free access of air during all determinations. The holes in the stainless steel sample holder had a capacity of 0·475 cm^3 and in view of reports that alumina does not behave in a completely inert manner when mixed with organic compounds, the samples were not mixed with nor sandwiched between alumina. All thermograms were reproducible. *N*-Acetyl-D-glucosamine, D-glucosamine hydrochloride, chitosan hydrochloride, and a chitodextrin [prepared by the action of phosphoric acid on *Jasus* chitin (Berger and Reynolds 1958)] were included as reference compounds. The differential thermograms are given in Figure 1.

Loligo, *Sepioteuthis*, and cuttlefish chitins were treated overnight with 6N HCl at room temperature, collected by centrifugation, and washed with water (till free of HCl), ethanol, and ether, and dried. The differential thermograms of these recovered chitins are also given in Figure 1. Addition of water to the 6N HCl supernatant from the chitins yielded a precipitate indicating that the chitins had undergone some degradation.

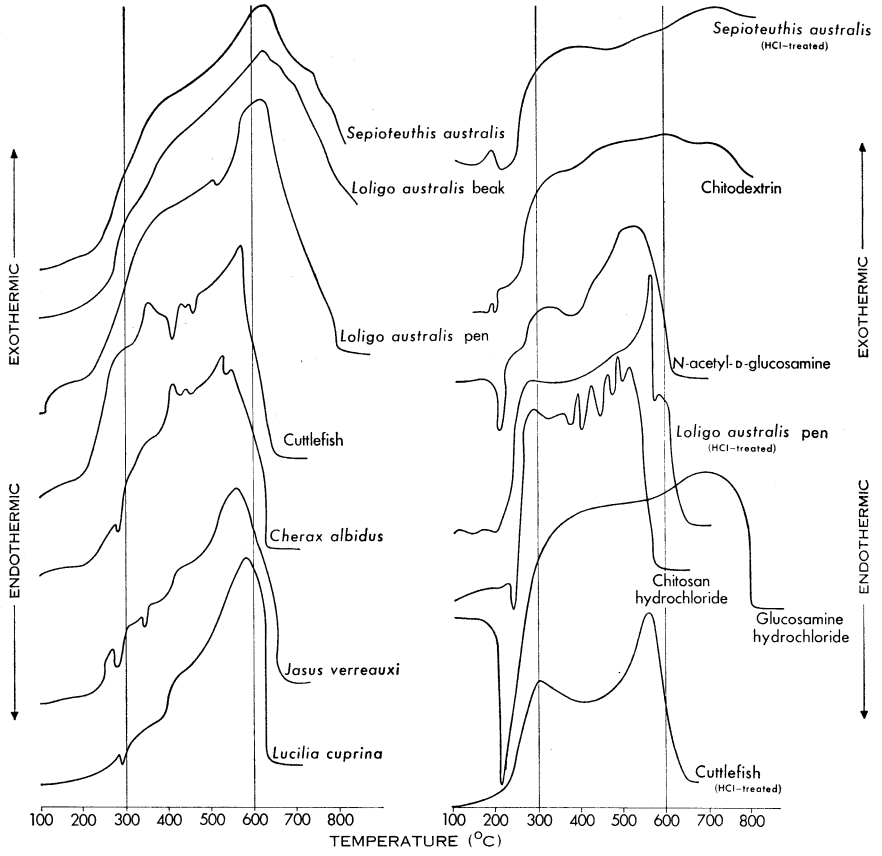

Fig. 1.—Differential thermograms of chitins and chitin degradation products.

(*f*) *Infrared Spectra*

Infrared spectra were measured by the KBr disk method with a Perkin–Elmer, model 221 double-beam spectrophotometer. The spectra for *Scylla*, *Loligo australis* pen, and cuttlefish chitins and for chitosan (free base) are given in Figure 2.

