THE FORMATION OF INCLUSION COMPOUNDS OF ALGINIC ACID IN AQUEOUS SOLUTION

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ABSTRACT. From the microscopic visualization of metal alginates, the alginate fine structure was found to be a regularly arranged network having many host spaces in its fiber net. Under acid solution such a fiber net took the rounded, buckled and twisted form as much as possible and finally changed to a porous cluster form. Then the binding properties of sodium alginate, calcium or cadmium alginate and granular alginic acid obtained from the treatment with hydrochloric acid to iodine, cholesterol and dyes were investigated. Sodium alginate and calcium or cadmium alginate formed adducts with iodine, cholesterol and dyes; accordingly the amount of these materials in solution was reduced by the addition of alginates. The high uptake of alginates by these materials were cleary observed under acid solution giving inclusion compounds. Granular alginic acid also showed high uptake of iodine and dyes, but not of cholesterol. The blue stained alginate-iodine compound formed at pH about 1 having maximum absorption at 587nm was found to be an inclusion compound judging from the distance of 3.13 Å for included iodine I-I and the number of uronic residues bound to the iodine molecule in the granular alginic acid-iodine compound. The binding mechanism for the high uptake behavior of alginic acid was clarified with the observation of inclusion compounds by scanning electron microscopy(SEM) and transmission electron microscopy(TEM).

INTRODUCTION

The effect of sodium alginate in reducing the uptake of strontium[1], cadmium[2] and cholesterol[3] absorption in man has been reported, but the binding mechanism of high uptake behavior of alginic acid has not yet been explained. Sodium alginate(1) is a binary heterogeneous copolymer of D-mannuronate(M) and L-guluronate(G) residues arranged in a blockwise pattern along the linear chain. There are three types of blocks, that is, M block, G block and MG block in which these two uronic acids occur in some sort of alternation.

The binding properties of alginate depend not only upon the uronate composition, i.e., the G/M ratio, but also upon the chain length, i.e.,

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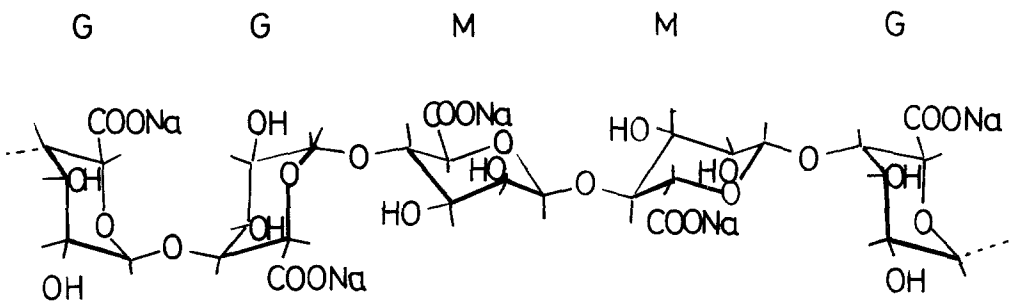


(1)

the number average degree of polymerization($\overline{DP}$). In general, guluronic acid-rich alginates(G-rich) were found to be more effective than mannuronic acid-rich alginates(M-rich) and degraded sodium alginates(m.w.= ca.30,000 for Sr) could bind strontium more strongly than undegraded ones[4]. To clarify these points, six kinds of sodium alginate samples (commercial sodium alginate, its degraded one, G-rich one, M-rich one, pure G one and pure M one) have been prepared and reacted with many ions (cadmium, calcium, strontium, zinc, lead and iron...Fe^{3+}) by single ion or by two ion mixed experiments. Metal alginate beads formed by the precipitation method and metal alginate films formed by the dialysis method have been obtained and then the number of metal ions bound to one uronic acid residue has been calculated by the amount of metal ions included in these metal alginates using the results obtained by EDTA titration or atomic absorption analysis in our previous work[5]. However, these binding experiments showed many exceptional facts. That is, the affinity of sodium alginates for heavy metals increased markedly in both dialysis and two ion mixed experiments. In a single ion experiment by the precipitation method, the number of metal ions bound to one uronic acid residue was not greater than 0.5 which indicated that for metal ions a tight interchain chelation of the divalent metal to the carboxylate groups on the interior faces of the dimer was present, but on two occasions the number reached about two. Then it was suggested that under certain conditions alginic acid possesses another binding force besides an ionic bond of its carboxyl group with a metal ion. The difference of affinity to cadmium and calcium between pure G one and pure M one was not found.

