Andrea Jeanne Hurlburt

Dept. of Experimental Medicine
Degree: M.Sc.

COMPOSITION OF ALGINATE IN RELATION TO STRONTIUM BINDING

Alginic acid from Laminaria hyperborea was partially hydrolysed and the non-dialysable degradation products were fractionated into several components, with different proportions of guluronic and mannuronic acid. Sodium salts of these products were examined by intubation technique in rats for their suppressive activity of intestinal absorption of radioactive strontium and calcium. *In vitro* binding capacity was tested by dialysis against water containing strontium and calcium. 'Poly-L-guluronic acid' was found to be most effective; its degradation products did not show any higher activity. These degradation products had a lower degree of polymerization than the parent compound and it appears that the maximal gel formation with strontium occurs. It was concluded that high biological activity is dependent on chemical composition, including guluronic acid content, viscosity, chain length. Inhibition of calcium absorption was invariably low and the various preparations did not show any significant difference in their effectiveness.

Title to appear along the spine of the bound copies:-

STRONTIUM BINDING BY ALGINATES

COMPOSITION OF ALGINATES IN RELATION TO STRONTIUM BINDING

by

Andrea Jeanne Hurlburt B.Sc.

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Experimental Medicine
McGill University
Montreal July, 1970.

ACKNOWLEDGEMENT

It has been a pleasure to work in the Department of Investigative Medicine and later the Department of Experimental Medicine. I wish to express my appreciation to Dr. S.C. Skoryna, Director of Research, for the opportunity to further my studies in the medical sciences and for his guidance and advice throughout the duration of this project.

Many thanks are due to Dr. Y. Tanaka for his supervision and generous assistance in the course of performing the laboratory work; to Dr. Dierdre Waldron-Edward whose suggestions and experience were of considerable help; and to Dr. K. Hong for his worthwhile criticisms and consultation.

Iam grateful to Dr. J.S.L. Browne for his assessment and appointment of the courses I have pursued in connection with the current endeavor. I have enjoyed my association with Dr. J.C. Beck and Dr. D.G. Cameron in the department of Medicine; Professor E.A. Hosein to whom I am indebted for his help and advice both in research and personal matters; and Professors L. Yaffe and M. Richter, whose lectures and demonstrations have proved very beneficial to my understanding of the problems facing those in research.

I also wish to thank Dr. Y. Kalinowski for preparing the animals and assisting in experimental procedures and Mr. I. Nikitin for his help in the radioactive room and care of the animals.

The U.S. Public Health Service provided the necessary funds to carry out the experimental investigations for which I am grateful.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

As a result of atomic energy released in reactors and fallout, a great number of radioactive isotopes are produced which constantly emit radiation, presenting a critical health hazard for biological systems. Radioactive isotopes follow the same metabolic processes in the body as the naturally occurring stable isotopes, and elements of the same periodic family often metabolize in a similar manner. Only a few radioactive isotopes from the fission process are potential hazards by virtue of their long-term internal irradiation. These fission products, however, constitute a relatively high percentage of fission yield and they localize chiefly in the bone tissue. In general, these isotopes, known as "bone seekers", have long half-lives and emit β radiation. Therefore, they will impair the bone tissue and particularly the bone marrow which is more sensitive to irradiation.(1) Radioactive strontium absorption can lead to leukemias, malignant lesions of bone, and degenerative bone damages.

In the process of releasing atomic energy, it is the production of radioactive isotopes of strontium that has stimulated attention in this regard. In many respects, the element, strontium, is chemically and physiologically analogous to calcium. Consequently, strontium displays similar metabolism to calcium in the organism and accordingly becomes concentrated and localized in the skeleton. Prevention of absorption of radioactive materials with long half-lives, such as Sr^{90}, which can result from atomic explosions or industrial contamination, is of prime importance to environmental health.

Since the realization of potential hazards arising from contact with radioactive materials, there has been an earnest quest for substances which may be utilized as decontaminants and for therapy in humans. Algae, which inhabit the sea, have the ability to extract metal ions from their environment and incorporate them into their skeletal structure. As a result of this unique characteristic of algae, experiments have been constructed which show that alginates extracted from brown seaweeds display selective binding of Sr.[2-8] Supplementing a diet with sodium alginates is known to inhibit the absorption of radioactive strontium from the intestine.

Previous investigations in the field of intestinal absorption of ^{45}Ca and ^{89}Sr have revealed significant differences between these two cations. Although these two elements are similar in chemical characteristics, their metabolism is unique and the specificity of the polyelectrolyte reaction to strontium and calcium is studied. In order to understand the alginate derivatives with respect to their biological activity it is important to consider their composition. It has been shown that alginate compounds of differing composition, i.e. unequal amounts of constituent acids, produce varied effectiveness.

It is the purpose of this current project to study the biological effectiveness of alginate compounds in relation to their chemical composition and chain length. As an example of the metal binding reaction of organic macromolecules, the reaction of polyuronates and radioactive strontium and calcium is studied in order to correlate the chemistry of these polymers and their in vivo effectiveness in binding the radio-isotopes.

CHAPTER II

RADIOACTIVE STRONTIUM CYCLE

1. Introduction.
2. Radioactive Strontium.
3. Pathways of Radioactive Strontium to the Body:
   (A) The Food Chain
   (B) Discrimination against Strontium
4. Transfer of Strontium and Calcium Ions across the Intestinal Membrane.
5. Metabolism of Calcium and Strontium.
6. Calcium and Strontium Deposition in Bone.
7. Attempts of Eliminating Radioactive Strontium from the Body.

CHAPTER II

RADIOACTIVE STRONTIUM CYCLE

1. Introduction:

With the conquest of nuclear energy, came nuclear weapons and nuclear power stations. This resulted in release of radioactive materials into the environment and a need arose for investigation of biological effects of ionizing radiation.

Radiation acts on the tissue at the molecular level and therefore on the cell and its constituents. The exposure to ionizing radiation may result in alteration of the highly organized molecular system, destruction of particular elements of the cells, possibly altering its function, or cell death. The biological effects manifest themselves in acute somatic effects such as radiation sickness, or chronic ones, comprising curtailed life span, aging, and malignancy. In addition, there may be genetic shortcomings from mutations transmitted from one generation to the next.[(9)] Several

TABLE I

ELECTROMAGNETIC			CORPUSCULAR	
X - rays	Light	Radiowaves	LIGHT PARTICLES	HEAVY PARTICLES
Gamma rays (usually ejected from atomic nuclei)	(infrared; visible; ultra-violet)	Microwaves	Electrons or Beta rays (e.g. Sr^{90}) Positrons	Protons Deuterons Heavy ions

studies on the mechanisms by which ionizing radiation produces biological changes and on the deleterious outcomes of radiation exposure have been pursued.[10,11]

The biological effects of exposure of living organisms to ionizing radiation depends on several factors, including the type of radiation.[12] (See Table I).

The source of radiation may be external (γ-rays, X-rays, or neutrons) or it can be internal as from radioisotopes retained within the body, emitting α, β , or γ radiation. Internally deposited radioisotopes have specific features. They irradiate the body continuously until they have decomposed. Depending on the element, the pattern of behavior in the body is uniquely determined, although it varies according to the rate of growth, physiological and nutritional state of the individual concerned. Radioisotope distribution is generally not homogeneous, but selective, e.g. strontium goes to the bone. Radioactive fallout is one of the largest contributors of internal irradiation through contamination of the atmosphere and the environment. Of the long term fallout products, Sr^{90} (a pure β emitter with a maximum energy of 0.544 Mev.)[13] is regarded to be the most hazardous with regard to biological consequences.

2. Radioactive Strontium:

Radioactive isotopes, about 200 in number, are associated with a nuclear chain reaction, whether it is controlled as in a reactor in industry or uncontrolled, as in the detonation of nuclear weapons. Many possess a very short biologically effective half-life, (T), defined by

$$1/T = 1/T_R + 1/T_B$$

where T_R is the physical half-life of the radioactive substance, and T_B is the biological half-life of the substance in a given organ or in the total organism. Other radioisotopes have little importance with respect to biological effectiveness and some are produced in such trace amounts that they cause no deleterious effect. Among the fission products of a thermonuclear explosion, an inert gas, Kr^{90}, is produced with a half-life of 33 seconds, which is subsequently converted to Rb^{90} with a half-life of 2.7 minutes. Rb^{90} is soon changed to Sr^{90}, which in the course of 28 years decays to $Ytrium^{90}$, with a half-life of 64 hours. In turn this produces the isotope, Zirconium (half-life of 0.8 sec.) which undergoes isomeric transition to the stable Zr^{91}. (14) Therefore, within an hour of an atomic fission explosion, radioactive Sr^{90} is present in considerable quantities.

