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# LIGHT-SCATTERING AND INFRARED-SPECTROPHOTOMETRIC STUDIES OF CHITIN AND CHITIN DERIVATIVES

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ABSTRACT

A sample of chitin isolated from the shell of the crab *Scylla serrata* had, when in lithium thiocyanate solution, an average, weight-average molecular weight (*1*) of $1.036 \times 10^6$ daltons, an intrinsic dissymmetry (*2*) of 1.93, and a Z-average radius of gyration (*3*) of 64.14 nm. Carboxymethylchitin and glycol chitin, in 0.5M sodium chloride, had, respectively, (*1*) 1.896 and $1.819 \times 10^6$ daltons, (*2*) 3.25 and 4.31, and (*3*) 143.49 and 251.57 nm. They had similar degrees of polymerization, they underwent dissociation as the concentration of sodium chloride was increased to 2.5M, and the molecular packing of the chains was essentially side-by-side. Chitin in 5.55M lithium thiocyanate and carboxymethylchitin in 2.5M sodium chloride had similar degrees of polymerization. It is concluded that a small but significient number of the amino groups in the chitin molecule are not acetylated.

## INTRODUCTION

Chitin occurs as a major cuticular or skeletal component in all arthropods, in some other invertebrata (*e.g.*, squid and cuttlefish), and in some fungi. It is always associated with protein, and at least part of it occurs in the form of a glycoprotein. Chitin is a polysaccharide of high molecular weight and is thought to consist of unbranched chains of (1→4)-β-linked 2-acetamido-2-deoxy-D-glucose residues, the unit being the biose. Three crystalline forms (α, β, and γ) of chitin are known, and these differ in the arrangements of the chains and the presence of bound molecules of water. Only α-chitin has been reported from arthropods. In α-chitin, equal numbers of chains run in opposite directions (antiparallel), the structure being stabilized by intra- and inter-chain hydrogen bonds. The crystal structure proposed by Carlström[1,2], based on a "bent"-chain configuration, is considered to be the most satisfactory, especially from a stereochemical viewpoint. This arrangement can only be true for the more highly ordered regions of the chains, which may account for less than one third of an α-chitin[3]. For reviews on the chemistry and structure of chitin, see Jeuniaux[4], and Ward and Seib[5].

Although chitin has been studied extensively, no definitive work has been published on its molecular weight. This lack of information is due undoubtedly to the intractable nature of chitin. Chitin is polydisperse, insoluble in all the common solvents, dissolves with degradation in concentrated mineral acids, but can be dispersed in hot, concentrated, aqueous solutions of certain mineral salts, *e.g.*, lithium thiocyanate. From viscosity data for solutions in nitric acid, Meyer and Wehrli[6] concluded that the molecular weights of cellulose and chitin were of the same magnitude. A number of derivatives of chitin have been prepared, and molecular-weight values have been reported[6–15] for some of them (*e.g.*, ethers of chitin, chitosan, and substituted chitosans). In the preparation of most, perhaps all, of these derivatives, degradation of the chitin chains took place, and so a wide range of molecular weights has been reported. The degrees of polymerization ranged from 20–1280 (corresponding to molecular weights of chitin ranging from ~4,000–261,000 daltons), much smaller than values given for cellulose.

This paper reports on a study by light scattering of the molecular parameters of size and shape, including molecular weight, of chitin, carboxymethylchitin, and glycol chitin. Infrared absorption spectra are also investigated. The chitin used was an α-chitin, prepared from the shell of a crab.

## EXPERIMENTAL

*Chitin.* — Chitin was prepared from hair-free pieces of the shell from fully grown, freshly caught crabs, *Scylla serrata* (Forskål) (Decapoda: Portunidae), collected in the coastal waters off southern Queensland. The shell was thoroughly cleaned to remove non-cuticular components, air-dried, and powdered in a Teema mill. That fraction which passed through a 150-mesh sieve was decalcified with cold M HCl, and the residue was collected and extracted with M NaOH for 24 h at 100°. The residue was again collected, and the extraction procedure with alkali repeated twice. The insoluble material (chitin) was collected, washed once with water, acidified with 2M HCl, dialysed against water till free of chloride, finally washed with ethanol and ether, and dried. The yield from powdered shell, on a water-free basis, was 9.3% (Found: N, 6.9; ash 0.83%).

