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Carboxymethylated Chitins and Chitosans

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SUMMARY

The carboxymethylation procedures developed to impart amphoteric properties to chitosan make use of monochloroacetic acid and of glyoxylic acid, for the preparation of O-carboxymethyl and N-carboxymethyl chitosans, respectively. Carboxymethyl chitin as well as N,O-carboxymethyl chitosans have also been synthesized. These water-soluble modified biopolymers find uses as medical aids, cosmetic ingredients and metal ion chelating agents.

INTRODUCTION

Early work on the carboxymethylation of chitin

Early reports on carboxymethyl chitosan date back 50 years (Rigby, 1937). By adding sodium hydroxide to a solution of chitosan in chloroacetic acid at 60°C for 12 h, a water-soluble derivative was obtained. While that product was not characterized, we can speculate today that the reaction could have led to variable extents of substitution at the primary hydroxyl group and, in view of the temperature used, at the primary amino group as well:

$$\mathrm{Chit(OH)} + x\,\mathrm{ClCH_2CO_2Na} + x\,\mathrm{NaOH} = \mathrm{Chit(OH)_{1-x}(OCH_2CO_2Na)_x} + x\,\mathrm{NaCl} + x\,\mathrm{H_2O}$$

$$\mathrm{Chit(NH_2)} + x\,\mathrm{ClCH_2CO_2Na} + x\,\mathrm{NaOH} = \mathrm{Chit(NH_2)_{1-x}(NHCH_2CO_2Na)_x} + x\,\mathrm{NaCl} + x\,\mathrm{H_2O},$$

where x is the degree of substitution (d.s.).

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The preparation of alkali chitin by Thor (1940) (see also Muzzarelli, 1973) opened the route to the carboxymethylation of chitin: carboxymethyl chitin (CM-chitin) was prepared by various authors in the course of the following decades (Capozza, 1975; Danilov & Plisko, 1961; Hackman & Goldberg, 1974; Hayashi *et al.*, 1968; Koshugi, 1980; Miyazaki & Matsushima, 1968; Okimasu, 1958; Trujillo, 1968).

CM-Chitin is typically prepared by mixing chitin (4 g) with sodium hydroxide (50 ml, 65%) and stirring the slurry for 1 h; then the excess solution is removed by pressing, the wet alkali chitin is added to 2-propanol (50 ml) containing monochloroacetic acid (6 g) and stirred for 1 h. Upon addition of water (200 ml), the pH is adjusted to neutrality with HCl. The degree of substitution is 1.

Most of this work was carried out with a view to preparing a water-soluble chitin which could be of use as a substrate for lysozyme and chitinases. While hydroxyethyl chitin (glycol chitin) has gained wider acceptance as an enzyme substrate (Yamada and Imoto, 1981), carboxymethyl chitosan (CM-chitosan) has attracted interest for a number of other reasons.

It should also be considered that much work has been done on the carboxymethylation of cellulose for which high degrees of substitution have been sought (Kloow, 1985; Nicholson & Merrit, 1985). The development of the relevant technology has stimulated research on CM-chitin. Carboxymethyl cellulose in more than 250 grades is commercially available for use in food additives, cosmetics, detergents, and basic pharmaceutical materials. Its worldwide sales are estimated to be about 250,000 tons per year. CM-Cellulose is generally prepared by the reaction of sodium monochloroacetate with alkali cellulose in aqueous medium.

Chowdhury (1924), Hayakawa & Morita (1958), Karabinos & Hindert (1954) and Sakurada (1928) tried to prepare CM-cellulose of high d.s. values, for instance by using 1 part (by weight) of cellulose suspended in 30 parts (by volume) of 88:12 (v/v) 2-propanol-water and then adding an excess of 50% aqueous NaOH and monochloroacetic acid, or of sodium monochloroacetate, under continuous stirring. Substitution could hardly exceed d.s. 1 in a single stage of operation. The use of 1,1,3,3-tetramethylurea plus dimethyl sulphoxide as a promoter of carboxymethylation of cellulose was also examined by Iwata *et al.* (1985).

In reviewing the work done on *O*-CM-chitosans, Muzzarelli (1977) pointed out 11 years ago that at that time 'no information was available on the interaction of these interesting derivatives with metal ions and

with other substances which are expected to easily react with them'. Because the situation has recently changed, the present review intends to focus on the chemical and biochemical significance of CM-chitosans, which are amphoteric polysaccharides possessing chelating, film forming and gel forming characteristics.

Tendency of chitin to yield soluble products

It would seem pertinent to mention here some aspects of the chitin chemistry, related to solubility in aqueous solution. Because the carboxymethylation process imparts solubility, among other characteristic properties, to an insoluble polysaccharide, it is important to use chitin/chitosan samples particularly suited to the production of water-soluble derivatives. In fact, if a water-soluble derivative is sought, it is appropriate to start from that chitin which is more prone to solubility. It would seem that shrimp and krill chitins are more convenient than crab and lobster chitins, from this standpoint, however a real survey has not yet been made. Several articles have been published on this topic in the past and some of their findings are cited here just to draw attention to this point.

In view of the preparation of water-soluble chitin, Sannan *et al.* (1975), isolated chitin from *Penaeus japonicus* shells (a shrimp) (200 g) by digestion with HCl (5 litres, 2 N) at room temperature, and deproteinized twice with NaOH (5 litres, 1 N) at 100°C for 6 h.