III. Discussion

X-ray diffraction studies on chitin show that α-chitins have a more compact type of chain grouping than β- or γ-chitins. In α-chitins the steric conditions are such that close packing of the chains (or portions of them) is possible and crystallites, stabilized by multiple hydrogen bonds, are formed. The chains of β-, α-, and γ-chitins

are reported by Rudall (1963) to be grouped in sets of one, two, and three respectively and in α-chitin the chains have an antiparallel arrangement. In α-chitin there is hydrogen bonding of the type N–H . . . O = C between the acetyl-amino groups of adjacent chains together with intramolecular hydrogen bonding between O_3 and O_5 of the next residue. The accessibility of various parts of the α-chitin structure will depend on the degree of order of the chains and the crystalline regions will be less readily penetrated by chemical reagents. The chemical reactivity of α-chitins will be related to the proportion of crystalline and non-crystalline (i.e. poorly organized

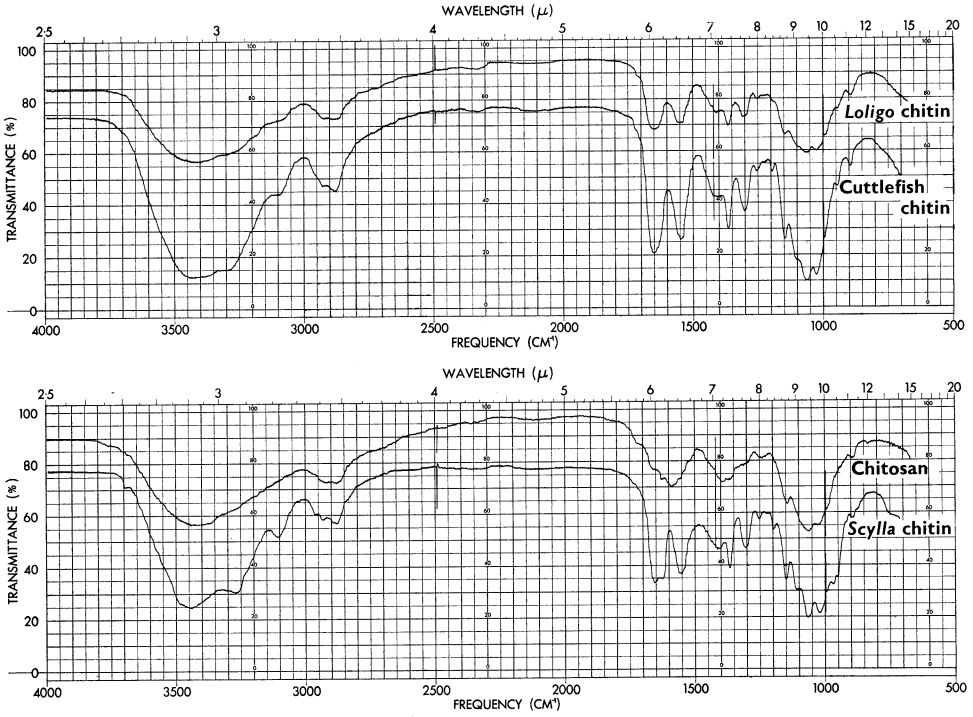

Fig. 2.—Infrared spectra of chitins and chitosan.

or amorphous) regions. However, inaccessibility to chemical reagents is not necessarily equivalent to "crystallinity" as measured by X-ray diffraction, the compactness of the chain structure also plays a part.

Enzymic digestion, acidic hydrolysis, and deuterium exchange have shown that the different chitins used in this study must have different structures in so far as accessibility of the chains to chemical reagents is concerned. It is also clearly shown that the *Loligo* and cuttlefish chitins (i.e. the β-chitins) used contained significant numbers of free amino groups.

The *Loligo* and cuttlefish chitins (β-chitins) were digested to a much greater extent by the *Lycoperdon* enzyme than were the insect or crustacean chitins (α-chitins). Obviously the chains of the β-chitins are more readily accessible to the enzyme than are those of the α-chitins. Clearly this behaviour is in accordance with the more open type of crystalline structure of β-chitins together with the concept of crystalline

and poorly organized regions in the chitins. Initial attack by the enzyme would occur in the poorly organized regions and the compactness of the chains in the crystalline regions of the α-chitins would make them less accessible to and so less readily degraded by enzymes. Although this is also true for β-chitins the open type of chain grouping in the crystalline regions is a complicating factor.