O-*Carboxymethylchitin.* — *O*-Carboxymethylchitin was prepared according to the method of Trujillo[14], except that the preliminary treatment with methyl sulphoxide was omitted because, with the above chitin, it did not increase the yield or degree of substitution (d.s.). The chitin was very finely divided, and it was not possible to reduce the wet weight of the alkali-swollen material below 41 g, starting with 8 g of chitin. The yield of sodium salt from 8 g of chitin was 8.54 g. The d.s. was 0.729, as determined by the acid-wash technique of Eyler *et al.*[16], and 0.735 by estimation of sodium (0.686% by atomic absorption); the degree of deacetylation[17] was 0.221.

*Glycol chitin* [O-(*2-hydroxyethyl*)*chitin*]. — The method used was essentially that supplied by Professor Y. Matsushima, Department of Chemistry, Osaka University College of Science, Japan. Particular attention was paid to temperature control.

Chitin (10 g) was mixed thoroughly with 42% (w/w) aqueous NaOH (120 ml), and the mixture was kept overnight at room temperature *in vacuo* to remove occluded air. Crushed ice (400 g) was added, the mixture was transferred to a strong, plastic vessel and cooled to 0°, aqueous NaOH (175 ml, 13% w/w) was added gradually at 0°, and the mixture was stirred at 0° until it became a uniform, highly viscous solution. Ethylene oxide (20 g) was added, the vessel was sealed, and the contents were mixed well and kept at 0° for 2 h. The vessel was removed from the cooling bath, kept overnight at room temperature and then for 2 h at 33°, again cooled to 0°, and opened, and the solution was neutralized with acetic anhydride at 0°. The precipitated glycol chitin was collected, dialysed against water to remove sodium acetate, washed with ethanol and ether, and dried. Yield, 9.3 g, degree of glycolation[18], 0.586; degree of deacetylation[17], 0.177.

*Light-scattering measurements.* — The photometer was a Brice–Phoenix instrument modified as described by Beattie *et al.*[19], and the galvanometer was replaced by a strip-chart recorder (Rikadenki B-140). All work was carried out at 20°, unpolarized light of wavelength 546.1 nm (interference filter) was used, and the methodology and the performance of the instrument were checked by determining the molecular weight of a series of polystyrene, molecular-weight markers (Schwarz–Mann). Agreement was within 2% of the reported values.

Carboxymethylchitin (sodium salt) and glycol chitin were dissolved in aqueous sodium chloride at approximately 1 mg/ml, and the concentration range of sodium chloride investigated was 0.01–2.5M. Chitin was dissolved in aqueous lithium thiocyanate (saturated at room temperature) at 95°, the cold solution was centrifuged at 26,600 $g_{av}$ for 1 h, and the supernatant was diluted with an equal volume of water [the solution then contained 3.85% of Li (w/v), *i.e.*, 5.55M LiSCN]. The solutions were equilibrated by dialysis (each with its respective solvent), and measurements were taken on the dialysed solutions, the dialysate being used as the reference solvent. Solvents and the dialysed solutions were clarified by filtration, respectively, through 0.2- and 3-$\mu$m filters (Millipore, except that Sartorius filters were used for the LiSCN solutions) under gravity or slight, positive pressure. The concentrations of the dialysed solutions were estimated by refractometry (Brice–Phoenix differential refractometer), and specific refractive-index increments of the two derivatives were determined on aqueous solutions of known concentrations. The specific refractive-index increment for chitin was assumed to be 0.164, the same as that of a lyophilized sample of hepta-*N*-acetylchitoheptaose, prepared from chitin by the method of Barker *et al.*[20].

Scattering intensities were measured at a series of angles between 45° and 135°, relative to the incident beam, in a Witnauersherr cell (3.6-cm diameter). For the two chitin derivatives, measurements were made at 5 concentrations, but for chitin, because of its low solubility, only 3 concentrations could be used. Dilutions were made by weight in the cell under dust-free conditions and were approximately in the ratios 1:0.8:0.6:0.4:0.2. Zimm plots were obtained in the usual way by plotting $Kc/R_\theta$ versus $\sin^2(\theta/2)+kc$. Extrapolation to zero angle (against concentration) and to zero concentration [against $\sin^2(\theta/2)$] gave linear plots. From these straight

lines, the average, weight-average molecular weights, the Z-average radii of gyration, and the second virial coefficients were calculated in the customary manner.