Alkali chitin is prepared as follows (Sannan *et al.*, 1975). A suspension of chitin (3 g) in NaOH solution (30 g NaOH in 45 g H_2O) is allowed to stand for 3 h at 25°C under reduced pressure. Then, the alkali chitin is dissolved by stirring vigorously with crushed ice (225 g) and cooling at 0°C.

The water-soluble regenerated chitin is then prepared according to Sannan *et al.* (1976): after standing at 25°C for 77 h, the alkali chitin solution obtained above (300 g) is cooled below 5°C and ice (210 g) is added followed by concentrated aqueous HCl under stirring until pH 9. The pH of the solution is adjusted to 8·7 with diluted HCl and then the solution is poured dropwise into an acetone/water mixture (5 litres, ratio 7:1, at 0°C with stirring. Cold acetone is added dropwise simultaneously to keep the acetone water ratio roughly constant. The white precipitate formed is filtered off and washed until chloride is removed. The degree of deacetylation is 46%.

While the deacetylation of chitosan is currently carried out in 40% NaOH aqueous solution, at temperatures above 100°C (heterogeneous

hydrolysis) it can be also performed at much lower temperatures over a more extended period of time (homogeneous hydrolysis). The products prepared by the two hydrolytic processes have different characteristics in terms of structure and of solubility in water, even when they have an equal degree of deacetylation (Kurita *et al.*, 1977).

The deacetylation under heterogeneous conditions seems to proceed preferentially from the amorphous region to give block-type copolymers of *N*-acetyl-D-glucosamine and D-glucosamine units as suggested from the presence of the upper limits in the deacetylation, the crystallinity change, and the solubility. On the other hand, the deacetylation under homogeneous conditions is considered to yield random-type copolymers of the two units, which result in solubility and lower degree of crystallinity.

The water-solubility of the samples with a deacetylation degree of *c.* 50% obtained by the homogeneous process was attributed to the structural randomness of the products, by Kurita *et al.* (1977, 1982), in agreement with modern views on water-soluble polymers (Glass, 1986).

Studies currently in progress indicate that chitosan obtained by the heterogeneous hydrolytic process contain a small proportion of scarcely deacetylated macromolecules. These come from the more crystalline regions of the polysaccharide which tend to remain aggregated in solution, and are therefore more resistant to chemical reactions conducted on chitosan. While this factor does not seem to have been taken into account for the preparation of samples so far submitted to carboxymethylation, it will certainly prove to be of importance.

O-CM-CHITIN, (METHOD OF TOKURA *et al.*, 1983*b*)

Preparation

Chitin (10 g) suspended in NaOH (40 ml, 60%) with sodium dodecylsulphate (2%) at 4°C is kept at −20°C overnight. The frozen alkali chitin is suspended in isopropanol (200 ml at 25°C) and monochloroacetic acid is added in portions under stirring until the mixture is neutral. The *O*-carboxymethyl chitin sodium salt is filtered and washed with ethanol and then is dissolved in water (2 litres) and reprecipitated with acetone (5 litres). The product can be obtained as a free acid by redissolving in 2 N HCl and dialysing against deionized water. The degree of carboxymethylation of chitin, contributing to the water solubility, was found to

be determined by the concentration of sodium hydroxide during the freezing process in the preparation of the alkali chitin.

Characteristics

The i.r. absorption spectra of *O*-CM-chitins (Nishimura *et al.*, 1984*a*) show an absorption band at 1735 cm^{-1}, which increases with d.s. This is attributable to carbonyl stretching of the carboxyl group. It was assumed from n.m.r. studies that the substitution site of the GlcNAc residue would be O6, the primary hydroxyl group (Tokura *et al.*, 1983*a*). The intensity of the C6 peak of GlcNAc (63·0 ppm) was decreased, and a new peak could be observed at 69·0 ppm attributable to the substituted carbon atom. There was no significant indication of a shift of C3 (76·8 ppm). Miyazaki & Matsushima (1968) reported also that 2-amino-3-*O*-(carboxymethyl)-2-deoxy-D-glucose hydrochloride is barely detectable by thin layer chromatography (t.l.c.) of the acid hydrolysate of CM-chitin when the degree of carboxymethylation was < 1.0. Attack at O6 seems to be particularly favoured because HO3 may be engaged by hydrogen bonds (Gardner & Blackwell, 1975), even though both hydroxyl groups at C6 and C3 can be substituted under other circumstances, for instance on hydroxypropylation with glycerol α-monochlorohydrin. The water solubility of CM-chitin becomes apparent when d.s. is over 0·6.

The degree of carboxymethylation was estimated as 0·6–0·8 under the reaction conditions given above from elemental analysis and potentiometric titration. The pK_a of the attached carboxyl group was found to be 3·40 (in agreement with the pK_a value for glycolic acid) from a potentiometric titration in 0·1 M NaCl and the content of the amino groups was estimated to be less than 6%.

CM-chitin shows characteristic polyelectrolyte behaviour in aqueous solution. The reduced viscosity when plotted against concentration showed a maximum when the sodium chloride concentration was quite low. The reduced viscosity became proportional to the concentration of CM-chitin at high sodium chloride concentrations. The intrinsic viscosity of CM-chitin was calculated to be 5·2 dl g^{-1} in a 0·1 M NaCl solution at 25°C from the intercept of the reduced viscosity/concentration plot. The number average molecular weight of CM-chitin was thus estimated to be $1{\cdot}63 \times 10^5$ by applying the viscosity equation proposed by Kaneko.