3. Pathways of Radioactive Strontium to the Body:

(A) The Food Chain:

Once released in the atmosphere, radioactive strontium can be transported by prevailing winds over a considerable distance, diffusing both laterally and vertically, returning to earth by gravity, or precipitation. Ingestion is one of the two routes by which radioactive strontium enters the body, while inhalation is the other. In regard to inhalation of radioactive strontium dust particles, two potential hazards exist:

(a) irradiation of pulmonary tissues from retention of radioactive Sr on the respiratory surfaces and deposition in bronchial lymph nodes,

(b) the possibility of translocation of radioactive Sr^{90} to its critical organ, the bone.

Considering fallout producing biospheric contamination, ingestion of radioactive Sr via the gastrointestinal tract is all-important. Food chain radio-contamination by Sr^{90} is represented in Fig. 1, with respect to its major terrestial routes.

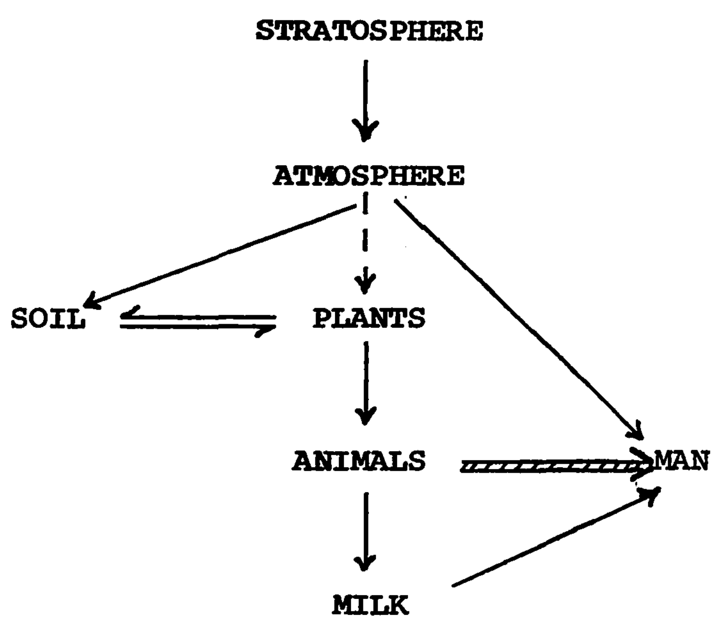


Fig. 1. Food chain radio-contamination by Sr^{90}

During active fallout, polution of the foliage, in particular the vegetables, is critical. Adsorption of radioactive Sr can contaminate food considerably, (lettuce 60%, carrots 87%). Plants can also absorb radioactive Sr through their aerial surfaces (leaves and stems) as has been demonstrated under experimental conditions for wheat, sugar, beets, potatoes, beans and cabbage.[15] As fallout subsides, the soil reservoir becomes a relatively more important source. Plants basic to the food chain (i.e. they supply nearly all of mans food either directly or indirectly) obtain most of their nourishment through their root system. Therefore, radioactive Sr may be easily accessible to growing plants as a result of soil retention from rain water which envelopes the radioactive material.

There are many conditions influencing the food chain pathway in so far as its significance and modification are concerned:[12]

(a) Time pattern of contamination, i.e. as time elapses after fusion of a nuclear mechanism, Sr^{90} becomes more important, because of the greater half-life, than those with a relatively short half-life.

(b) Climatic conditions.

(c) Properties of soil; such as type, acidity, content.

(d) Methods of agriculture used: fertilization, plowing depth, barn feeding or pasture feeding.

(e) Food technology, including washing contaminated cans containing food, proper washing of foods to remove adsorbed Sr.

(f) Nutritional customs, such as degree of leafy vegetables eaten or amount of milk consumed in diet.

(g) Plants with entwined roots may delay Sr reaching soil for dilution with soil Calcium, thus permitting relatively higher concentration of Sr to be absorbed by the plant.

This latter factor introduces the similarities of Sr and Ca as can be expected from their close relationship in the same family within the periodic chart. It is not only because of the amount contained in fallout and its long half-life, that Sr^{90} is regarded as one of the most important of all fission products in stratospheric fallout; but in particular, because it is metabolized by the animal and human body and deposited in bone tissue in a similar manner to calcium.[16] (Strontium exposure occurs largely as a result of its ingestion, along with calcium in the diet).

(B) Discrimination against Strontium:

The Sr/Ca relationship is meaningful since the amount of radioactive Sr incorporated from the soil to man is interrelated to the simultaneous absorption of calcium. The Sr/Ca ratio expresses the amount of strontium retained in a living organism in comparison to that of calcium. Although strontium and calcium are chemically analogous and both of their metabolic pathways in the body relate to their accretion and resorption by bony tissue; when both elements are available, plants, animals and humans discriminate between strontium and calcium absorption, with a particular preference for calcium.[17]

When Sr^{90} is released from the biosphere, it is deposited on the plants and on the soil. The Sr to

Ca ratio found in plants can be half that of the soil from which the elements are absorbed; although there is an apparent difference with respect to Ca and Sr discrimination between plant species.(17) This differentiation can also be influenced by the conditions effecting the food chain which were described previously. For the most part, the hazard is in the food chain where it moves from plants to animals. This discrimination occurs initially in the animals that consume radioactive strontium, in which the intestinal absorption of strontium is relatively less than that of calcium. Both secretion of strontium in milk and reabsorption of strontium in the renal tubules is less than that of calcium.(18) The concentration of radiostrontium in dairy products (milk, cheese, etc.) from these farm animals can be less by as much as a factor of 10 than that in the grass upon which the cattle graze.(19) This reduces the ingestion of radiostrontium in humans in general, but animals that are to be used for human consumption, and which are fed on contaminated areas of land, remain a ready source of health hazard to man. Once strontium is ingested, the same physiological processes in man provide further protection.[18] Greater amounts of calcium are absorbed from the alimentary tract compared to the amount of strontium absorbed when both are ingested in comparable amounts. Urinary excretion displays a more efficient kidney tubular reabsorption of calcium than strontium. There is a significant difference in calcium and strontium secretion from blood to milk thus protecting the foetus and infant to an even greater extent. The discrimination factor for Sr^{90} with respect to Ca in its passage through the animal organism is

$$\frac{Sr^{90}/Ca \ (diet)}{Sr^{90}/Ca \ (bone)} \qquad (1)$$

and in humans is more pronounced than in plants and animals, varying from 2 to 4.[18]

Of the radiocontaminating sources, including inhalation of radiostrontium, water intake and food, that of food consumption appears to be the principal contribution of this deleterious element. Once incorporated into the skeleton, strontium is not readily replaced by calcium.[20]

4. Transfer of Sr & Ca ions across Intestinal Membrane:

Absorption, a complex phenomenon, is the ultimate process by which products of digestion can fulfill their metabolic role. After the oral ingestion of food, it is processed throughout the digestive tract and the absorption of most elements occurs almost entirely from the small intestine.[21] All ingested strontium is subjected to the discrimination processes during absorption from the gastrointestinal tract, whereby calcium is assimilated in preference to strontium; so less strontium is absorbed and more strontium is excreted than calcium. This indicates that the intestinal absorption of strontium is most significant in the overall selection of this element. The rate of resorption, i.e. the speed by which the radioactive isotope enters the blood stream and thus comes into contact with its associated organ - in the case of radiostrontium, the osseous tissue - depends on chemical composition, particle size, solubility, ionic charge, etc. As a result of these overall chemical properties, divalent cations like Sr^{++} and Ca^{++} are not readily absorbed across the gut wall.[20] Investigations on surviving intestinal segments suggest that the membrane is metabolically active. The columnar epithelium of the small intestine is specifically adapted for absorption of products of digestion by the villi. It is by this active transport that the nutritionally essential calcium ions are absorbed across the mucosal wall against a concentration gradient from the intestinal lumen to the plasma. This brings about net transfer against an electrochemical gradient and is an energy requiring process. On the other hand, Sr^{++}, is transferred proportionately more rapidly when in low concentration by passive diffusion.[22,23] This mechanism enables quick passage of solutes without demanding energy, as it is not capable of net transfer against a potential gradient.

5. Metabolism of Calcium and Strontium:

The fate of strontium depends on the state of equilibrium between its free and bound forms. Strontium which is complexed, i.e. in the form of a precipitate, is insoluble and is therefore eliminated from the body Once absorbed, the metabolism of divalent Ca^{++} and Sr^{++} cations is associated with bone tissue, and they are relatively slowly eliminated by urinary and faecal excretion.