On digestion with *Lycoperdon* enzyme, cuttlefish and *Loligo* chitins (prepared by the use of hot alkali) gave an oligosaccharide which appeared to be a triose in which one or two of the amino groups were not acetylated. It could be selectively *N*-acetylated when it became indistinguishable from *N*,*N*,*N*-triacetylchitotriose. Nitrogen analyses of the intact chitins indicated that the triose contained two free amino groups (see also discussion on deuterium exchange below). From cuttlefish chitin the triose was obtained in a maximum yield of about 25%. This triose was formed during the first 24 hr digestion and in this same period of time about an equal weight of *N*-acetyl-D-glucosamine was produced. Further digestion gave more *N*-acetyl-D-glucosamine but little if any more of the triose. The triose was not detected in digests of the α-chitins (i.e. *Lucilia*, *Scylla* or *Agrianome*), in digests of a chitin prepared from cuttlefish shell by the use of EDTA or in digests of powdered *Loligo* pen. The failure to detect the triose in these preparations and the failure of the *Lycoperdon* enzyme to deacetylate *N*-acetyl-D-glucosamine shows that the enzyme had no deacetylase activity. The difficulty experienced in digesting the powdered *Loligo* pen has its parallel in the concept of "free" and "bound" chitin as discussed by Jeuniaux (1963). Bound chitin is chitin with which protein is associated and the chitin is degraded by chitinases only after the protein has been removed, e.g. by alkaline digestion.

The results given in Table 1 for cuttlefish chitin show clearly the effect of particle size of the substrate on the rate of enzymic digestion. In 5 hr 50% more digestion occurred with a sample of chitin which passed through a 200-mesh sieve than occurred with a sample which passed through a 150- but not a 200-mesh sieve. A highly swollen preparation such as that described by Hackman and Goldberg (1964*a*) is completely digested in the same time. Waterhouse, Hackman, and McKellar (1961) report a small increase in the amount of *N*-acetyl-D-glucosamine liberated from crab chitin which passed a 300-mesh sieve when compared with that liberated from a sample which did not pass a 150-mesh sieve. The smaller increase in digestion was no doubt brought about by the use of an α-chitin and of an enzyme preparation of low activity.

The α- and β-anomers of *O*-alkylglycosidic derivatives of *N*-acetyl-D-glucosamine are hydrolysed by HCl at different rates, the β-anomer being hydrolysed more rapidly (Foster, Horton, and Stacey 1957). However, as far as is known all chitins are built up of residues linked in the 1,4-β manner so the differing rates of acidic (HCl) hydrolysis must reflect the ease with which the hydronium ion can penetrate the chain structure. The chains of *Loligo* chitin are penetrated much more readily than are the chains of *Lucilia* or *Scylla* chitins. Half of the amount of hydrolysis recorded for *Lucilia* chitin took place in the first hour, but it took several hours for a similar situation to be reached with *Loligo* chitin. These results are consistent with

the view that *Loligo* chitin has an open chain structure, even though crystalline, while *Lucilia* and *Scylla* chitins have closely packed crystalline regions together with poorly organized regions. Chitin from the poorly organized regions would be hydrolysed rapidly and chitin from the surfaces of the crystalline regions would be hydrolysed slowly. This would give a continuous but slow increase in the amount of chitin hydrolysed with time after the initial rapid reaction was completed.

Deuteration is an established method in the study of the crystalline–amorphous structure of cellulose and this technique has now been applied to chitin. The deuterium exchange reactions (Table 3) also divide the chitins into two groups—the three α-chitins in one group and *Loligo* and cuttlefish chitins in the other. Again this is to be expected from what is known about the structures of the different types of chitin.

If chitin were a poly-*N*-acetyl-D-glucosamine then each residue would contain three hydrogen atoms available for exchange with deuterium, i.e. there would be available 14·78 mg-atoms of hydrogen per gram of chitin. For the α-chitins the amount of exchange which took place was less than this but for *Loligo* and cuttlefish chitins it was appreciably more. These results were reproducible in experiments over 12 months on samples which, although drawn from the same bulk preparations, were dried on separate occasions. *Loligo* and cuttlefish chitins on enzymic digestion gave in addition to *N*-acetyl-D-glucosamine, a partially deacetylated triose and glucosamine. The free amino groups in the residues which these products represent would make available further hydrogens for exchange with deuterium. Similarly the small amounts of glucosamine obtained on enzymic digestion of the α-chitins indicates the presence of some free amino groups in the chitins which could raise the amount of exchangeable hydrogen to something near 15 mg-atoms per gram of chitin.