In this paper, the fine structure of these metal alginates described above(a part of data presented) was at first investigated by SEM. The alginate fine structure was found to be a regularly arranged network having many host spaces in its fiber net. It was considered that if the high uptake behavior of alginic acid depends on this network, metal alginate might produce many inclusion compounds under some conditions. From this point of view, the binding properties of cadmium and calcium alginates(Cd-alg., Ca-alg.) to metal ion(data not shown), iodine, cholesterol and dyes were investigated. At acidic pH, metal alginate beads or films become very small and gave the blue stained alginate-iodine compounds with iodine and the alginate-dye compounds with dyes, and sodium alginate also gave them. If the formation of these

compounds depends on changing the structure of the alginic acid fiber itself,isolated alginic acid(granular alginic acid) obtained from the treatment with hydrochloric acid should also include them. Then the results of binding experiments on granular alginic acid to iodine, cholesterol and dyes are also presented.

MATERIALS AND METHODS

Materials

Sodium alginate...Sodium alginate(Wako Pure Chemical Industries, Ltd) was obtained commercially. This was named CM or sample 1. G/M of sample 1 is ca.0.91 and $\overline{DP}$ is 468-473. From sample 1 insoluble fraction(degraded one, sample 2), pH 2.85(G-rich, sample 3), pH 1.5(M-rich, sample 4), G(pure G, sample 5) and M(pure M, sample 6) were obtained by partial hydrolysis with 1M oxalic acid and by anion exchange chromatography.

Granular alginic acid...Sample 1 was suspended in 0.1, 1, 5 and 10N hydrochloric acid solution, respectively, with stirring slowly for 2hr. The precipitate was collected and suspended again in a fresh solution of the same normality. After centrifugation, the granular precipitate was washed repeatedly by suspension in fresh water until the rinsing no longer gave a chloride ion reaction. After centrifugation, the granular ppt was treated with alcohol and ether to dry.
All other commercially available chemicals used were of the highest purity.

Method

SEM investigation...Film form metal alginate——In a typical experiment, 10ml of 0.1-0.4% sample 1 was pipetted in a cellulose bag(Visking, dialysis tube 36/32) and dialyzed in 100ml of 0.01-0.02M cadmium or calcium nitrate for 24hr. And the bag was washed twice with water and alcohol. The film formed in the bag was attached to the sample plate of the SEM with silver paste or for a fractured surface pasted on the side of a cupper plate placed on the sample plate. Critical point drying(C.P.D.) and Pt-Pd ion sputtering were followed. Bead form metal alginate——In a typical experiment, 25ml of 0.1-0.4% sample(1-6) sodium alginate was added dropwise to 50ml of 0.02M metal nitrate with stirring. After standing for 2-20hr at 30°, alginate beads were separated by glass filter or nylon cloth. The bead form sample was fixed with 2% glutaraldehyde and subsequently with osmium tetroxide. For a fractured surface of the bead form sample, after freezing with liquid nitrogen, it was cut and immersed in absolute alcohol. After exchanging of alcohol with iso-amyl acetate, bead form samples were dried by C.P.D. and sputtered with Pt-Pd ion. Film form and bead form samples were investigated by Hitachi HFS-2RS SEM or S-430 SEM.