Principal factors which may influence strontium metabolism include the following:(24)

(a) The physiological state, i.e. the age, health and metabolism of the individual concerned.

(b) Conditions in the intestinal tract at the time Sr^{++} ions are present, in particular, the presence of food, the physico chemical characteristics of the ingested substances and the rate at which this ingesta is transported through the gastrointestinal tract.

(c) Dietary constituents may also be important with respect to presence or absence of minerals, amino acids, carbohydrates, polysaccharides, vitamins, etc.

Subject to usual conditions, strontium occurs only in trace amounts in mammalian tissue. A proposed model for strontium metabolism offered by Dolphin and Eve[25] is represented in Fig. 2 . According to these investigators, strontium metabolism follows the calcium model and is subject to the same qualifications and limitations. Because strontium is present in trace amounts in food, it is obvious that the concentration of stable strontium ingested and transported to the sections varies in degree to calcium. Discrimination occurs in the transport constants comparative to calcium; fractional transfer from gut to plasma is only half that of calcium and fractional urinary excretion from the plasma is 4 times greater than for calcium, also excretion in the form of sweat is 1.5 fold that of calcium. Fractional transfer from plasma to gut and bone are similar to calcium. In general, the transfer from gut to bone is 4 times less for strontium than for calcium, and in the case of radiostrontium the Sr^{90}/Ca ratio is 4 times less in bone than in the dietary intake.

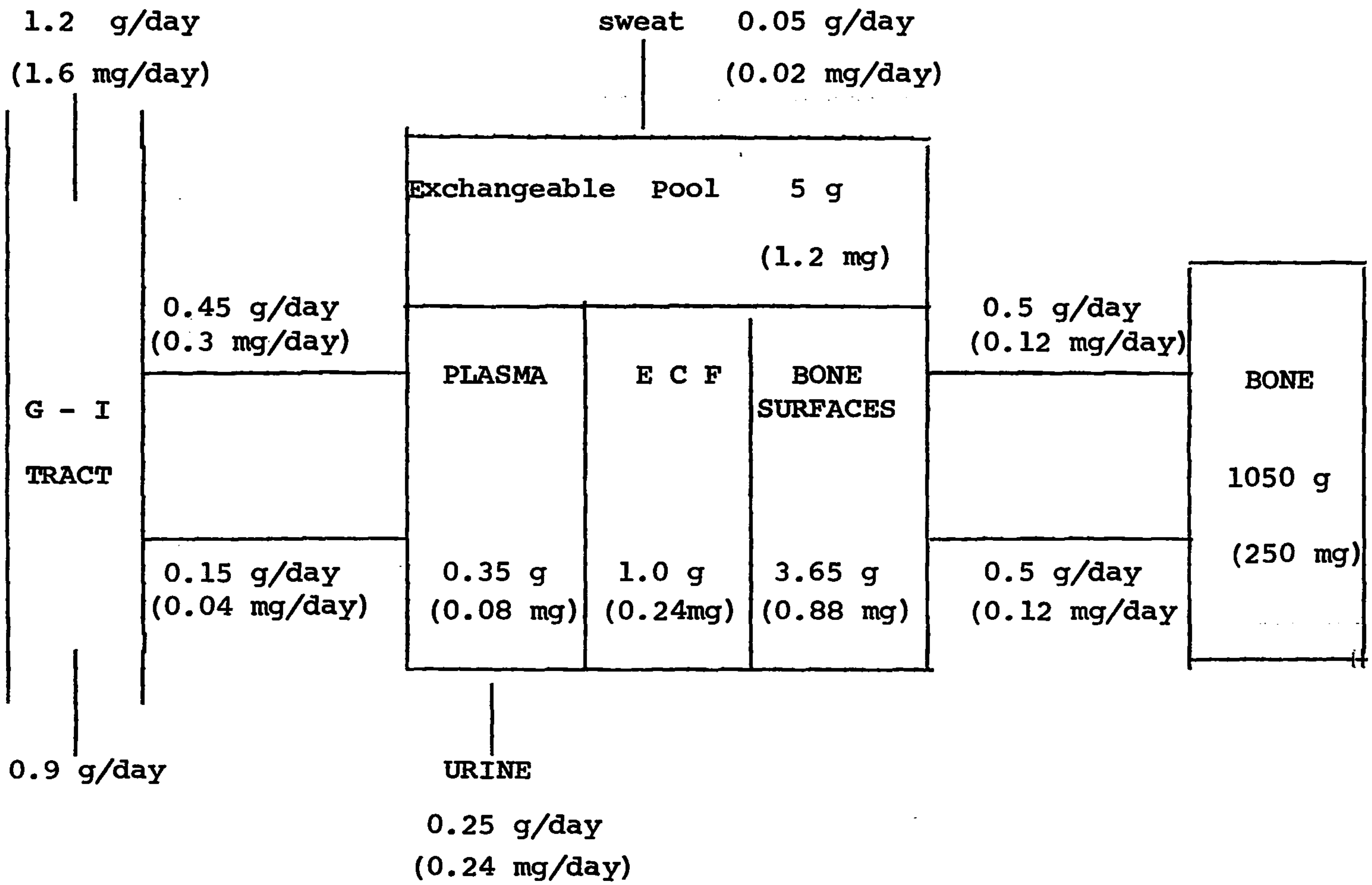


FIG. 2. Model for the metabolism of Calcium and Strontium (no. in brackets) adopted from Dolphin, G.W. and Eve, I.S. Phys. Med. Biol., 8(2): 193 (1963).

6. Calcium and Strontium Deposition in the Bone:

Once the radioisotope, strontium, is present in the blood circulation, its activity may be identified in all tissues, gradually accumulating in the hard tissue. This is particularly true for elements such as Ba^{++}, Ca^{++}, Sr^{++}, Ra^{++}, which are specifically attracted to the skeletal structure; at the same time reduction in their concentration is noted in the soft tissues. Radioactive Sr is deposited in particular, in the areas of current bone growth, e.g. metaphysis. Evidence shows that calcium requirement demanded in active osteogenisis significantly affects the skeletal accumulation of strontium. This implies that radioactive strontium is a major hazard to the young during the period of bone formation and development.

Calcium and strontium are present in the blood plasma in two forms. (1) protein bound (2) complexed and free.[26,27] Calcium and strontium ions exchange readily between these two forms. The Sr ions are associated with the bone minerals similar in molecular structure to $Ca_{10}(PO_4)_6(OH)_2$, which occurs in very small crystallytes. (17) The apatite particles have a large surface area and, due to their small size, possess the ability to attract various types of ions by adsorption. The strontium ions may also be incorporated into the apatite crystals of the bone tissue. Once integrated into the bone tissue, radioactive strontium is not readily removed.

7. <u>Attempts of Eliminating Radioactive Strontium from the Body</u>:

Due to chronic effects in humans, observed after trace doses of Sr^{90}, there has been considerable effort to decrease Sr^{90} deposition in the body, particularly in children. This has been attempted by reducing the Sr^{90}/Ca ratio on a nutritional basis, by adding stable strontium and calcium to the diet or by use of exchange resins in order to remove Sr^{90} from milk.

The effects of different materials and compounds have been followed with respect to the fate of radiostrontium. These materials include, Calcium glucuronate, NH_4Cl, Mg^{++}, $CaNa_2$citrate, etc. Among these methods, chelating agents such as EDTA and hormonal influence have been used, but without much success. The fact that the food can be contaminated with radiostrontium, makes apparent the advantage of binding strontium in the gastrointestinal tract before it is absorbed and retained. The in vivo binding characteristics of naturally occuring macro-molecular acidic polysaccharides and synthetic ion exchange resins have been investigated. Sodium alginate has received most attention because of its capacity to inhibit intestinal absorption of radioactive strontium and because it satisfies such prerequisites as easy availability, lack of toxicity, palatability and non-absorbability.

C H A P T E R I I I

A L G I N A T E S

1. Effects of Alginates
2. Chemical Composition
3. Chemical Properties
    (A) In general
    (B) Ion Exchange Properties
4. Pilot studies on toxic metal, Lead.

CHAPTER III

ALGINATES

1. Effects of Alginates:

The most significant polysaccharide found in brown seaweeds is Alginic acid, which represents a unique agent for the selective binding of strontium; thus providing a possible source of material which can prevent intestinal absorption of radioactive products of atomic fission.