While chitosan is known not to bind calcium to a significant extent (Muzzarelli, 1973, 1977), CM-chitin was found to adsorb calcium ions to an extent depending on the proportion of the molecule in the hydro-

gen or sodium form. Since the moles of adsorbed calcium correspond to one half of the moles of carboxyl groups, the binding seems to involve two carboxylate ions and perhaps one acetamido and one hydroxyl group simultaneously (Tokura *et al.*, 1983*b*). The strikingly high affinity of CM-chitin toward Ca^{2+} seems to suggest the formation of a chelate complex between CM-chitin and the calcium ion because of the large difference in selectivity constants of CM-chitin and CM-cellulose. The selectivity constant of CM-chitin toward Ca^{2+} is 230 times higher than that of CM-cellulose (H-type) and 17 times higher than that of CM-cellulose (Na-type). The net capacity of the CM-chitin sodium salt was estimated to be 1·6 mg Ca g^{-1}. Calcium is the only alkali-earth element that can be collected to such an extent. Manganese was also reported by Tsutsumi *et al.* (1986) to interact with CM-chitin, this being another point of difference between chitin/chitosan and CM-chitin.

While chitin was found to interact with bovine blood proteins, the affinities of these proteins for *O*-CM-chitin tended to be depressed. Bovine serum albumin (BSA) showed the highest affinity among the proteins applied to CM-chitin (d.s. 0·35), their K values (adsorption coefficients) being 20 for bovine serum albumin, 1·96 for bovine gamma globulin and 1·2 for bovine fibrinogen. The binding site of BSA for CM-chitin was assumed to be regulated not only by the cationic groups of BSA but also by other factors such as the recognition capacity of BSA to bind GlcNAc residues in CM-chitin. Binding is maximal in the pH range 5–7 where both surfaces should be charged negatively as the pK_a of the carboxyl group of CM-chitin was reported to be 3·4, and the isoelectric point of BSA is known to be 4·8. The pH dependence of the adsorption of protein would also suggest the presence of a type of driving force to overcome such an electrostatic repulsion between BSA and CM-chitin (Nishimura *et al.*, 1984*b*).

Immunoadjuvants

Chitin from Queen crab shells and various kinds of derivatives of chitin have been examined from the immunological standpoint, to define the activation of peritoneal macrophages *in vivo*, suppression of growth of Meth-A tumour cells in syngeneic mice and stimulation of non-specific host resistance against *Escherichia coli* infection, and to develop new immunopotentiating agents affecting host resistance to tumour invasion and bacterial infection (Nishimura *et al.*, 1984*b*).

Thirty per cent deacetylated chitin (30% DA-chitin), 70% DA-chitin and CM-chitin induced cytotoxic macrophages most effectively. Chitosan, hydroxyethyl chitin, dihydroxypropyl chitin (DHP-chitin) and

DHP-chitosan had moderate activities. Phosphorylated, sulphonated or acetylated chitins, however, were less effective. Both 70% DA-chitin and DHP-chitosan were most active on the suppression of Meth-A tumour growth in BALB/c mice, and 30% DA-chitin had a moderate effect. For the stimulation of non-specific host resistance against *E. coli* infection, 30 and 70% DA-chitin were effective.

CM-chitin was as effective as 70% DA-chitin at inducing cytotoxic macrophages. In contrast, no cytolytic activity was observed in macrophages from mice injected with phosphorylated and sulphonated chitins, but potent cytostatic and H_2O_2 releasing activities were present. A particular reduction of activity was observed with O-CM-chitosan compared with CM-chitin, especially in cytolytic activity.

The activation of macrophages by 70% DA-chitin or CM-chitin was examined in the dose range 20–2500 μg mouse^{-1}. The maximum cytolytic activity was induced by 70% DA-chitin in the range from 100 to 2500 μg and by CM-chitin at the dose of 500 or 2500 μg mouse^{-1}. Potent cytostatic activity was induced by both polysaccharides at every dose.

A close correlation was observed among three activities, tumouricidal activity of macrophages, tumour-suppressive and protective activity against *E. coli* infection in mice treated wtih 30% DA-chitin and 70% DA-chitin, whereas chitosan had a moderate effect on macrophage activation but no effect on host resistance, and chitin was not effective at all. Macrophages play a very important role in host resistance to tumour cells and bacteria. The efficacy of partially deacetylated chitins on tumour-suppression and bacterial infection may be explained by the activity of macrophages induced by them.

6-*O*-Substituted derivatives of chitin assumed different aspects in relation to macrophage activation and host resistance. Even effective derivatives for macrophage activation, such as CM-, HE- and DHP-chitin, had no effect on host resistance against tumour cells and bacteria. In this case, activation of macrophages was not reflected in the host resistance. The properties of macrophages treated with partially deacetylated chitins are different from those treated with 6-*O*-substituted derivatives of chitin, because, the cytolytic activity of macrophages induced by CM-chitin was drastically reduced. Potent cytotoxicity was shown by 70% DA-chitin-induced macrophages.

The solubility in water and the susceptibility to lysozyme of CM-chitin parallel the degree of carboxymethylation at C6 (Nishimura *et al.*, 1986*a*). The above results could lead to an adjuvant having reasonable solubility, biodegradability and unique pharmacodynamics by chemical modifications of chitin (Tokura *et al.*, 1983, 1987).

Liposomes

The development of artificial red blood cells substitutes, useful for all recipients regardless of their blood groups, has been attempted by many investigators. The necessity to develop such substitutes has become increasingly important, because man-to-man transfusion may be one of the causes of many immunologically infectious diseases, including AIDS (Kato *et al.*, 1985*a*, *b*).