*Infrared spectra.* — Infrared spectra were measured by the potassium bromide disc method with a Perkin–Elmer, Model 221 double-beam spectrophotometer. Carboxymethylchitin and glycol chitin were incorporated into KBr by lyophilization from aqueous solutions.

## RESULTS AND DISCUSSION

The chitin used for the preparation of carboxymethylchitin and glycol chitin was very finely powdered and, as judged by its uptake of aqueous sodium hydroxide, became more swollen in alkali than did the chitin used by earlier workers. Substitution would have therefore occurred under milder conditions and yields would have been increased, though some deacetylation could have taken place. The yield of carboxymethylchitin (86%) was very much higher than that reported by Trujillo[14], and indicates that little degradation took place. The low degree of hydroxyethylation of chitin explains the low solubility of this glycol chitin in water (~2.25 mg/ml) and is consistent with the results published by Senju and Okimasu[21]. Again, the high yield (85%) indicates that little, if any, degradation took place. In view of the work of Hara and Matsushima[22] and Hara *et al.*[23], this glycol chitin would be a mixture of ethers. Clark and Smith[24] have shown by X-ray examination of the chitin precipitated from solution in aqueous lithium thiocyanate that no hydrolysis took place even when the solution of chitin had been kept for several months. Dissolving chitin in acidic solutions is known to bring about a shortening of the chains. Light-scattering measurements were, therefore, made on solutions of chitin in aqueous lithium thiocyanate, but the concentration of lithium thiocyanate used was less than that used by Clark and Smith. Although some chitin dissolved in 11M and 5.5M lithium thiocyanate at room temperature and also at 50°, the amount dissolved was too low to give sufficient light-scattering data to enable Zimm plots to be drawn. The low solubility is presumably due to a low degree of swelling. The concentration of chitin in aqueous lithium thiocyanate solution could not be measured accurately, consequently the specific refractive-index increment of chitin could not be determined. Hepta-*N*-acetylchitoheptaose has a density value close to that of chitin, and gives X-ray diffraction patterns and infrared absorption spectra similar to those of chitin[25,26]. So, it is reasonable to assume that its specific refractive-index increment is close to that of chitin.

Attempts to determine the d.p. of carboxymethylchitin and glycol chitin by measuring the reducing-sugar values, using the neocuproin method of Dygert *et al.*[27], were unsatisfactory because solutions of glycol chitin brought about the immediate formation of a yellow colour at room temperature. The intensity of the colour finally developed corresponded to a molecular weight of less than 10,000 daltons, which is obviously in error, in view of the results reported below and the results reported by others for glycol chitins[7–9] and glycol chitin derivatives[10]. Consequently, no reliance could be placed in the results obtained for carboxymethylchitin.

Figs. 1–3 illustrate some of the Zimm plots obtained for carboxymethylchitin (Na salt), glycol chitin, and chitin. The molecular parameters calculated from these and other Zimm plots are given in Table I. In Fig. 4, $1/P_\theta$ versus $\sin^2(\theta/2)$ has been plotted for each of the compounds. The i.r. spectra for chitin, carboxymethylchitin, and glycol chitin are given in Fig. 5.