After ingestion of radioactive strontium, the metal is absorbed through the intestinal wall and is deposited, via the blood stream, in the bone. If the ingestion of strontium occurs in the presence of sodium alginate, strontium binds with the alginate through an ion exchange process:

$$\underset{\text{Ingested}}{Sr^{++}} + \underset{\text{sodium alginate}}{2\ Na\ Alg} \rightleftharpoons \underset{\text{Excreted}}{Sr(Alg)_2} + 2\ Na^{+}$$

$$\text{Ingested} \rightleftarrows \text{Blood } Sr^{++} \rightleftarrows \text{Bone } Sr^{++}$$

The strontium alginate is not absorbed and passes through the intestinal tract, thus providing an effective means of eliminating the undesirable metal.

This finding has been supported by other reports involving different species (7,28,29) as well as man. (30-32) In fact, radioactive strontium already absorbed, can be bound with the alginate as it is resecreted into the gut. This was demonstrated by Stara and Edward, (33) who maintained cats on long term radioactive strontium dosage and an alginate diet. There is a linear relationship between the bone uptake of strontium and the intake of alginate, thus enabling adjustment in the dosage administered.

The ideal situation, in the use of alginates with respect to inhibition of undesirable metal absorption requires that essential elements are unaffected in their metabolic pathways. Studies have shown that, in fact, there is no reduction in absorption of Na, K, Ca, Mg, Fe, or Zn in rats maintained on a 10% manucol (commercial alginate) diet over a period of a year.(34)

Although calcium and strontium are chemically very similar, the specificity of the reaction is displayed by the greater affinity of alginate towards strontium, thus reducing bone uptake of the element. The absorption of calcium and its subsequent deposition in the bone is not altered to a significant degree.

It is also noteworthy, that recently, calcium alginate was reported to be as efficient a Sr^{89} binder as the corresponding sodium salt of the same alginic acid.(35) By means of inhibiting the absorption of radioactive strontium, calcium alginate has two significant advantages as opposed to the corresponding sodium alginate. In extended periods of experimental feeding, sufficient calcium may be acquired and at the same time excess sodium intake is avoided.

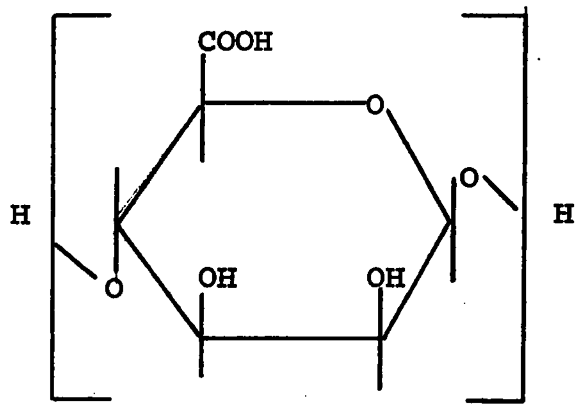
It is therefore apparent that the polysaccharide, alginic acid, is of considerable importance, not only with respect to prevention of metal poisoning, but as a means of therapeutic elimination of toxic substances.

In order to understand the alginate derivatives with respect to their biological activity, it is important to consider their composition and their chemical properties. For instance, it was shown recently that alginate compounds of differing composition, i.e. unequal amounts of mannuronic acid and guluronic acid, produced varied effectiveness in rats.[36]

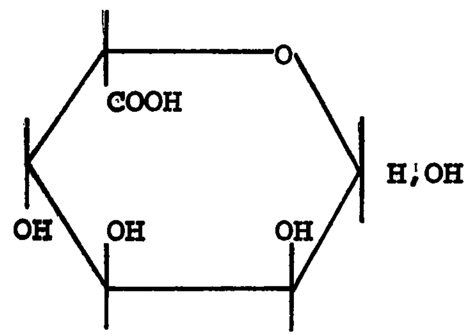
2. Chemical Composition

Alginic acid is a polysaccharide which occurs in largest quantities in most Phaeophyceae (brown marine algae) forming the main structural component of the cell wall of these seaweeds. First discovered by Stanford in 1881,[37] alginic acid was extracted from brown algae as a mixed salt. Forty-five years later it was confirmed by independent groups that as a polyuronide, alginic acid is comprised of uronic acid units.[38,39] Mannuronic acid was found as a constituent of alginic acid.[40-42] The nature of the bonds between the uronic acid residues was established by methylation of partially degraded alginic acid and isolation of 2,3 dimethyl-D-mannuronic acid,[43] confirming the proposed structure[44] of Alginic acid as similar to cellulose and pectic acid with 1,4 linkages between pyranose units. (Fig.3.I) Support of this hypothesis evolved from the following experiments: (a) resistance of the alginic acid to acid hydrolysis, thereby eliminating the possibility of C_1 to C_5 bonds and furanoside configuration. (b) periodate oxidation by which mannuronic acid residues were oxidized between C_2 and C_3 , yielding a mixture of erythraric acid and L-threaric acid,[45] and (c) methylation on a more impure alginic acid,[46] producing similar results to previously described methylation products. It was concluded that alginic acid consisted of a straight chain of 1,4 linked β-D-mannuronic acid units, (Fig.3.I), i.e. a chemically homogeneous compound. The configuration of C_1 was predicted as β , as a result of negative optical rotation of alginic acid.

STRUCTURE OF ALGINIC ACID COMPOUNDS:



1,4 -D-Polymannuronic acid
Fig. 3.I



L-Guluronic acid
(Fischer & Dorfel, 1955)
Fig. 3.II

Monomeric units of mannuronic acid (I) and guluronic acid (II) are joined together by glycoside links to form the naturally occuring polymer.

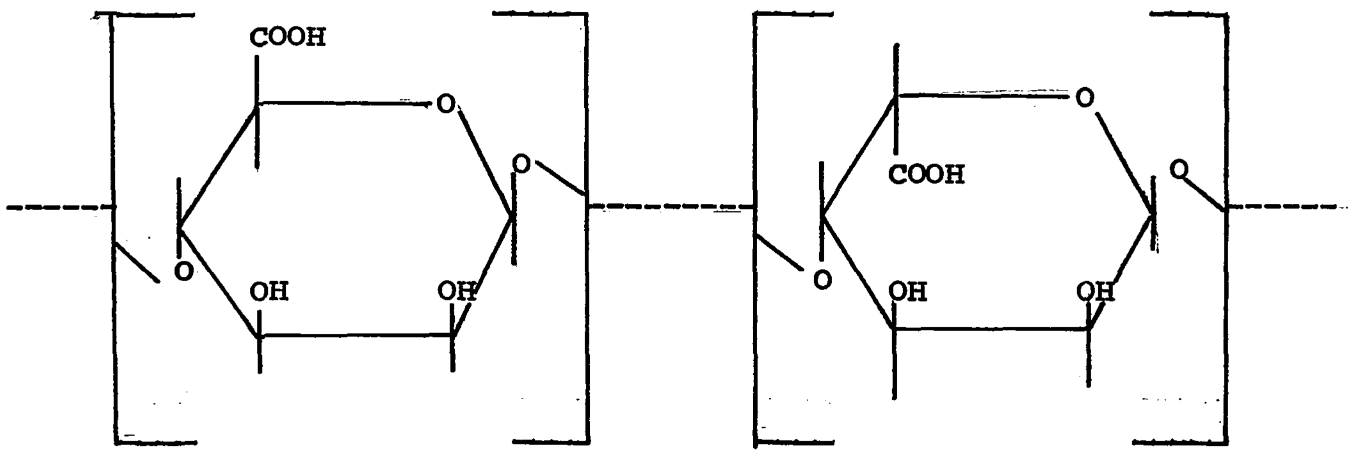


Fig. 3.III

ALGINIC ACID (Haug, Larsen and Smisrød, 1966)[78]

The experimental results of these investigators indicated that Alginic acid consists of separate mannuronic acid-rich and guluronic acid-rich chains.

In 1955, Fischer and Dörfel[47] isolated, by means of paper chromatography, two uronic acids from acid hydrolysates of alginic acid, D-mannuronic acid and L-guluronic acid. (Fig.3) The ratio of the two monomeric units differed with respect to raw material and environmental conditions such as tidal flow and changes in temperature. Whistler and Kirby[48] later confirmed this study.

The discovery of L-guluronic acid as a component of alginic acid motivated examination of the homogeneity of the polymer. In order to show whether alginic acid is a mixture of polymannuronic and polyguluronic acid,(Fig.3.III) or contains both uronic acids in the same molecule, partial hydrolysis of the polymer was performed. This partial hydrolysis of the alginic acid molecule yielded oligouronic acid fractions, all containing both uronic acids.[49] In further experiments by Hirst et al.,[50,51] a disaccharide, mannosylgulose, containing both mannose and gulose, and in addition, a β-1,4 mannobiose, was isolated from an acid hydrolysate of reduced alginic acid. i.e. the molecule was reduced with diborane to the neutral polysaccharide before hydrolysis. This demonstrated, without doubt, that the corresponding uronic acids are linked together to some extent in the alginic acid molecule, and that at least some of the polymers contain adjacent mannuronic acid units.