The results in Table 3 show that deuterium oxide was able to penetrate a large part of the structure of the α-chitins — from about two-thirds to three-quarters of the chain structure depending on the source of the chitin. This leaves one-third to one-quarter of the α-chitins which can be considered to be in a highly crystalline form.

Loligo and cuttlefish chitins behave as though their chain structures permit of free access of deuterium oxide, a result which confirms Rudall's (1963) observation that a dominant factor about β-chitin is that water penetrates the crystalline structure freely. It has been shown (Hackman and Goldberg 1964*a*) that on complete enzymic digestion cuttlefish chitin gives 73·5% *N*-acetyl-D-glucosamine and 2·2% glucosamine, the remainder being a triose. Nitrogen analyses indicates that this triose contains only one acetyl group and so there would be available 16·54 mg-atoms of hydrogen per gram of chitin for exchange with deuterium. This result is in good agreement with the result reported in Table 3 and so this cuttlefish chitin contains one glucosamine residue for every four *N*-acetyl-D-glucosamine residues. The amount of hydrogen exchanged for deuterium in the *Loligo* chitin indicates that there are 2·2 glucosamine residues for every 3·2 *N*-acetyl-D-glucosamine residues and this is in agreement with the nitrogen analyses, with the amount of triose produced on enzymic digestion, and with the infrared absorption spectrum.

The infrared absorption spectra also support the conclusion that the *Loligo* and cuttlefish chitins contain a considerable number of free amino groups. As shown in Figure 2 the spectra of these two chitins are similar and differ considerably from that of *Scylla* chitin in the region 3000–3700 cm^{-1}. In this respect they resemble the spectrum of chitosan (free base). Darmon and Rudall (1950) and Pearson, Marchessault, and Liang (1960) have shown that during the formation of chitosan (i.e. the deacetylation of chitin) there is progressive weakening of those bands associated with carbonyl and amide groups (in particular those at 1625, 3100, and 3265 cm^{-1}). Darmon and Rudall also report the appearance of a new peak at 3365 cm^{-1} which is assigned to $-NH_2$ groups. A comparison of the spectra of *Loligo* and cuttlefish chitins with that of *Scylla* chitin shows differences of the same kind—*Loligo* and cuttlefish chitins are in fact partially deacetylated, *Loligo* chitin containing more free amino groups than cuttlefish chitin.

It is of interest to compare the results obtained on hydrolysis with HCl (Table 2) with those of deuterium exchange (Table 3). In both instances the molecular size of the reactant is small. The ratios of *Loligo* chitin hydrolysed to *Lucilia* chitin hydrolysed and of *Loligo* chitin hydrolysed to *Scylla* chitin hydrolysed approximate the respective ratios of the same chitins for the amount of hydrogen exchanged for deuterium. Also when Tables 1, 2, and 3 are compared the chitins are in the same order for increasing enzymic digestion, HCl hydrolysis, and deuterium exchange.

Differential thermal analysis has been applied with limited success to organic compounds and in particular to polysaccharides. Morita (1956*a*) observed that cellulose, in contrast to starch, gave differential thermograms which were nearly identical regardless of the botanical source of the samples. This led him (1956*b*) to conclude that the differential thermal reactions which occur depend on the type of glycosidic link. It is to be expected that the types of reactions which take place would be those involving dehydration and depolymerization leading to decomposition which would produce ultimately a broad exothermic peak.

As shown in Figure 1 the chitin thermograms have the same general shape regardless of their origin. All the α-chitins (i.e. those from insects and Crustacea) showed a small but reproducible endothermic reaction between 280 and 295°C which was not present in the squid or cuttlefish chitins. Chitin from the beak of the squid *Loligo australis* did not give this endothermic reaction but gave a thermogram very similar to that obtained from the chitin of the pen of the same animal. Rudall (1955) has reported that the X-ray diffraction patterns of chitin from the beak of a squid (*Loligo* sp.) show it to be an α-chitin whereas that of the pen is a β-chitin. Cuttlefish chitin gave a very small but reproducible endothermic reaction at 235°C which could not be shown on the curve in Figure 1. β-Chitins are converted to α-chitins by treatment with 6N HCl (Rudall 1963). When *Loligo* pen and cuttlefish chitins were treated with 6N HCl at room temperature overnight, degradation occurred and the differential thermograms of the insoluble materials did not resemble those of α- or β-chitins but rather those of chitin degradation products.