Iodine...Metal film was formed as follows. The bottom petri dish in which 10ml of 0.4% sample 1 was filled was covered and sealed with a cellulose membrane, turned upside down and immersed in 20ml of 0.02M

cadmium or calcium nitrate filled in the upper petri dish for dialysis. After 20hr the gel film formed on the membrane was slightly dried with filter papers and then immersed in iodine-potassium iodide solution (I_2-KI soln.). Addition of acid to the solution gave a blue stained film and potassium iodide(KI) and the iodine content of the KI-I_2 soln. had large effects on the intensity of the film extinction. In typical experiments, the solution was adjusted to pH 1 with hydrochloric acid and several parallel experiments on different concentration of KI in the solution were carried out. Because the equipment for a film sample was not available, the extinction measurement was made on a spectrophotometer(Hitachi 340, Hitachi 100-10). Iodine in the alginate-iodine compound was determined by the absorbance at 587nm and the iodine of the solution both by absorbance at 285nm and with a titration value($Na_2S_2O_3$ soln.).

For X-ray diffraction analysis the dried film was attached to an X-ray sample window and measured by Rigaku Co. D-3F meter using copper Kα radiation and 0.15mm slit under 15mA, 30kV. For every run of iodine experiments a blank film was made and measured simultaneously.

Cholesterol...To 1-20ml of 0.01M cadmium or calcium nitrate, 0.5-10ml of 0.4% sodium alginate(CM) was added dropwise. After standing for 2hr at 30°, the beads formed were collected with nylon cloth(first step bead). The beads were suspended in 20ml of 0.02M cadmium or calcium nitrate for 2hr at 30° and then separated with nylon cloth or glass filter(second step bead). After drying with filter papers, first step beads or second step ones were suspended in 30ml of acetic acid containing cholesterol(0-17mg) for more than 30min at room temp. The cholesterol content of the solution was determined by ferric chloride reaction ; an aliquot(3.0ml) was taken and reacted with 'cholesterol detectable solution'(2.0ml)(the solution...8ml portion of which 100ml of phosphoric acid containing 2.5g of ferric chloride($FeCl_3 \cdot 6H_2O$) was diluted by concentrated sulphuric acid to 100ml.). At each run of experiments the absorption measurement was carried out at 560nm with the calibration for cholesterol.

Dyes...Dyes used were Food color Blue No.1, Green No.3, Red No.104 and No.105 and 5-iodo-eosine. Because all of them were slightly soluble in water and even in alcohol, quantitative experiments were impossible. Cd-alg. or Ca-alg. beads formed as described above(first step ones) were added to each color solution(aqua, alcohol). After a day standing at 30° hydrochloric acid to be adjusted at pH 1 and alcohol were added to the solution.

Granular alginic acid...$\overline{DP}$ measured by periodate oxidation is as follows. Granular treated with 5.0N HCl...ca.8.7, 1.0N HCl...ca.8.2 and 0.1N HCl...ca.12.5(the yield was reached ten times more than CM taken.)

TEM or SEM examination of inclusion compound...The blue stained (alginate-iodine compound) film sample and the red stained(alginate-Food color Red No.105 compound) bead sample were stained negatively with 5% uranyl acetate, made by the usual method and observed by 200kV TEM (Hitachi H-800). The shrunk and hard bead separated from acetic acid (alginate-cholesterol adduct) sample was prepared in the same manner described above(for SEM sample) and observed by SEM(Hitachi S-430).

RESULTS

Microscopic examination of metal alginate...Typical appearances of metal alginates are shown in Figure 1 and Figure 2 respectively. Although different metals give different shapes and sizes, the network structure of metal alginate is clearly observed in each figure.