The isolation of a mixed, unsaturated triuronide (O-4-deoxy- -L-erythrohexopyranosyl-4-enyluronic acid-(1$\longrightarrow$4)-O- D-mannopyranosyluronic acid-(1$\longrightarrow$4)-L-guluronic acid) from alginic acid by Yoshikawa and Kiyohara (74), by means of enzymatic degradation, confirms that there is a chemical bond between the two uronic acids. Therefore, alginic acid represents a general term for polymers of (1,4)- linked β-D-mannuronic and L-guluronic acids in different ratios. The configuration of the molecule, L-guluronic acid probably is of the α form. Both uronic acids are linked together by glycoside links.

The alginates have been shown to occur only in the Phaeophyceae, a single family or the marine plants, however, it is interesting to note that an alginic acid - like polymer has recently been isolated from mucoid produced by a bacterium of the Pseudomonas species.[75]

Fractionation experiments[52,53] on partially hydrolysed alginic acid have shown that the molecule is chemically heterogeneous; i.e. alginic acid contains molecules with different uronic acid composition. The sequence of uronic acids was then investigated by partial hydrolysis with oxalic acid.[54] Results suggest that the linear alginic acid molecule consists of two polymers, one of mannuronic acid units, the other, guluronic acid residues, which are joined by an alternating sequence of the uronic acids. The relative proportions of these three fractions can be determined by partial acid hydrolysis and subsequent fractional precipitation. In 1969, Haug examined brown algae with attention focussed on the different structural components of the plant to determine the uronic acid content of each. The alginate structure varied in different parts of the plant and isolation of a pure uronic acid from any section of the plant was not possible. This indicates that alginates of different composition occur in the same plant tissue.(55)

3. Chemical Properties

(A) In General:

For a natural polysaccharide, alginic acid has certain unique characteristics, which are mentioned below. Itself insoluble in water, alginic acid forms soluble salts with alkali metals, displaying polyelectrolyte behavior. The water soluble salts of alginates are extremely viscous at low concentration, an important property with respect to some of its applications - as a stabilizer and emulsifier in the food and cosmetic industries. When solutions of sodium salt are treated with salts of polyvalent metals, (heavy metal ions and alkali earths), an insoluble alginate salt is formed as a precipitate or gel, due to its ability to exchange cations with the solution.

It is a well known fact, that the viscosity of polyelectrolyte solutions such as alginate, can be markedly reduced by the addition of salts. In all, the properties of alginic acid derive from its high molecular weight, from its linear structure, and from its acidic nature.

(B) Ion Exchange Properties:

Of all its chemical properties, those of ion exchange, which have been extensively examined by several investigators, are primary.[56-58] Divalent metals such as calcium and strontium form insoluble salts on reacting with alginates. The ion exchange properties of alginates, depend upon their relative content of mannuronic and guluronic acids. On slow addition of metal cations into an alginate solution, a gel can be produced which is birefringent in nature and of a smaller volume.[56] Assuming analagous treatment, these properties depend on the metal used, as observed from its order in the ionotropic series of Thiele, where Pb > Cu > Cd > Ba > Sr, Ca > Co, Ni > Zn > Mn in shrinking and birefringent capacities. The gel formation can be represented by the following expression:

$$\underset{\text{gel}}{M(Alg)_2} + \underset{\text{liq.}}{2\,Na^{+}} \rightleftharpoons \underset{\text{gel}}{2\,Na\,Alg} + \underset{\text{liq.}}{M^{++}}$$

where M = divalent cation concerned
Alg. = alginate
liq. = liquid

This ionic exchange reaction achieves equilibrium and the selectivity coefficient of the metal is obtained from the following formula:

$$\text{At equilibrium,} \quad K = \frac{[M\ (gel)]\ [Na\ (liq.)]^2}{[M\ (liq.)]\ [Na\ (gel)]^2}$$

The concentrations in the alginate gel are expressed as equivalent fractions and those in the solutions as normalities.[76,77]

In general, the selectivity coefficients of metals are in accordance with the ionotropic table of Thiele. However, recently Haug and Smisrød[77] observed difference in selectivity coefficients depending on the varying uronic acid composition of the alginates. Guluronic acid rich alginates attract the strontium ions to a greater extent than those of calcium. This work was supported in 1966 [59] and 1967 [60] by two groups of investigators who have shown a marked decrease in absorption of Sr^{85} in rats by supplementing their diet with alginate derivatives. Calcium absorption was reduced only slightly. This differential suppression of strontium uptake as compared with calcium is dependent upon the uronic acid composition, i.e. the greater the guluronic acid content of the alginate preparations used, the greater the inhibition of strontium uptake relative to calcium.

Recent work by Tanaka et al., has shown inhibition of absorption of Sr^{89} and Ca^{45} in relation to uronic acid content of alginate preparations extracted under different conditions from various seaweeds. [61] Table II . Increased proportions of guluronic acid in the degraded alginate, i.e. a lower M/G ratio, in general, correspond to a decrease in radiostrontium

TABLE II

	Degradation: Acid concentration	Degradation: Time (hrs.)	Degradation: % Yield	M/G	Percentage Inhibition ^{89}Sr	Percentage Inhibition ^{45}Ca
1. Sodium alginate						
Batch No. 742974..	--	--	--	2.54	46.2	3.0
(a) H_2SO_4...........	1N	6	51	2.01	60.5	33.8
(b) H_2SO_4...........	1N	12	24	2.21	65.2	43.1
(c) H_2SO_4...........	2N	6	22	2.00	71.0	30.2
(d) $(COOH)_2$.........	1M	6	13	1.51	77.2	33.7
(e) HCl/MeOH........	1N	6	52	2.03	54.8	26.5
2. Sodium alginate						
Lam. hyperborea...	--	--	--	1.00	68.9	42.8
(a) H_2SO_4...........	1N	6	77	0.34	89.3	52.0
(b) H_2SO_4...........	1N	12	65	0.55	84.4	32.7
(c) H_2SO_4...........	2N	6	67	0.45	85.8	54.0
(d) $(COOH)_2$.........	1M	6	75	0.50	84.8	49.9
(e) HCl/MeoH........	1N	6	26	0.26	50.4	58.9
3. Sodium alginate						
Lam. digitata.....	--	--	--	1.53	71.2	56.9
(a) H_2SO_4...........	1N	6	44	0.62	87.6	11.3
(b) H_2SO_4...........	1N	12	41	0.70	72.4	36.9
(c) H_2SO_4...........	2N	6	43	0.72	56.8	26.9
(d) $(COOH)_2$.........	1M	6	45	0.67	72.4	29.2
(e) HCl/MeOH........	1N	6	48	1.05	62.6	32.3

Inhibition of Absorption of ^{89}Sr and ^{45}Ca as Measured by Radioassay of Rat Femur. 2 μC Radioisotope + 0.5 mM $SrCl_2$ or $CaCl_2$ with 20mg Degraded Alginate was given to 12 rats in each group.

absorption. Ca^{45} uptake is only slightly reduced, thus enhancing the discrimination factor, (expressed as the fraction of Sr^{89}/Ca^{45} taken up in the femur), against strontium in favour of Ca.

In 1967, this calcium-strontium selectivity of alginates was studied in vitro by Haug et al. Their experiments indicate that strontium selectivity of the alginates is directly related to the concentration of guluronic acid present.[62]

4. Pilot Studies on toxic metal, Lead:

Recently, application has been made of the ion exchange properties of alginates to the suppression of absorption of other toxic metals such as lead.[63,64] With reference to the ionotropic series, lead has a greater affinity to alginate than strontium, the absorption of which has been markedly decreased in man by supplementing a diet with guluronic acid rich preparations of the alginate.[61]

Preliminary studies in rats have shown that the effect of alginate on lead, Pb^{203} absorption is small, depending on dietary intake.(63) Further experiments, in man, indicated no change in Pb^{203} absorption by alginate supplemented diets.(64) These results are contrary to those expected, considering the position of lead in the ionotropic table and its consequent greater affinity to alginate than strontium.