The origin of the free amino groups in the *Loligo* and cuttlefish chitins is of considerable interest. On enzymic digestion of the chitins *N*-acetyl-D-glucosamine and a partially deacetylated triose were produced together at first but later *N*-acetyl-

D-glucosamine was the only product formed. Since the enzyme did not have any deacetylase activity this indicates that there are free amino groups in that part of the chain structure which is initially accessible to the enzyme. The failure to detect the triose in the digests of powdered *Loligo* pen or of cuttlefish chitin prepared by the use of EDTA may be explained in either of two ways: there are no free amino groups present or there are free amino groups but the parts of the chain in which they occur are protected from enzymic attack by the protein associated with the chitin. If there are no free amino groups present then their presence in the isolated chitins can be explained as follows: either the free amino groups represent the points of attachment of protein which is subsequently removed by hot alkali, or deacetylation occurs during the purification of the chitin.

The results given in Tables 2 and 3 show that quite a significant proportion of the α-chitin structure is readily penetrated by hydronium ions and by deuterium oxide. If deacetylation of the *Loligo* and cuttlefish chitins occurred during purification then some deacetylation of the α-chitins would have been expected although it is possible that the different structures of α- and β-chitins confer on them different stabilities to alkali. Except for the small amount of glucosamine no unacetylated compounds were detected in enzymic digests of the α-chitins. Similarly there was no indication that removal of the protein associated with α-chitins led to the formation of free amino groups.

Thus it can be concluded for the *Loligo* and cuttlefish chitins that either (i) there is a significant number of free amino groups along the chains but confined to the less highly organized regions and that the protein which is associated with these chitins as they occur naturally protects those parts of the chains in which the free amino groups occur, from attack by chitinase, or (ii) the manner in which the protein is associated with these chitins differs markedly from that occurring in α-chitins. The differences are such that considerable numbers of free amino groups are formed when the protein is removed. In both cases it would also follow that the linking of the protein to the chitin occurs in the less highly organized regions of the chitin.

These observations indicate some of the factors which must be taken into consideration when discussing the structure of chitin. Most work to date relates to "purified" chitin in which the intra- and intermolecular bonding between the chains would be expected to differ considerably from that which is present in the chitin–protein associations which occur naturally (i.e. in native chitin). Carlstrom's (1957, 1962) structure of α-chitin is probably the best so far described and it incorporates extensive intra- and intermolecular bonding. The deuterium exchange reactions described above show that α-chitins are not uniform in their chain structure but have what may be called crystalline and poorly organized regions, the latter predominating. There is evidence (see Darmon and Rudall 1950) to show that α-chitins contain at least two kind of C=O groups, one of which is more readily removed. Again Rudall (1963) has suggested that α-chitins may contain significant amounts of glucosamine residues and firmly bound water (but not available for deuterium exchange). The bound water would be associated with the free amino groups of the glucosamine residues and as it were replacing the acetyl groups to maintain the correct density and crystallographic parameters. However, the absorption spectra

given in Figure 2 show that for the *Scylla* chitin the ratio of free amino groups to acetylated amino groups must be considerably less than 1 to 5 or 6 as suggested by Rudall for α-chitins. Suggestions such as these would require modification of the accepted structure of α-chitins.

For β-chitin much less information is available and this has been reviewed by Rudall (1963). β-Chitin has to date been considered to be a poly-*N*-acetyl-D-glucosamine. It has now been shown that two "purified" β-chitins contain considerable numbers of free amino groups. It is not certain that these free amino groups occur in the native β-chitins but if they do not then groupings are present which readily give rise to them on alkaline digestion as discussed above. Moreover, β-chitins from different sources differ significantly, e.g. in the ratio of glucosamine to *N*-acetyl-D-glucosamine residues in the chain and in the behaviour on heating (differential thermal analysis). Also cuttlefish chitin became more highly swollen when immersed in deuterium oxide than did squid pen chitin. For this reason a lesser amount of cuttlefish chitin had to be used in the deuteration experiments. Differences such as these were not observed among α-chitins. Perhaps these differences indicate that squid pen and cuttlefish chitins are not both β-chitins but the differences may be correlated with each other.

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V. References

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