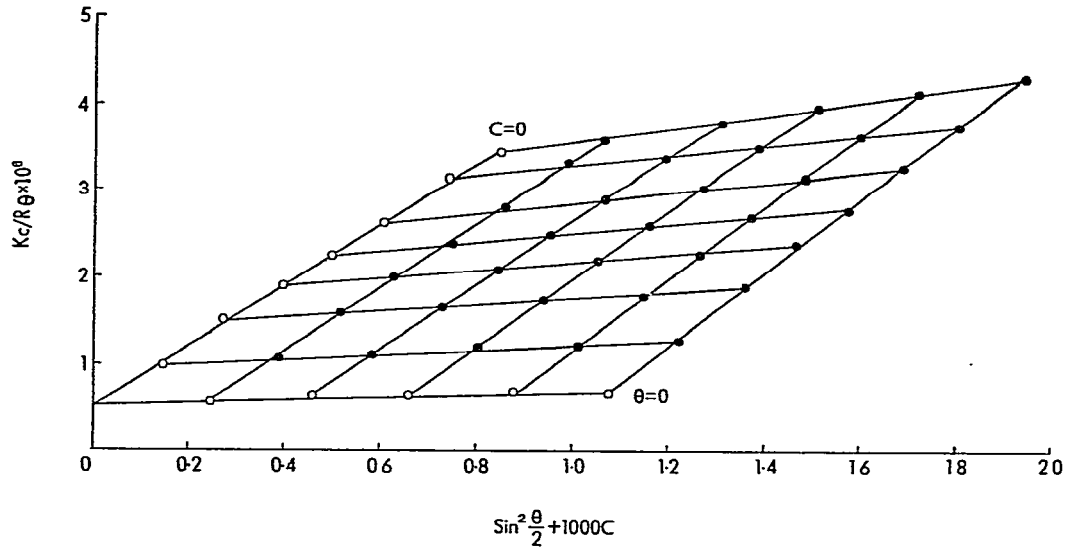


Fig. 1. Zimm plot for carboxymethylchitin in 0.5M NaCl.

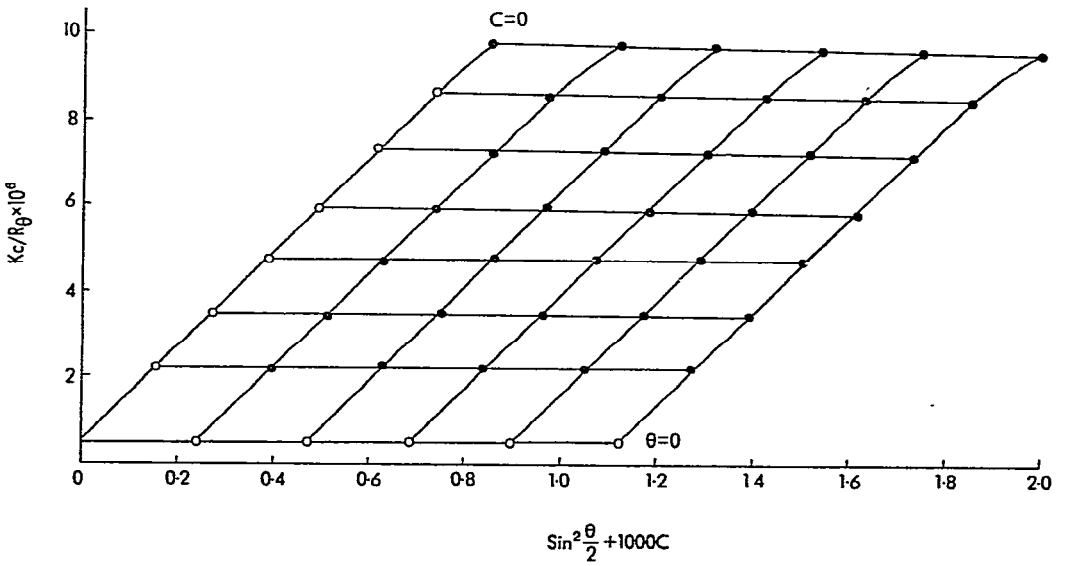


Fig. 2. Zimm plot for glycol chitin in 0.5M NaCl.