CHAPTER IV

METHODS AND MATERIALS

1. Extraction of Alginates from Algae.
2. Fractionation of Alginate.
3. Quantitative Determination of Uronic Acid Composition of Alginate Preparations.
4. Qualitative identification of Uronic Acids.
5. Determination of Viscosities.
6. Biological Assays.
7. In Vitro Tests.

CHAPTER IV

METHODS AND MATERIALS

1. Extraction of Alginates from Seaweed.

Examination of the chemical composition of alginates necessarily involves the isolation of the alginate from the raw material. The preparation of alginate from brown algae has been studied extensively in the literature and Stanford's[(65)] main principles are still followed. The nature of the extraction process involves an ion exchange reaction and diffusion process as is apparent from continuous swelling of algae in the study of cation exchange reactions.[(66)] Alginate occurs in brown algae as a mixed salt with a high proportion of calcium. If one assumes that alginate occurs as an insoluble calcium salt the extraction of alginate may be viewed as a transformation of insoluble alginate into the soluble form which diffuses into the solution.

The standard procedure in obtaining Alginic acid involves demineralization of the seaweed with acid and consequent conversion of the polyuronide to insoluble alginic acid, which may then be extracted with sodium carbonate solution according to Stanford, producing soluble sodium alginate. The sodium salt is then precipitated with alcohol or converted to insoluble calcium salt by treatment with $CaCl_2$.

2. Fractionation of Alginate:

Since a remarkable reduction in the absorption of radioactive strontium from the intestine of rats was demonstrated when sodium alginate was supplemented to the diet contaminated with the radioisotope, more extensive studies were required.

In order to further these tests, polymannuronate and polyguluronate were obtained in a similar manner to Haug[(67)] with the exception that only one hydrolysis in the procedure was followed in order to avoid the possibility of losing valuable material. Laminaria Hyperborea (Fig. 4)

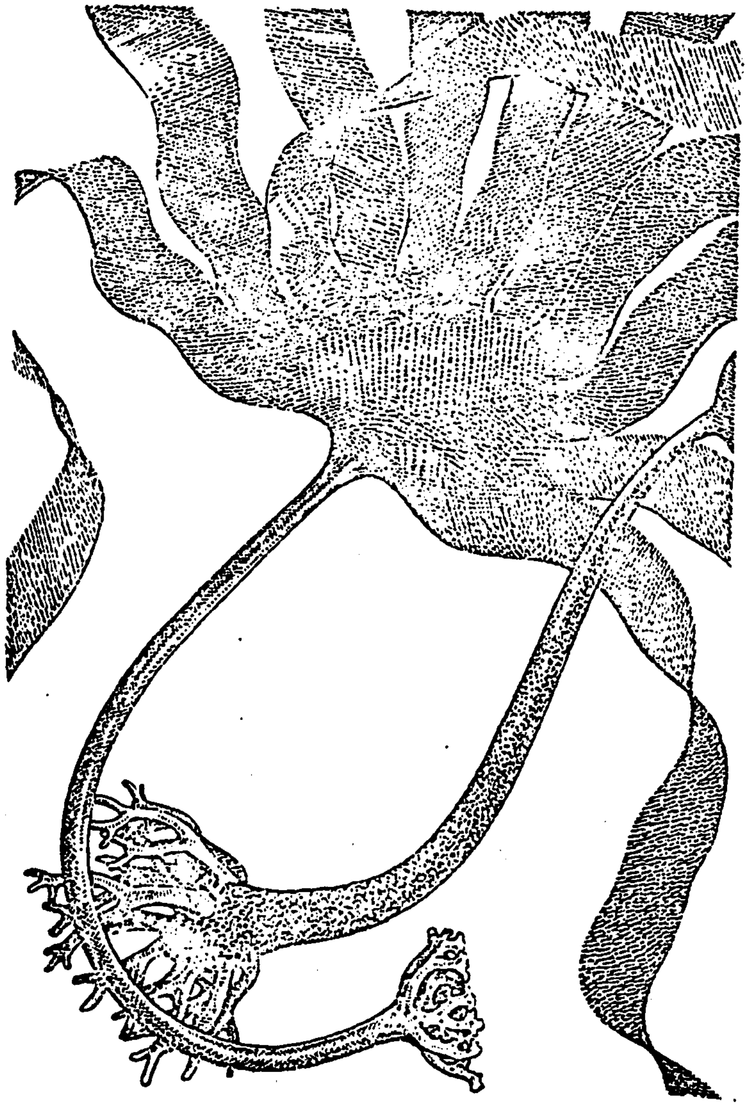


Fig. 22. *Laminaria digitata*, left ($\times \frac{1}{6}$); *Laminaria hyperborea*, right ($\times \frac{1}{8}$).

FIG. 4. The plants of Laminaria hyperborea are attached by strong branching haptera more or less in vertical rows forming a steep-sided cone. Other specific characters are the tapering stipe and the presence of mucilage ducts on the stipe. At one time, there was no distinction made between the plants of Laminaria hyperborea and Laminaria digitata. (Reference No.80)

was chosen as a source of alginic acid. This seaweed contains guluronic acid and mannuronic acid in comparable amounts. Partial hydrolysis of alginic acid with 1M $(COOH)_2$ at a temperature of 100°C for a twenty hour interval was carried out yielding insoluble matter which was dissolved in alkali and subsequently dialysed against distilled water to rid the material of small degradation products. The remaining partially degraded sodium alginate was further separated into soluble and insoluble fractions by preparing a 0.5% sodium alginate solution to which dilute 2.0 N and 0.05 N HCl was added until a pH of 2.85 $\pm$ 0.05% was reached. The fractions were filtered in order to separate the precipitate or polyguluronic acid from the polymannuronic acid remaining in solution. The fractions were further neutralized and precipitated with alcohol. Sodium polyguluronate possesses greater ability to suppress strontium absorption than polymannuronate. However, due to the polyguluronate fraction still containing some mannuronic acid, it was again hydrolysed under the same conditions and repeated fractionation in accordance with the foregoing procedure was performed on the degraded polyguluronate.

3. <u>Quantitative Determination of Uronic Acid Composition of Alginate Preparations</u>:

In order to obtain relative ratios of mannuronic acid and guluronic acid, sodium alginate (80 mg.) was hydrolysed with 80% sulfuric acid (1ml/100mg), in stoppered test tubes for 18 hours in a water bath at 25°C. The hydrolysate was diluted with water to a sulfuric acid strength of 2 N, the test tubes were sealed and hydrolysis at 100°C, for 5 hours, in a boiling water bath was carried out. After neutralization of the acid with a slight excess of calcium carbonate, the precipitate was filtered off and washed with water and the filtrate was concentrated in vacuo to a smaller volume. Before applying it to the column, a few drops of NaOH were added to the hydrolysate and it was allowed to stand for approximately thirty minutes at a slightly alkaline pH of 8-9, to convert the lactone to the corresponding sodium salt. Column chromatography using a Dowex 1 -XB (acetate form) column (20 x 350 mm) and 0.5 N - 2 N acetic acid as the gradient solvent system enable fractionation of the sodium salt mixture into mannuronic acid and guluronic acid. These fractions were collected on a fraction collector and examined by the Orcinol test,[(68)] in duplicate, from which the relative ratios can be calculated. At least two analysis were performed on each sodium alginate preparation. It was necessary to hydrolyse greater amounts of the sodium alginate derivatives (as much as 300 mg.), adjusting the amount of acid, when the content of either mannuronic or guluronic acid was extremely low.

4. Qualitative Identification of Uronic Acids:

Further evidence of the identity of the uronic acid fractions collected, aside from the Orcinol test, was obtained by thin layer chromatography on micro - crystalline cellulose and paper chromatography. The chromatographic medium of Fischer and Dörfel was used for the separation of uronic acids: ethyl acetate - acetic acid - pyridine - water (5:1:5:3 by volume).(47)

5. Determination of Viscosities:

The determination of the viscosities of sodium alginate and its derivatives was carried out using Ostwald No. 100/68 viscosometers in a water bath at 25°C. Ten ml. of a 1 % solution of all substances were prepared and their relative viscosities were calculated with respect to that of water under the same conditions.

6. <u>Biological Assay</u>:

Biological assays on rats are used to test the inhibition of intestinal absorption of radioactive strontium and calcium by sodium alginate. Animals can be maintained on an alginate diet with simultaneous addition of radioactive tracers. However, due to the very high viscosity of some alginate solutions there may be interference in proper mixing of the alginate and tracers, which is necessary in order to obtain optimum experimental results.