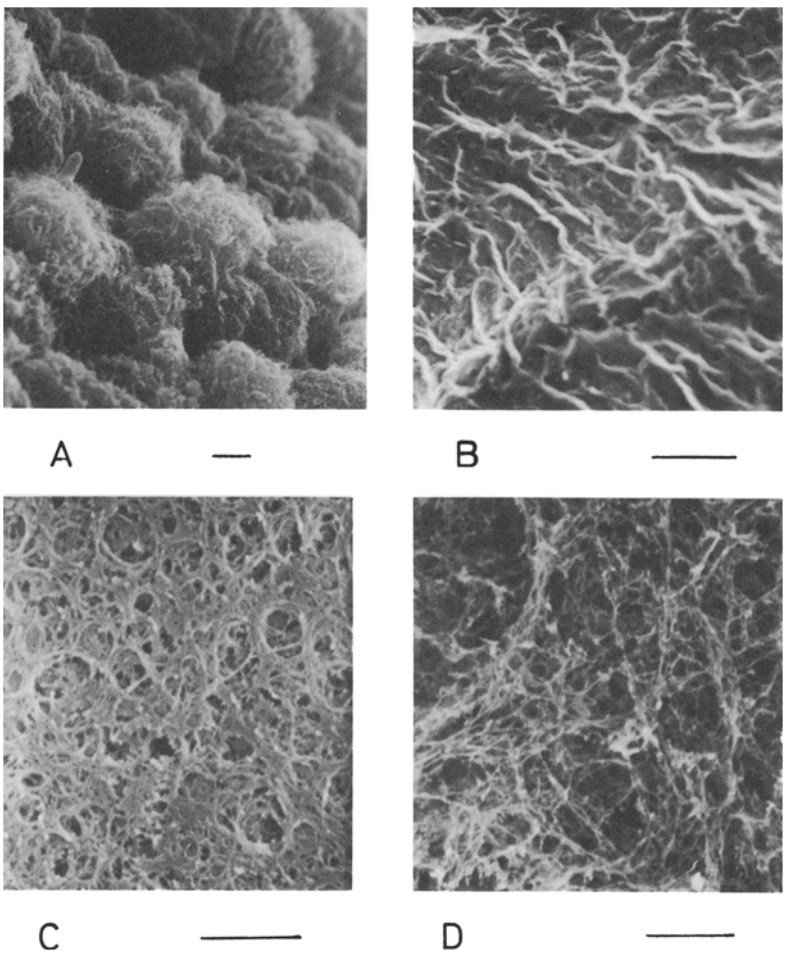


Figure 1. The network structure of bead metal alginate(sample 1).
A: Ca-alg. surface, no fixation B: Pb-alg. surface, fixation
C: Sr·Cd-alg. fractured surface, Fixation D: Fe·Cd-alg. fractured surface, no fixation each Bar=1μm

The structural feature of the surface of metal alginate is the fiber network arranged regularly and closely, but that of the fractured surface is the large and spacious network having round cages or channels

surrounded by the fiber chain nets. The fiber network of the film form is more closely packed than that of bead form and dimer fiber chains are obviously seen. Fixation is usually assumed not to change the sample shape, but the treatment with osmium tetroxide makes the sample small and the fiber chain takes a somewhat fixed but changed form.