Liposome-type artificial red blood cells (ARBC) containing human haemolysate using non-toxic materials such as lecithin and CM-chitin have been prepared by a two-step emulsification technique. Chitin and its derivatives are expected to be usable for biomaterials owing to their non-toxic and enzymatically biodegradable properties (Kato *et al.*, 1985*a*; Nishimura *et al.*, 1986*b*; Pangburn *et al.*, 1981).

From the therapeutic viewpoint, liposomes containing drugs, enzymes or solutes are promising intravenous carriers. However, phosphatidylcholine liposomes survive poorly *in vivo* when they are exposed to biological fluids such as serum or plasma constituents (Allen & Cleland, 1980). Moreover, it is reported that conventional liposomes hardly retain stability to aqueous globular proteins such as haemoglobin (Kim & Martin, 1981). In contrast, the preparation of lipsomes using CM-chitin as a stabilizer yields very stable liposomes containing human haemolysate. ARBC were shown to have a remarkable stability to various plasma proteins and nonionic surfactants, and good flow properties in plasma or plasma expander solutions. An electron microscopy study on these vesicles by Wehrli *et al.* (1984) suggested that CM-chitin molecules would form a mesh-like structure on the surface of the ARBC and this structure contributes greatly to the stability of the cell integrity. Egg yolk phosphatidylcholine vesicles stabilized with NCMC are also stable against disintegration by surfactants (Izawa *et al.*, 1986).

Liposome-type ARCB stabilized with CM-chitin were prepared by Kato *et al.* (1984) in the following way. Egg yolk lecithin was dissolved in dichloromethane (50 mg ml^{-1}) under nitrogen. CM-chitin was dissolved in a phosphate buffer solution (pH 7·4; ionic strength 0·154, concentration 0·2%). To human haemolysate (10 ml) was added an equal volume of the lecithin solution, and the mixture was agitated for 30 s to give a water-in-oil emulsion. The emulsion obtained was quickly added with stirring to the CM-chitin solution (100 ml) to yield a water-in-oil-in-water complex emulsion. After 10 min stirring, another 100 ml of the CM-chitin solution was added to the complex emulsion and the stirring was continued until the dichloromethane was completely evaporated.

Lysozyme and chitinase produce almost the same degrees of increase in the reducing power of CM-chitin up to a substrate concentration of 0.5 mg ml^{-1}. Therefore, these enzymes are likely to exert almost the same levels of hydrolytic action on CM-chitin molecules in the phosphate buffer solution until the substrate concentration reaches this value.

Acute toxicity of the ARBC to mice was investigated by means of intravenous injection and it was calculated that *c.* 1 litre of the ARBC suspension at 10% (v/v) particle concentration can be used in transfusion for a recipient who weighs 70 kg.

O-CM-CHITOSAN (METHOD OF PARK *et al.*, 1986)

Preparation

Chitin, suspended in NaOH (42%) is reacted with sodium monochloroacetate obtained by adding monochloroacetic acid (30°C for 5 h) the final concentration of NaOH being 18%. The pH is adjusted to 7 with HCl and the solution is dialysed for 3 days against deionized water.

This procedure differs from the one given above in that isopropanol is not used and the alkali treatment is made at higher temperature (30°C) thus permitting the deacetylation of chitin. This method, which refers to Okimasu's previous one (Okimasu, 1957) combines the advantage of omitting the organic solvent and starting with chitin to yield *O*-CM-chitosan.

Characteristics

The pK_a of the carboxyl group in *O*-CM-chitosan was estimated to be much lower than the pK of glycolic acid, 3·8, because of intramolecular electrostatic attraction between $-COO^-$ and $-NH_3^+$ groups. The group titrated between the two inflection points at pH 5·1 and 8·9 of the alkalimetric curve was assumed to be $-NH_3^+$ and therefore the amine content in *O*-CM-chitosan, was estimated to be 1·39 meq g^{-1} corresponding to a degree of deacetylation of *c.* 33%. The pK_a of the amino group was 7·0, between the pK_a values of the same group in chitosan (6·2) and in D-glucosamine (7·8).

The sodium ion content of basic *O*-CM-chitosan was 1·42 meq g^{-1}. This value was assumed to correspond to the $-COO^-$ group in the polymer. The *O*-CM-chitosan sample prepared in this investigation was soluble in water over the pH range 3–11. The sharp increase in η_{sp} at

pH 8 reflects the expansion of the polymer chain due to the deprotonation of the $-NH_3^+$ group. This pH dependence is characteristic of a polyelectrolyte and matches the titration data. Also, the virtual invariance of viscosity at pH 4–5 supports the assumption that neither $-COO^-$ nor $-NH_3^+$ group in the *O*-CM-chitosan is titrated in that pH range. Addition of NaCl leads to large decrease in viscosity of the solution; especially above pH 6, this being a typical salt effect on the viscosity of polyelectrolyte solutions.

The capacities of *O*-CM-chitosan for various metal ions were calculated from the concentrations inside and outside cellulose bags after equilibrium dialysis at ionic strength of 0·1 M and were found to be between 0·05 and 0·3 mmol g^{-1} for Cd, Ni, Pb and Cu. In all cases, the capacities increased with pH, and the decreasing order of binding affinity was $Cu^{2+} > Pb^{2+} > Ni^{2+} > Cd^{2+}$. These capacities appear to be low, and could have been limited by the experimental conditions and set-up selected.