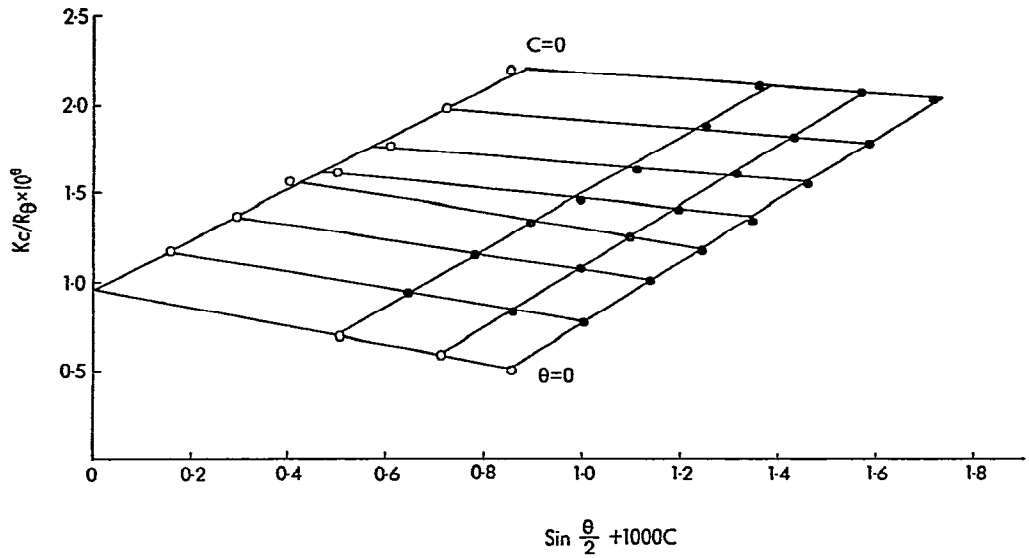


Fig. 3. Zimm plot for chitin in 5.5M LiSCN.

TABLE I

MOLECULAR PARAMETERS OF CARBOXYMETHYLCHITIN, GLYCOL CHITIN, AND CHITIN

| *Compound* | *Mol. Wt. of repeating unit (daltons)* | *Mol. Wt. (Av. $M_w \times 10^6$) (daltons)* | *Wt.-average d.p.* | *Intrinsic dissymmetry* | *Z-average radius of gyration (nm)* | *Effective bond-length (nm)* |
|---|---|---|---|---|---|---|
| Carboxymethylchitin (Na salt in 0.5M NaCl) | 252.7 | 1.896 | 7503 | 3.35 | 143.49 | 3.31 |
| Carboxymethylchitin (Na salt in 2.5M NaCl) | 252.7 | 1.338 | 5295 | 3.18 | 131.69 | 3.62 |
| Glycol chitin in 0.5M NaCl | 221.6 | 1.819 | 8207 | 4.31 | 251.57 | 5.57 |
| Chitin in 5.55M LiSCN | 199.0 | 1.036 | 5206 | 1.93 | 64.14 | 1.80 |

The results given in Table I show that the d.p. of carboxymethylchitin and glycol chitin in 0.5M sodium chloride are similar, which is further evidence that the methods used for preparing these two derivatives did not degrade the chitin chains. For carboxymethylchitin, as the concentration of the sodium chloride in the solution was decreased so the intrinsic dissymmetry increased, showing that the size and shape of the envelope of the scattering particle increased. In 0.01M sodium chloride, the intrinsic dissymmetry was 6.3. On the other hand, as the concentration of the sodium chloride was increased from 0.5M, there was little change in the intrinsic dissymmetry, but in 2.5M sodium chloride, the average mol. wt. had dropped from

$1.896 \times 10^6$ to $1.338 \times 10^6$ daltons. The change in the radius of gyration was also small, so it is concluded that dissociation occurred, a chain (or chains) of mol. wt. $0.558 \times 10^6$ daltons (total) was removed, and the packing had been essentially side-by-side. Similar changes occurred with glycol chitin but were not investigated in detail. Both carboxymethylchitin in 2.5M sodium chloride and chitin in 5.5M lithium thiocyanate had d.p. of ~5,200. That these two compounds in such different solvents had the same d.p. indicates that the average mol. wt. of this sample of *Scylla* chitin is $1.036 \times 10^6$ daltons. This value is higher than published data for chitin derivatives, but it approaches the values given for celluloses[28,29].

The second virial coefficients for the three compounds in the solvents used, as calculated from the Zimm plots, were low, which indicates little interaction between these polymers and the solvents. For carboxymethylchitin, glycol chitin, and chitin, the values were $6.64 \times 10^{-5}$, $-4.36 \times 10^{-6}$, and $-2.65 \times 10^{-4}$ cm$^3$.mole.g$^{-2}$, respectively.