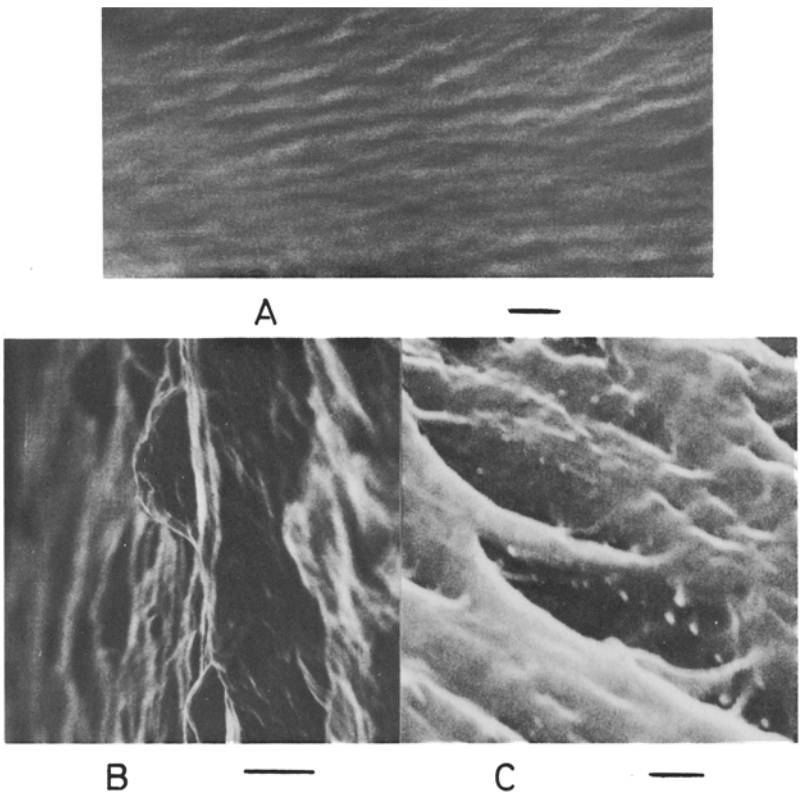


Figure 2. The network structure of film metal alginate(sample 1). A: Ca-alg. surface, no fixation Bar=100nm B: Ca-alg. fractured surface, no fixation Bar=1μm C: Cd-alg. fractured surface, no fixation Bar=100nm

Figure 3 shows the porous structure of a metal alginate. According to the report by Simionescu et al[6] helicoidal portions of alginate chain will be made up of mannuronic acid under hydrochloric acid. In Figure 3 it is clearly observed that under the acid metal alginate fiber net takes the rounded, buckled and twisted form as much as possible and finally changes to a porous cluster form. Sample 4=M-rich is the precipitate at pH 1.5 adjusted with HCl.

Iodine-metal alginate compound...Sodium alginate reacts with iodine and under acid gives a blue stained adduct like a starch-iodine inclusion complex. In this paper results for film form metal alginate are presented. The effect of pH on the formation of a blue stained adduct is shown in Figure 4A. At about pH 1 the formation reaches the maximum. The amount of the adduct shows an increase with iodine concentration and

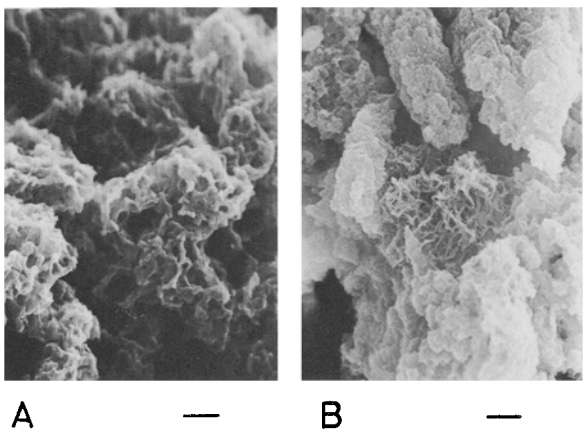


Figure 3. The porous structure of cadmium alginate(sample 4). By precipitation method, A bar=1μm, B bar=2μm.

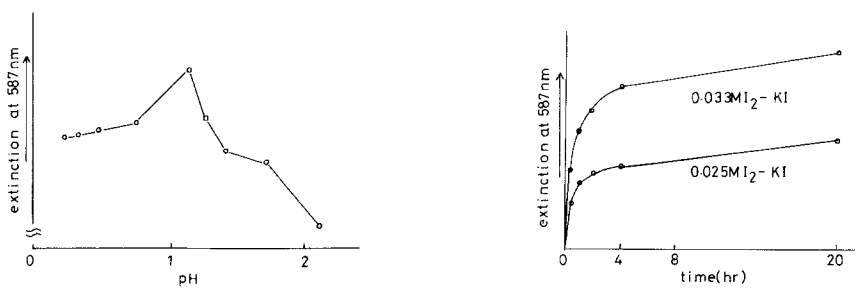


Figure 4A The effect of pH on formation of alginate-iodine compound. Ca-alg. film immersed in 30ml of 0.028M I_2-KI was measured by spectrophotometer. Figure 4B. The effect of immersing time on formation of alginate-iodine compound. Ca-alg. film