At acidic pH, the spectra of the copper chelate of *O*-CM-chitosan display an absorption peak at 238 nm. This being the typical carboxylate to copper charge transfer band indicates that Cu^{2+} ions bind mainly to $-COO^-$ and the binding increases with pH. At high pH, for example pH 9·7, the spectrum shows another band near 270 nm, and this can be attributed to the involvement of the $-NH_2$ groups to the copper complexes. This spectral change with pH confirms that both $-COO^-$ and $-NH_2$ groups in *O*-CM-chitosan serve as binding sites to Cu^{2+}, depending on pH of the solution.

N-CM-CHITOSAN (METHOD OF MUZZARELLI, 1982)

Preparation

This method for the *N*-carboxymethylation of chitosan consists of reacting the free amino groups of chitosan with glyoxylic acid to produce a soluble aldimine and then reducing the product with a suitable reducing agent. This preparation is very readily effected under homogeneous conditions and does not require warming or cooling. It produces an *N*-carboxymethyl chitosan, containing acetyl, carboxymethyl, and free amino groups in proportions readily controlled through the choice of the starting chitosan (in terms of degree of deacetylation and molecular weight) and the amount of glyoxylic acid used. When the chitosan is highly deacetylated, the reaction product can be called glycine glucan,

because nearly all of the side groups are glycine residues (Muzzarelli, 1985, 1986*a*,*b*; Muzzarelli & Zattoni, 1986; Muzzarelli *et al.*, 1985, 1987).

Glyoxylic acid is added to an aqueous suspension of chitosan to cause immediate dissolution yielding a clear solution that is more or less viscous according to the concentration of the biopolymer. The resulting pH is 3·2 when glyoxylic acid (0·57 g) is added to the chitosan powder (1 g) suspended in water (30 ml) to give a molar ratio 1·0 between glyoxylic acid and 2-amino-2-deoxy-D-glucose residues.

As soon as sodium borohydride is introduced, the pH rises to about 4·0 and, at the end of the reduction step, the pH is 4·8. Depending on the characteristics of the chitosan used, the *N*-CM-chitosan obtained will be either soluble or insoluble.

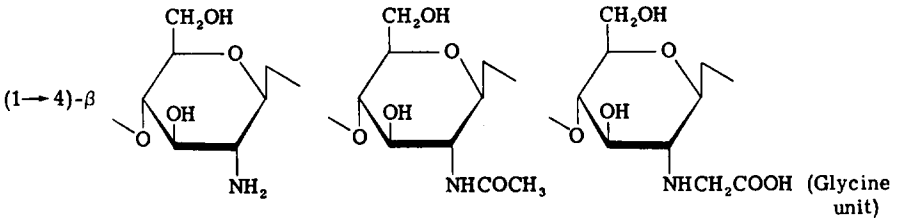
Characteristics

In the i.r. spectra of the variously substituted polymers conditioned at pH 1, the band at 1730 cm^{-1} (—COOH) increases as the degree of substitution increases. It also appears that the absorption at 1500 cm^{-1} ($—NH_3^+$) decreases on progressing from chitosan (pH 1) to the fully substituted *N*-CM-chitosan.

The samples treated with an excess of glyoxylic acid were found to be fully carboxymethylated, to the extent allowed by their degrees of deacetylation.

The differential enthalpy of protonation for *N*-CM-chitosan ($\Delta H_p = -2{\cdot}3$ kcal mol^{-1}) is in agreement with the values for glycine, *N*,*N*-dimethylglycine and *N*-tris(idrossimethyl)methane glycine, thus giving indication that the carboxymethyl group is essentially free from interaction with the polysaccharidic backbone. The ΔH_p for polyglucosamine ($-2{\cdot}6$ kcal mol^{-1}) being far from the values reported for the protonation of aliphatic amines (-9 to -15 kcal mol^{-1}) indicates that the primary amino groups are partly engaged in hydrogen bonds (Muzzarelli & Delben, 1987).

The n.m.r. spectra for chitin and chitosan have been discussed by Casu (1985) and the ^{13}C n.m.r. spectrum of *N*-CM-chitosan has been published by Muzzarelli *et al.* (1985, 1986). Signals attributed to the *N*-carboxymethyl substituent ($—COO^-$ and *N*-CH_2) are at 168·7 and 47·7 ppm, respectively. A downfield shift (*c.* 6 ppm) is observed for the carbon bearing the *N*-carboxymethyl group (C2′), with respect to the corresponding carbon of unmodified residues (C2, 57·8 ppm). From the area ratio between signals C2 and C2′ (as well as the corresponding



Typical *N*-Carboxymethylchitosan

MW:	161.15	203.19	219.19
Percent:	29 ± 2	42 ± 4	29 ± 2
Av. MW:	-	195.64	-

Fig. 1

anomeric carbons C1 and C1′), *N*-acetylation is confirmed to be *c.* 40% and *N*-carboxymethylation more than 50%. The *N*-CM-chitosan produced with stoichiometric ratio of 1:1 for the amino group glyoxylic acid shows that the primary amino groups of chitosan react nearly quantitatively even when the degree of deacetylation is relatively low and interchain hydrogen bonds exist.