A more successful physiological approach involves administration of radioisotopes by the method of intubation using rats.[69] Radioactive Sr^{89} in HCl of low concentration is diluted with inactive $SrCl_2$ solution to the required concentrations. Standard grade, dry sodium alginate is dissolved in these radioactive solutions. 20 mg. of sodium alginate can be administered directly into the stomach of the rats by this procedure, with a standard dose (2μc) of Sr^{89} and Ca^{45}, with 0.5 micromole of inert Sr or Ca chloride respectively. The animals are fasted for twenty-four hours preceding the introduction of 1 ml. of radioactive test solution into the stomach via an orogastric tube. Twenty hours later a femur from each rat is assayed for radioactivity. The decrease in uptake of radioactive material by the bone indicates greater efficiency of the binding of sodium alginate to Sr, and so enhancing suppression of absorption of the radioactive material in the intestine.

7. In Vitro Tests

In order to clarify the binding properties of the *in vivo* experiments, determination of the calcium and strontium bound to alginate in vitro was obtained.(62) Two ml. of 1 % sodium alginate solutions were dialysed against water containing $CaCl_2$ and $Sr(NO_3)_2$ in appropriate concentrations to permit atomic absorption spectrophotometry analysis. The amount of Ca and Sr bound to the alginate is recorded in Table V, and was obtained by subtracting the calcium and strontium content respectively of the bathing solution after dialysis from its original concentration. The difference represents the Ca and Sr which bound to the alginate samples.

C H A P T E R V

R E S U L T S

1. Extraction of Alginates.
2. Fractionation of Alginates and Uronic Acid Composition.
3. Qualitative Identification of Uronic Acids.
4. Determination of Viscosities of Preparations.
5. Bio - Assay of Binding of Strontium and Calcium by Alginates.
6. *In vitro* tests of Binding Capacity.

C H A P T E R V

R E S U L T S

1. Extraction of Alginates:

In brown seaweeds, the alginic acid exists as an insoluble mixed salt, and the extraction of alginate as a soluble material represents an ion-exchange process. Since alginic acid is fully neutralized at a pH less than 7, alginate was extracted under neutral or slightly acidic conditions. The sodium alginate was readily isolated from the extract by precipitation with alcohol. This takes place in the form of the following reaction:

Assuming alginate occurs as an insoluble calcium salt (Chapter lV):

$$Ca\,(Alg)_2 + 2\,H^+ \rightleftharpoons 2\,H\,Alg + Ca^{++}$$

insoluble alginate — acid — Alginic acid insoluble

+

Na_2CO_3

neutralizer

↓

$$Ca\ salt \leftarrow CaCl_2 \quad + \quad Na\,Alg + Ca^{++}$$

insoluble — Sodium alginate soluble

+

EtOH

↓

Na salt

insoluble

Fig. 5.

2. Fractionation of Alginate:

The ratio of guluronic acid to mannuronic acid increased considerably on degradation of Alginic acid with 1 M Oxalic acid, yielding partially degraded sodium alginate with a G/M ratio of 2.04 compared to that of 0.98 of the starting material. Fractionation of this degraded product with dilute HCl at a pH of 2.8, yielded poly-L-guluronic acid with a maked increase in G/M ratio of 3.7. This indicates a purer form of the guluronate fraction than its parent molecule, however, it still contains approximately 20 % mannuronic acid. As a means of eliminating the mannuronic acid content still present in poly-L-guluronic acid, hydrolysis and fractionation was repeated as previously described. As expected, the degraded polyguluronate contained less mannuronic acid than the polyguluronate obtained from the first fractionation procedure. This was determined by evaluation of the ratio of the two uronic acids: G/M ratio was 3.7 for polyguluronic acid and 7.14 for degraded sodium polyguluronate, thus showing a decrease in the mannuronic acid content.

On separation of this degraded 'polyguluronate' with dilute acid at pH of 2.85, the resulting insoluble polyguluronate had a G/M ratio of 4.35, again considerable lower than the G/M ratio of 7.14 of degraded sodium polyguluronate, whereas the soluble polymannuronate contained less mannuronic acid indicated by G/M ratio of 8.25 as compared to that G/M ratio of 7.14, stated above. These findings suggest that the structure of alginic acid is more complex than that suggested by Haug.[53]

HYDROLYSIS AND FRACTIONATION OF ALGINIC ACID:

ALGINIC ACID

G / M 0.98

1 M $(COOH)_2$

INSOLUBLE HYDROLYSATE

SOLUBLE HYDROLYSATE

Na_2CO_3

PARTIALLY DEGRADED SODIUM ALGINATE

G / M 2.04

pH 2.8

Poly-D-mannuronic acid

Poly-L-guluronic acid

G / M 0.09

G / M 3.70

Fig. 6

HYDROLYSIS AND FRACTIONATION OF Poly-L-Guluronic Acid:

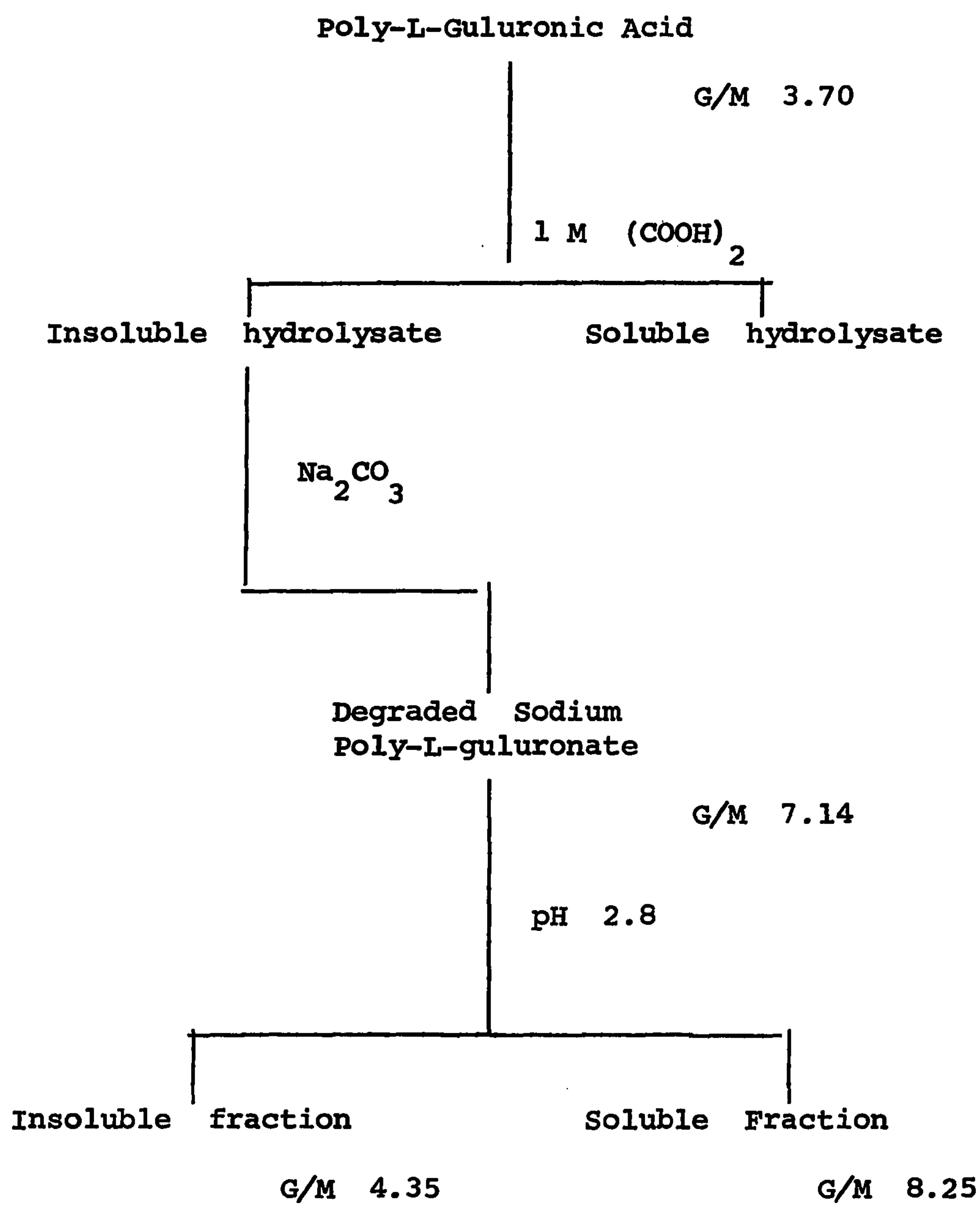


Fig. 7

3. Qualitative Identification of Uronic Acids:

The identity of the constituent uronic acids which have been fractionated from alginate was verified by thin layer chromatography on micro crystalline cellulose. The solvent system of ethyl acetate - acetic acid - pyridine - water in the ratio of 5 : 1 : 5 : 3 yielded Rf values for the uronic acids as follows:-

Mannuronic acid	0.3 (acid)	0.7 (lactone)
Guluronic acid	0.2 (acid)	0.9 (lactone)

4. Determination of Viscosities:

As can be observed from Table III, the viscosity of the sodium alginate molecule is reduced by increased hydrolysis. The original material, before hydrolysis, has a very high viscosity (greater than 7), compared to its degradation products. This value of 7 is markedly lower after one hydrolysis, varying in significance from 1.15 to 1.27. On additional degradation, the polymer and its constituent fractions have an even more decreased viscosity, estimated in the range of 1.11 to 1.17.