The lines drawn in Fig. 4 are the theoretical curves for random-coil structures, polydisperse in molecular weight with a polydispersity parameter of 1, calculated according to the formula given by Geiduschek and Holtzer[30]. For each compound, the observed values of $1/P_\theta$ coincided with the theoretical curves, so each had a

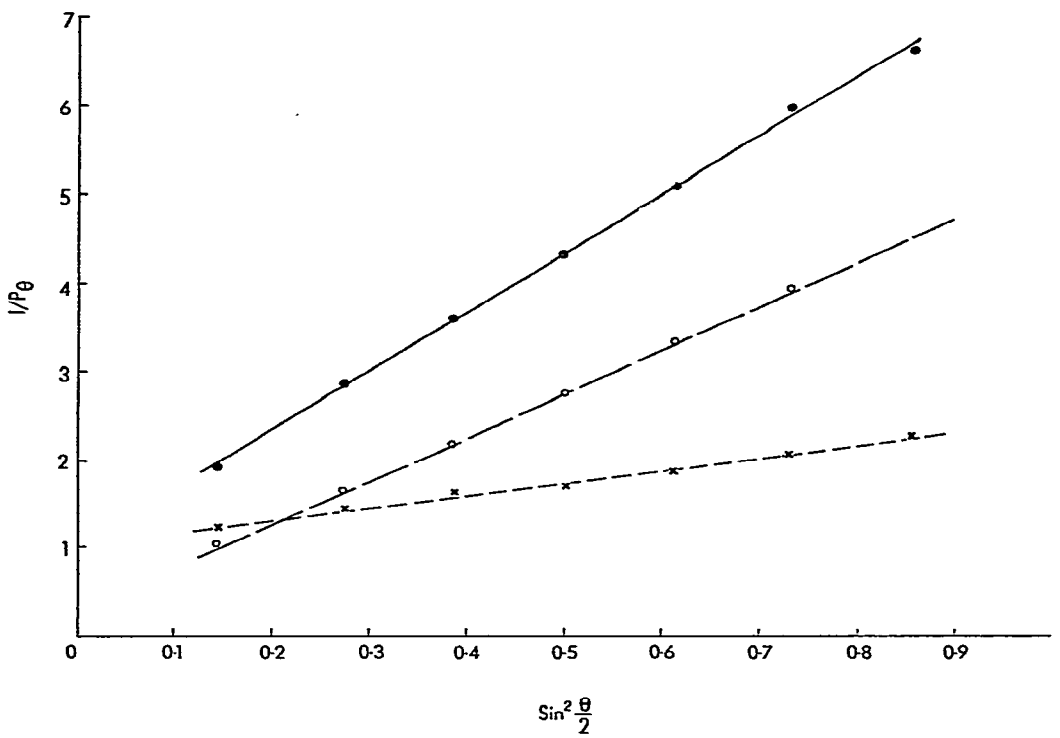


Fig. 4. Plot of $1/P_\theta$ versus $\sin^2(\theta/2)$ for carboxymethylchitin (●), glycol chitin (×0.25) (○), and chitin (×).

random-coil structure in solution. None of the theoretical curves for the other shapes fits the data. The molecular-weight distribution for each compound, therefore, corresponded to one characterized by Mz:Mw:Mn = 3:2:1. Thus, the effective bond length $b$ can be calculated by using the formula $b^2 = \bar{r}^2/N$ and substituting the appropriately averaged d.p. $N$ and root-mean-square end-to-end dimension $\bar{r}^2$. The latter value is calculated from the radius of gyration, assuming the molecules to have a random-coil structure. The bond lengths are given in Table I, and their magnitudes confirm the fact that rotation about the C–O bonds is restricted. Clearly, in sodium chloride solution, glycol chitin has a more extended coil configuration than has carboxymethylchitin. By comparison, chitin in lithium thiocyanate solution has a compact coil configuration. The effective bond length of carboxymethylchitin in sodium chloride solution is of similar magnitude to that of carboxymethylcellulose in sodium chloride solution, as reported by Schneider and Doty[31].