The weight average molecular weight of *N*-CM-chitosan was measured by Muzzarelli *et al.* (1987). For the *N*-CM-chitosan obtained from glyoxylic acid under reducing conditions at pH 4·8, a weight average molecular weight (M_w) of 545 300 g mol^{-1} was obtained, in agreement with the value calculated for the *N*-CM-chitosan on the basis of the *Euphausia superba* chitosan for which laser light scattering (l.l.s.) indicated 464 990 g mol^{-1} and gel permeation chromatography gave 450 000 g mol^{-1} (Table 1). This corresponds to the complete reaction of the available free amino groups, leading to a modified polymer containing 58% of its units in the carboxymethyl form and 42% in the original acetamido form. This also confirms that the reaction of glyoxylate with chitosan proceeds to 100% yield, as indicated by n.m.r.

As far as the Schiff bases are concerned, the data obtained in the three cases listed in Table 1 are much higher than expected. For the glyoxylic acid aldimine, a M_w of 1 075 000 g mol^{-1} was recorded. The molecular weight of the α-ketoglutaric acid, 592 530 g mol^{-1}, points to 99% substitution which is not in agreement with the 30% substitution measured by titration. For the 3,4-dihydroxybenzaldehyde chitosan aldimine, the values are also high, 896 490 g mol^{-1}, and while this could possibly be explained in other ways, the general behaviour of these three Schiff bases would indicate a tendency to association which is not shared by water-soluble *N*-CM-chitosan; the other two compounds, namely glutamate glucan and dihydroxybenzyl chitosan, being insoluble.

TABLE 1

Properties of Krill (*Euphausia superba*) Chitosan and its Carboxymethylated Derivatives, as Measured by Laser Light Scattering Spectrometry (Muzarelli *et al.*, 1987)

Chitosan or chitosan derivative	*Molecular weight*	*Radius of gyration (nm)*	*2nd virial coefficient* (g^{-2} *mol* cm^3)
Chitosan from krill	464 990	75·0	$1{\cdot}20 \times 10^{-3}$
Water soluble derivatives			
N-Carboxymethyl chitosan	543 300	70·2	$0{\cdot}83 \times 10^{-3}$
Glycol *N*-carboxymethyl chitosan	316 000	62·9	—
N-Carboxymethyl chitosan 6-sulphate	16 000	—	—
Water soluble Schiff bases			
Glyoxylate	1 075 000	113·8	$1{\cdot}80 \times 10^{-3}$
α-Ketoglutarate	592 530	83·1	$0{\cdot}93 \times 10^{-3}$
3,4-Dihydroxybenzaldehyde	896 490	112·9	$1{\cdot}39 \times 10^{-3}$

One of the characteristic properties of *N*-CM-chitosan is its ability to chelate transition metal ions, thus yielding insoluble chelates, which readily settle as hydrated solids within minutes after mixing. Even relatively dilute solutions (0·2 mM) may be conveniently treated with solutions of *N*-CM-chitosan to collect transition metal ions: the supernatant solution may be removed by filtration or centrifugation.

The fully substituted *N*-CM-chitosans were particularly suitable for precipitating transition metal ions; less substituted *N*-CM-chitosans formed precipitates that did not settle quickly and remained gelatinous. The insolubilization of metal ions by chelation with soluble *N*-CM-chitosan is pH dependent. With Co, Cu, Zn, Hg and Pb, chelation maxima are observed at neutrality (pH 6–7), whereas Ni and Cd show maxima at 7·5 and U at 5·5.

A linear dependence of the binding percentage on the concentration of *N*-CM-chitosan was observed in almost all instances, even with low metal ion concentration (0·2 mM). In the concentration interval studied, *N*-CM-chitosan completely scavenged Co, Ni, Cu, Cd, Pb and U from 0·2 mM solutions; Cu, Hg, Pb and U could also be completely removed, even when present at higher concentrations (0·3–0·5 mM).

Dilatometric titrations showed that the solvation sphere of *N*-CM-chitosan is disturbed as a consequence of chelation and that *c.* 20 and 6 mol of water are released per 1 mol of bound Cu and Pb, respectively. Reaction enthalpies for the chelation of Cu, Pb and Ca are exothermic whilst endothermic values are commonly found for polycarboxylate

polymers and carboxylic acids. This fact points to the involvement of other electron donating groups in the chelate formation, as confirmed by circular dichroism analysis. The latter technique also gives evidence of the lack of calcium chelation by *N*-CM-chitosan.

Cross-linked chelating polymers

Glycine glucan (1 g) prepared as above from glyoxylic acid and polyglucosamine, was kept stirred in suspension in 40% NaOH (5 ml) to which isopropanol (25 ml) was added. In this way, the alcohol groups became sodium alcoholate groups. After *c.* 60 h, a mixture of dioxane (90 ml), water (52·5 ml) and epichlorohydrin (2·5 ml) was added. The reaction required *c.* 72 h for completion. Small beads of a colourless, transparent, and rigid gel were formed; they were submitted to dialysis against water and isolated as a product containing 90–92% water (Muzzarelli, 1987).

The cross-linked glycine glucan was contacted with cobalt, nickel and copper solutions at pH values between 5 and 6. The initial concentration ranges were: Co and Cu, 50–1000 mg litre^{-1} and Ni 50–1000 mg litre^{-1}. After 16 h the capacities were measured and plotted against the equilibrium concentrations. The results showed that the capacities for Co and Cu at equilibrium concentration 0·6 mg litre^{-1} were 14 and 30 mg g^{-1} (1·4 and 3·0% by weight) respectively, while at 750 mg litre^{-1} were 140 and 240 mg g^{-1} (14 and 24% by weight) respectively. The polymer exhibited typical colours, pink, green and blue, for Co, Ni and Cu, respectively. In view of the high capacities for transition metal ions, it is a suitable chromatographic support and filtration aid.