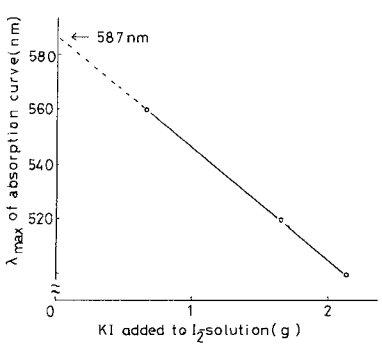
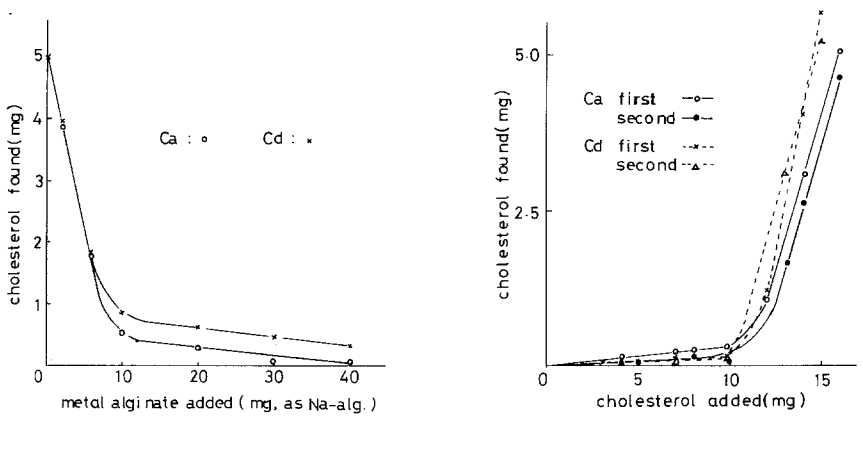


Figure 5. The effect of KI on formation of alginate-iodine compound. Cd-alg. film was immersed in 30ml of 0.015M I_2-KI at pH 1 for 24hr.

immersing time as shown in Figure 4B. But the addition of potassium iodide inhibits the formation. The maximum absorption wavelength of the compound is 587nm of which potassium iodide concentration corresponds to zero(shown in Figure 5).

X-ray fiber diffraction data of film form metal alginate showed broad peaks corresponding to around 7.2Å, 8.7Å. The characteristic fiber peak observed in the blue stained film prepared at pH 1 corresponds to 3.13Å. This value is similar to 3.06Å for I-I distance of iodine packed in helical cylinder of starch-iodine inclusion complex.

Cholesterol...The reduction of cholesterol in acetic acid by increasing the amount of metal alginate beads is clearly seen in Figure 6A. Figure 6B also shows the effect of Cd-alg. or Ca-alg. on cholesterol concentration in acetic acid. The addition of metal alginate beads formed from 40mg of Na-alg.(CM) helped to exclude 5mg of cholesterol.



A B

Figure 6. The effect of metal alginate on cholesterol in acetic acid. A: First step beads were suspended in 30ml of acetic acid containing 5mg of cholesterol for 24hr at 30°. B: Metal alginate beads formed from 40mg of Na-alg. were suspended in 30ml of acetic acid containing cholesterol

Dyes...Each dye used in this work was absorbed by the gel of CM or Cd-alg. or Ca-alg. But a part of these absorption phenomena occurred by packing with the fiber net. Because some blocks of dye powder were packed in the fiber net cage without dissolving in water or in alcohol, quantitative experiments for dye were impossible. By the addition of acid to the mixture of dye solution and the colored alginate gel, the color of solution died away, but the gel did not lose color at all. After treating with acid the color of the gel did not dissolve in water and in alcohol.