As yet, it is not possible to estimate accurately the content of free amino group for an α-chitin. Enzymic digestions[32] of chitin from the shell of *S. serrata* produce small amounts of 2-amino-2-deoxy-D-glucose, which appear to arise from residues containing free amino groups. The results indicate that as much as 10% of the amino groups in the chitin chain are not acetylated, and this was for chitin which had not been treated with aqueous sodium hydroxide. Similar results have been obtained by the enzymic digestion of a β-chitin[3], but in this instance a partially deacetylated trisaccharide, which was not further degraded by the enzyme, was also formed. Together, the 2-amino-2-deoxy-D-glucose and the trisaccharide accounted for all the free amino groups (on 20.6% of the residues). Only a relatively small percentage of an α-chitin has been degraded enzymically, so it is conceivable that the undegraded part contains free amino groups, and in fact the presence of free amino groups may prevent further enzymic attack. Enzymic digests may only give a minimum figure for free amino groups. Studies with enzymes have established the presence of 2-amino-2-deoxy-D-glucose in α-chitin, but they do not enable a true estimation of the number of residues to be given. Histochemical studies (especially the periodic acid–Schiff reagent) have suggested to many workers that some of the residues in chitin are deacetylated, but such tests are too unreliable to lead to any definite conclusions. Nevertheless, they add to the general body of evidence. Lipke and Geoghegan[33], in a study of *Sarcophaga bullata* puparial cases, detected up to 3% of 2-amino-2-deoxy-D-glucosyl residues, but again this may well be a minimum figure. Hunt's failure[34] to detect any free amino groups in blowfly-cuticle chitin by a dinitrophenylation technique could be explained by the fact that chitin is an insoluble material in which the amino groups may be prevented from reacting by steric hindrance.

Giles *et al.*[35] have proposed that 16% of the residues in α-chitin are deacetylated and that the molecule contains bound water. Caution must be exercised in accepting these conclusions, based on elementary analyses of chitin, because experience has shown that such analyses, although reproducible, are likely to be unreliable. Chitins are not always completely burnt, they sometimes leave a tar which is mistaken for

ash. Rudall[36] mentions the anomalous, meridional reflections in the X-ray diffraction patterns given by chitins. He comments that it is customary to discount these in order to support an exact, twofold screw axis in the fibre direction of the chain and that they may be related to the presence of some significant number of 2-amino-2-deoxy-D-glucose residues. In the same publication, Rudall has discussed the structure of α-chitin, using X-ray diffraction data, i.r. spectra, and density measurements. An interpretation of the data is one in which acetyl groups have been replaced by water molecules.

Ideally, the i.r. spectrum of chitin should indicate the presence of free amino groups. However, it is doubtful if the free amino groups would be revealed should less than 10% of the amide groups be deacetylated. Since it is not possible to say that, in any given sample of chitin, all the amino groups are acetylated, the true spectrum