In contrast to chitosan, the cross-linked glycine glucan gives a totally amorphous X-ray diffraction pattern and the same can be said for the cobalt- or copper-loaded polymer. The absence of crystallinity seems to act synergistically with the powerful glycine groups in chelation, and explains the exceptionally high capacities.

Blood anticoagulants

The sulphation of partially depolymerized *N*-CM-chitosan in pyridine and chlorosulphonic acid mixture leads to derivatives carrying sulphate groups on primary and secondary alcohol functions as well as on the residual amino groups (Muzzarelli *et al.*, 1984). The *N*-CM-chitosan trisulphate thus prepared was characterized as a beta(1-4)glucan carrying *N*-carboxymethyl, sulphamido, acetamido and sulphate ester groups on C3 and C6 (sulphur content 11·0–15·2%).

The results confirmed that the sulphation on C6, while essential for activity, should be preferably accompanied by sulphation on other positions; moreover the sulphamido group does not need to be present to a high degree of substitution; and finally, the carboxymethyl group, which can be conveniently introduced on the amino group, adds to the biological activity. Carboxymethylation seems to impart activity if directed to the amino group rather than to the C6 hydroxyl group. Enhanced activity could be obtained by defining the optimum molecular weight and by reducing polydispersity.

The most interesting aspect of that work (Muzzarelli & Giacomelli, 1987) is that the samples studied specifically interact with the factors of the intrinsic coagulation pathway, while they do not interact with those of the extrinsic and common pathway. Their behaviour is distinctly different from that of heparin. The characteristic properties of *N*-CM-chitosan trisulphate open the perspective of the use of blood anticoagulants which selectively act on only one branch of the coagulation cascade, thus leaving unaffected an alternative route to coagulation.

Cosmetic uses of CM-chitin and *N*-CM-chitosan

Low molecular weight chitosan (6000 g mol^{-1}) has found application as an ingredient for shampoos and lotions because of its cationic nature associated with its film-forming ability and antistatic properties. If emulsion-type cosmetics for skin care were to be formulated, the acids which must be used to dissolve chitosan would impart undesirable properties to the cosmetic creams; however, the water-soluble derivatives of chitin/chitosan are of use in cosmetics.

Chitin and its derivatives have a number of favourable biochemical and physiological properties, which are briefly mentioned here.

Chitin is an absorbable suture material which finds wide acceptability because tissue reaction is not specific and the good healing which ensues provides evidence for a satisfactory biocompatibility. Toxicity tests, including acute toxicity, pyrogenicity and mutagenicity are negative; and no other adverse effects have been found (Muzzarelli *et al.*, 1987).

Lysozyme plays an important role in the *in vivo* degradation of chitin/chitosan and carboxymethylated derivatives. Oligomers are further hydrolysed by exo-glycosidases such as beta-*N*-acetylglucosaminidase and beta-*N*-acetylhexosaminidase. The generated *N*-acetyl glucosamine, a common aminosugar in the body, enters the innate metabolic pathway to be incorporated into glycoproteins or to be excreted as carbon dioxide. Thus, the chitin and its degradation product are natural and safe.

Chitosan may be used to inhibit fibroplasia in wound healing but also to promote tissue growth and differentiation in tissue culture. Chitosan provides a non-protein matrix for three-dimensional tissue growth and suppresses contamination by microorganisms in tissue culture. Vascular graft implantation, wound healing, bone repairs and cell tissue cultures have been demonstrated to be beneficially affected by the presence of chitosan (Muzzarelli *et al.*, 1986). Chitosan shows a biological attitude to stimulate cell proliferation and hystoarchitectural tissue organization. It can play the role of a biological primer for cell tissue proliferation and reconstruction. Chitosan can be considered a polysaccharide with biochemical similarities with other extracellular glycoprotein carbohydrates such as glycosaminoglycans and therefore it might play similar morphogenetic functions. Histological evidence indicates (Muzzarelli *et al.*, in preparation) an orderly organized connective matrix in chitosan-treated tissue that is the result of chitosan influence on mesenchymal elements, as already documented for glycosaminoglycans.

As indicated in other sections of this review, chitin and chitosan share some of these attractive properties with carboxymethylated chitins and chitosans. The latter, in fact have further similarities with the extracellular matrix polysaccharides, i.e. water solubility, anionic functionality high viscosity, large hydrodynamic volumes, cation binding characteristics, large osmotic pressures and gel-forming capabilities. Modified chitin/chitosan could also function as a selective permeability barrier, as is the case for hyaluronate, which forms a protective layer around the human ovum.

One could also speculate that CM-chitin and *N*-CM-chitosan can provide some aid against senescence of the skin. Longas *et al.* (1987) have presented evidence that in man dermatan sulphate and hyaluronate undergo *N*-deacetylation between the ages of 60 and 75 years; thus if one of the mechanisms of skin senescence is the deacetylation of *N*-acetylglucosamine to glucosamine, it would be preferable to use CM-chitin and *N*-CM-chitosan which contain large quantities of *N*-acetylglucosamine; furthermore, in *N*-CM-chitosan those units which are not *N*-acetylated carry glycine groups which could be of particular significance in view of the fact that glycine is most abundant in skin.