Granular alginic acid...Granular alginic acid also showed high uptake behavior to iodine and dyes, but not to cholesterol at all. Table 1 shows the amount of iodine bound to granular alginic acid. The values showed a relatively fixed number under the same conditions. The experiment was repeated changing the KI content of the solution and the val-

ues obtained were plotted against the KI content. The number extrapolated to zero mg of KI was around six.

TABLE I The number of alginic acid residues bound to one iodine molecule

Granular alg. taken(treated with 10N HCl)	g	0.300	0.500	0.700	0.900	1.100
	residue $\times 10^{-3}$	1.69	2.82	3.95	5.08	6.21
I_2 bound to granular alg.	mol $\times 10^{-3}$	0.115	0.224	0.333	0.387	0.402
Ratio of granular alg. to iodine(residue/mol)		14.7	12.6	11.9	13.1	15.4

Microscopic examination for inclusion compound...The fine structure of the inclusion compounds was observed by TEM and SEM(shown in Figure 7.). Confirmatory evidence for the inclusion compounds was not obtained, because the included materials(iodine, cholesterol) were freed from the alginate samples in the process of making samples for TEM or SEM. Alginate-dyes compound was the only stable one. In Figure 7 shrunk and rounded fiber chains(A,B) and regularly arranged round holes surrounded by fixed fiber are clearly observed. The alginate-cholesterol bead was very hard and the fractured surface sample was not obtained.

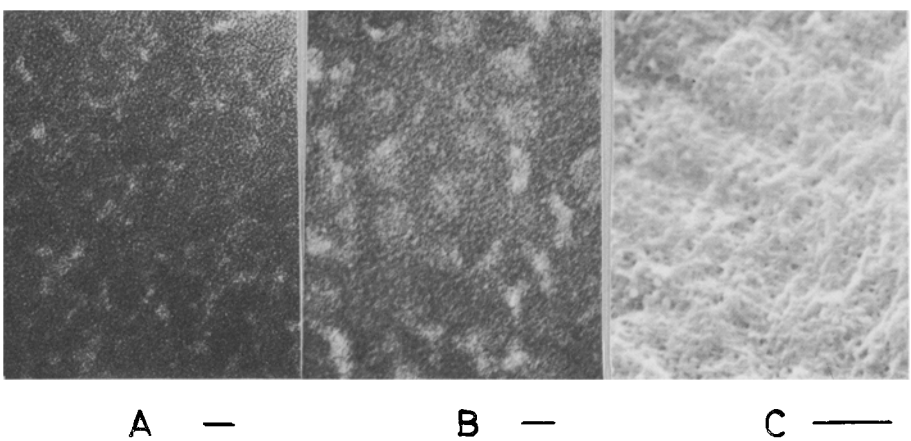


Figure 7. Microscopic examination for inclusion compounds of alginate. A: Ca-alg.-iodine film, by TEM, Bar=10nm B: Ca-alg.-color(Red No.105) bead, by TEM, Bar=10nm C: Ca-alg.-cholesterol bead(first step) suspended in 30ml of acetic acid containing 10mg of cholesterol, surface, fixation, Bar=1μm.

DISCUSSION

Microscopic results indicate that alginates form three types of packing under acid solution: Cage(shown in fractured surface of alginate

bead) or channel(shown in fractured surface of alginate film) type where the guest position is surrounded by an alginate fiber net, round hole (shown in alginate-cholesterol bead) type where dimer alginate fiber makes a ring and rounded cylindrical space(supposed figure of alginate -iodine or alginate-dye). The third one depends on the so-called helical cylindrical chain of alginic acid. Data for alg.-iodine compound (X-ray: 7-8Å and TEM: 20-50Å, 3.13Å and ratio of alg.reg. to iodine mol: 6) support this observation. Because binding depends strongly on the host structures, the high uptake behavior of alginic acid depends on these three types of packing under acid.

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