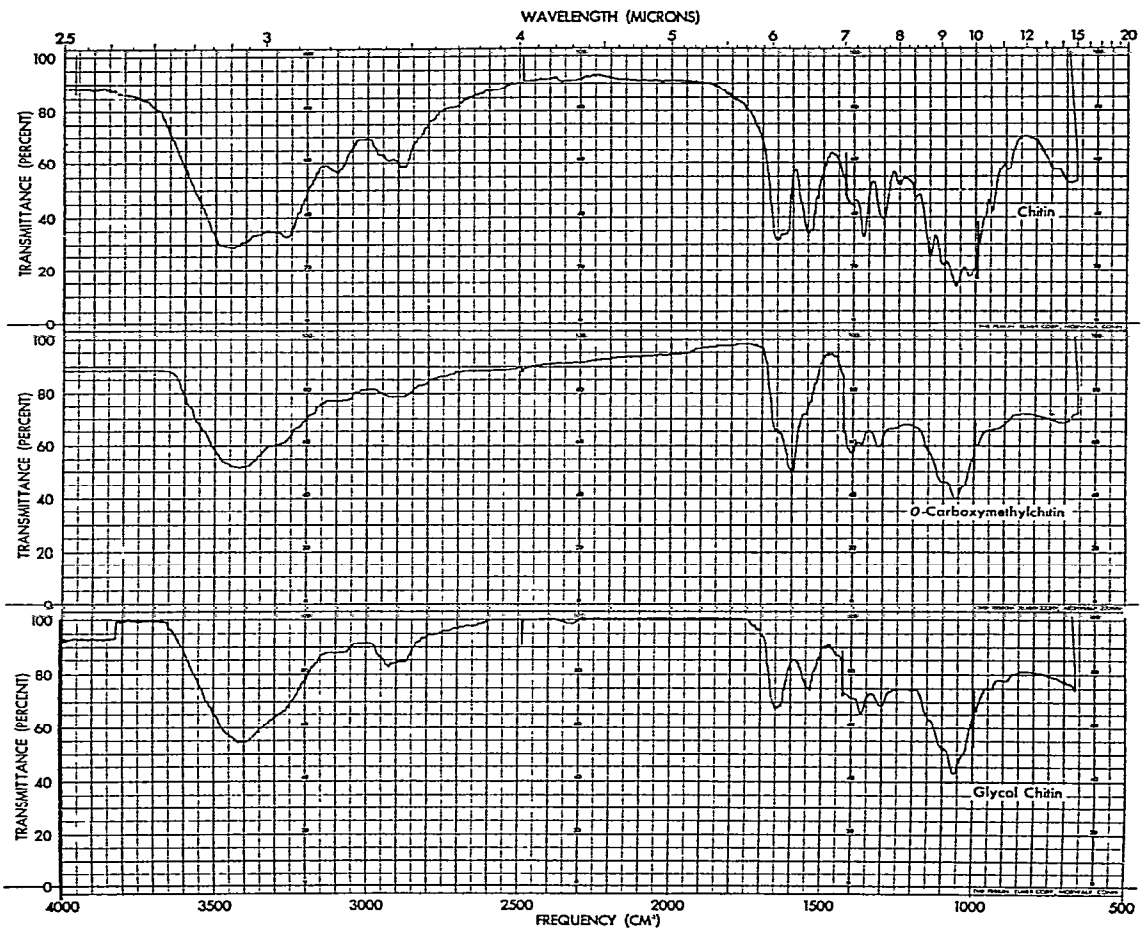


Fig. 5. Infrared absorption spectra for chitin, carboxymethylchitin, and glycol chitin.

of a poly-(2-acetamido-2-deoxyglucose) may not be known. Considering the region 3100–3300 $cm^{-1}$, the bands at 3100 and 3265 $cm^{-1}$ in the spectrum of chitin have been assigned to NH stretching[26,36]. As the amino groups become deacetylated (*e.g.*, in the glycol chitin and carboxymethylchitin; see also spectra for chitosan and chitins from cuttlefish and squid[3]), these bands are weakened, and a new band appears at $\sim$3400 $cm^{-1}$ which Pearson *et al.*[26] assign to the free amino group. The presence of this band in the chitin spectrum would not be apparent unless sufficient deacetylation had taken place, because of the presence of the strong O–H stretching vibrations at 3450 and 3480 $cm^{-1}$. So the spectrum for chitin given in Fig. 5 would be consistent with the chitin containing some amino groups which are not acetylated but, obviously, the number must be fewer than that present in the glycol chitin, *i.e.*, less than 17%. A similar conclusion can be drawn from the i.r. spectra given by Hackman and Goldberg[3], *viz.*, that *Scylla* chitin contains less than 20% of free amino groups, this value being the free amino content of the cuttlefish chitin. So, for the light-scattering data given in Table I, an arbitrary figure of 10% free amino groups has been assumed, this value being consistent with both the enzymic and infrared studies on *Scylla* chitin. This assumption has the effect of reducing the average molecular weight of the repeating unit from 203.2 to 199.0 daltons.

From the above work, it is concluded that the average, weight-average molecular weight of the sample of chitin isolated from the shell of the crab *Scylla* is $1.036 \times 10^6$ daltons, and that a small but significant number of the amino groups in the molecule are not acetylated. It is not known if the free amino groups occur in the chitin as such, or if they are formed during its isolation. However, the work of Waterhouse *et al.*[32] indicates that at least some of the free amino groups occur naturally. The presence of amino groups along the chain would have important ramifications when considering the biological role of chitin. Chitin has a molecular weight of the same magnitude as cellulose. The techniques used when isolating cellulose probably always cause a shortening of the chain length, even exposure to the atmosphere can bring about depolymerization of the molecule[28]. Chitin is less readily attacked by chemical reagents than is cellulose, it is a more stable molecule, and so should undergo depolymerization less readily.

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