While the above aspects relate to the regeneration of the skin components and deserve further investigations, other aspects of a more immediate significance to the cosmetologist have led to the proposal of *N*-CM-chitosan and CM-chitin as ingredients for creams. For instance, 0·25% aqueous solutions of *N*-CM-chitosan and CM-chitin were found to be comparable to a 20% aqueous solution of propylene glycol in terms of moisture-maintaining capacity and the viscosities of the solu-

tions containing minor amounts of *N*-CM-chitosan or CM-chitin were higher than those of other moisture-maintaining agents and almost equal to that of hyaluronic acid.

While many polysaccharide solutions show various changes on heating (some solutions gel, some harden, some form films and some become less viscous), the viscosity of a 0·25% aqueous solution of CM-chitin is very stable: it does not gel nor harden nor form a precipitate over a wide range of temperatures and times. Furthermore, solutions are stable over a wide pH range (4·0–10) and remain clear though a slight decrease in stability is observed below pH 4·0. Consequently, CM-chitin can be used as a viscosity builder or a moisture-maintaining agent in liquid products, like clear lotions, in basic cosmetics like creams and milky lotions which require heat treatment or heat sterilization and in liquid products for cold permanent wave with high pH. Of course, these properties would prevent its use for the preparation of gels, which, on the other hand, are easily prepared with *N*-CM-chitosan. As far as the stability of the emulsions containing *N*-CM-chitosan is concerned, particle sizing by l.l.s. has given evidence of an improvement in stability.

N-CM-chitosan possesses bacteriostatic activity which is notably absent in CM-chitin as well as in sulphated *N*-CM-chitosan. The bacteriostatic activity of *N*-CM-chitosan is particularly high on bacteria typical of the oral cavity, and therefore *N*-CM-chitosan can be used as an ingredient for toothpastes.

CM-chitin was assessed for safety to the skin in animal experiments in which primary skin irritancy, accumulated irritancy, contact allergy, phototoxicity and primary eye irritancy were tested; the results obtained in every test were negative. Human patch tests with the liquid undiluted preparation of *N*-CM-chitosan and CM-chitin were performed and no abnormality was observed.

N,O-CM-CHITOSANS (METHODS OF HAYES, 1986 AND MUZZARELLI, 1987)

Preparations

To chitosan (20 g) suspended in isopropanol (200 ml) under stirring, NaOH (50·4 ml, 10 M) is added in six equal portions over a period of 20 min. The alkaline slurry is stirred for an additional 45 min, then solid monochloroacetic acid (24·0 g) is added in five equal portions at 5-min intervals. Heat is then applied to bring the reaction mixture to a temperature of 60°C for 3 h. Next cold distilled water (17 ml) is added to the mixture and its pH adjusted to 7·0 by addition of glacial acetic acid. The

reaction mixture is then filtered and the solid product washed with a 70% methanol/water mixture (300 ml) and then with anhydrous methanol. The resultant *N,O*-CM-chitosan (30 g) is dried in an oven at 60°C (Hayes, 1986).

This procedure yielding a carboxymethyl derivative at both C6 and N2 of chitosan, differs from the Park's procedure because the latter introduces into the polysaccharide a certain degree of deacetylation but does not take advantage of the generated free amine, while Hayes starts from a deacetylated compound the amino groups of which are reacted with monochloroacetate at 60°C. Thus monochloroacetate combines with both the primary alcohol and the primary amine.

An alternative route to *N,O*-CM-chitosan is the following, proposed by Muzzarelli (1987): chitosan, suspended in NaOH (42%) is reacted with monochloroacetate (20°C, 24 h) and then neutralized with HCl. Glyoxylic acid is added to the *O*-CM-chitosan solution in the ratio glyoxylate/amine close to 1·6/1·0. The resulting solution (pH 3·5) is treated with sodium borohydride to pH 4·8 over a period of 24 h and then dialysed against deionized water, and liophilized to yield *N,O*-carboxymethyl chitosan sodium salt.

This procedure has a number of advantages, because it does not require heating, and the synthesis is conducted totally in aqueous media. Both of these *N,O*-CM-chitosans are still to be fully characterized; they appear to be insoluble in the pH range 4–6.

CONCLUSIONS

The carboxymethylation of chitins and chitosans imparts water solubility to these insoluble polysaccharides. The carboxylate group is readily-solvated by water and inhibits intermolecular association through electrostatic repulsion (Glass, 1986). The substitution also introduces or enhances non-uniformity in the repeating structure. Chain rigidity is also alleviated, being progressively lower in going from chitin to chitosan, 6-*O*-CM-cellulose, *N*-CM-chitosan and polyglucosamine. All of these factors lead to interesting water-soluble derivatives which appear to be reproducible and well characterized.

Certain characteristic properties of CM-chitins and CM-chitosans are shared with some synthetic polymers; for instance, polymers with carboxylate functionality have been found to elicit a broad range of biological activities (Ottenbrite, 1985). Polymers carrying the —NH—CH_2—COOH group have chelating ability; others, such as the Carbopol® polymers, are used in cosmetic formulations. As far as CM-chitins and

CM-chitosans are concerned, their original structure based on *N*-acetyl glucosamine enhances their performances and makes them more biocompatible, more hydrophilic, more biodegradable and more amenable to various physical forms than synthetic polymers.

Ten years of research activity have brought a significant increase in our understanding of carboxymethylated chitin and have established simple procedures for their preparation; other procedures are also being investigated (Sachetto & Bedert, 1985). Still many physico-chemical aspects of their behaviour remain to be studied; however, these substances will possibly become a very versatile class of water-soluble chitins.

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