TABLE III

ALGINATE DERIVATIVE	VISCOSITIES
Undegraded Na Alginate	7.07
Degraded Na Alginate	1.20
Na polymannuronate	1.15
Na polyguluronate	1.27
Degraded Na polyguluronate	1.11
Insoluble fraction	1.17
Soluble fraction	1.14

5. BIO - ASSAY OF BINDING:

Biological assays involving intubation using rats were performed in order to test the suppression by alginate of radioactive strontium and calcium absorption respectively in the intestine. The reduction of bone uptake, measured in the femur of rats, dosed with the alginate derivatives as compared with the controls is expressed as the percentage inhibition. (Table IV).

1. The biological effectiveness of undegraded alginate was markedly and positively influenced on partial hydrolysis. This may due in part as a result of the decrease in viscosity of the polymer upon degradation, thus permitting more available groups on the alginate to bind with the metal ions.

2. Attempted isolation of the two polymers produced polyguluronate with a greater potential to inhibit the intestinal absorption of strontium ions than the corresponding polymannuronate.

3. Also the polyguluronate possessed a greater activity than the parent alginate polymer.

4. Additional hydrolysis of the polyguluronate produced a purer derivative, but one of slightly lower inhibitory activity than sodium polyguluronate.

5. Another point of interest is that the fractions obtained, both insoluble and soluble, while displaying similar biological activity were very different with respect to their chemical composition. i.e. G/M ratio.

6. Because of clinical importance the inhibition of calcium absorption was also tested and compared to that of strontium. The results indicate the more the sodium alginate is hydrolysed there is a greater decrease in the inhibitory characteristics concerning calcium absorption as opposed to the improved inhibition of strontium absorption in the intestine. A critical outcome of the reduced strontium absorption and enhanced calcium absorption is the uninterrupted maintenance of calcium equilibrium in the body.

TABLE IV

INHIBITION OF INTESTINAL ABSORPTION OF RADIOACTIVE STRONTIUM AND CALCIUM BY SODIUM ALGINATE AND ITS DEGRADATION PRODUCTS

INHIBITOR	G / M	VISCOSITY	% INHIBITION Sr^{89}	% INHIBITION Ca^{45}
Undegraded Na alginate	0.98	7.07	63	36
Degraded Na alginate	2.04	1.20	77	35
Na polymannuronate	0.09	1.15	64	32
Na polyguluronate	3.70	1.27	85	30
Degraded Na polyguluronate	7.14	1.11	78	32
Insoluble polyguluronate fraction	4.35	1.17	72	32
Soluble polymannuronate fraction	8.25	1.14	72	-

6. IN VITRO TESTS OF BINDING CAPACITY:

The results of the *in vitro* tests to determine the amounts of calcium and strontium bound by the different alginate derivatives are shown in Table V. The 1 % alginate samples were derived from Laminaria Hyperborea and the table shows that the observed, in vitro results, are in fair agreement with the in vivo experiments. That is, there is a greater affinity of strontium ions to alginate than calcium ions, and guluronic acid rich alginate preparations tend to bind strontium to a greater extent than their corresponding mannuronic acid rich preparations. In addition, the Sr / Ca ratio increases with additional degradation. i.e. Sr/Ca ratio of Na polyguluronate is 3.49 compared to that of the further degraded guluronate rich polymer, the 'insoluble fraction' which has a ratio of 4.07. Also, the Sr/Ca ratio of Na polymannuronate increases from 1.44 to 3.64 for the corresponding fraction obtained from further hydrolysis and fractionation, the 'soluble fraction.

TABLE V

IN VITRO TESTS OF BINDING CAPACITY

ALGINATE SAMPLE	mg. Sr bound to alginate.	mg. Ca bound to alginate	Sr/Ca ratio
Degraded Alginate	6.52	1.74	4.06
Na polymannuronate	4.24	2.24	1.44
Na polyguluronate	7.06	1.87	3.49
Degraded Na polyguluronate	6.70	2.12	1.91
Insoluble polyguluronate fraction	6.59	1.62	4.07
Soluble polymannuronate fraction	6.59	1.81	3.64

CHAPTER VI

DISCUSSION

The evaluation of biological activity of alginates in relation with their chemical composition is of practical significance in preventing absorption of radioactive strontium from the gastrointestinal tract as well as their use as possible decontaminants. Previous investigations including those of Skoryna et al.[70,71] and Harrison et al.[59,72] have indicated that a salient role in determining effectiveness relates to the ratios of the constituent uronic acids in alginate derivatives: mannuronic acid and guluronic acid.

It is evident from Table IV, that all the alginate derivatives manifest greater biological potency in sequestering radiostrontium absorption from the intestine, in comparison to their closest parent molecule.

NATURE OF THE BINDING PROCESS:

The suppression of intestinal absorption of the alkaline earths depends on the ion exchange properties of alginates. Divalent metals, such as Ca and Sr form insoluble gels with alginate.

$$\text{E.g.} \quad \underset{\text{gel}}{M(\text{Alg})_2} + \underset{\text{sol.}}{2\,Na^+} \rightleftharpoons \underset{\text{gel}}{2\,Na\,\text{Alg}} + \underset{\text{sol.}}{M^{++}}$$

where M = metal
Alg = alginate
sol. = solution

The alginate polymers are polyelectrolytes, which when dissolved in water, dissociate into many carboxylate ions. The discrepancy in biological effectiveness of mannuronate and guluronate units in alginate derivatives is a result of the different chemical reactions of the carboxyl groups, i.e. the ion exchange groups in these two units. The chemical reactivity of these functional groups is influenced by their accessibility to the metal ions and their particular position within the polymer itself.

Studies have indicated differences in selectivity coefficients according to the M/G ratio; strontium showed a high affinity for alginates, rich in guluronic acid. Further work by Thiele and Andersen,(56a) and others,(43, 73) revealed that Sr^{++} had a greater affinity than Ca^{++} for combining with alginic acid, to form a gel.

In vivo the following reactions take place in the intestinal tract:-

$$2\,H\,Alg + Sr^{++} \rightleftharpoons Sr(Alg)_2 + 2\,H^{+}$$

$$2\,Na\,Alg + Sr^{++} \rightleftharpoons Sr(Alg)_2 + 2\,Na^{+}$$

$$Ca(Alg)_2 + Sr^{++} \rightleftharpoons Sr(Alg)_2 + Ca^{++}$$

The equilibria of the above reactions are greatly in favour of strontium alginate.

Haugs' experiments on the ion exchange properties of sodium alginates revealed a correlation between chemical composition of alginic acid, i.e. the ratio of mannuronic acid and guluronic acid with respect to its ion exchange properties.[53,62] Assuming that a similar relationship occurs in the gastrointestinal tract, it is possible to relate the effectiveness of alginate to the Mannuronic acid / Guluronic acid ratio.

DEGRADATION OF ALGINATES:

Isolation of the uronic acid fractions by repetitive degradation and fractionation, did not progress such as to yield a monomeric unit; fractions consisting of several units were obtained. Nor was it possible to obtain a pure mannuronic acid or guluronic acid preparation, i.e. a preparation in which either mannuronic acid or guluronic acid dominated. A detectable concentration of the corresponding acid was always present. The proportion of mannuronic and guluronic acid represented by their G/M ratio (Table IV), increases with degradation in favour of a guluronic acid rich polymer. At the same time, the biological efficiency of the alginate derivatives, defined as the number of mg. theoretically required to give 100 % inhibition, (assuming an inverse linear relationship between the dose of alginate and the bone uptake of radiostrontium, Chapter III), can be compared with the corresponding G/M ratios (Table IV).

Due to high viscosity of alginate solutions it was thought that their effectiveness may be enhanced if the viscosities were lowered. It was thought the less viscous the solution, the more efficient is the polyelectrolyte activity of the alginate derivative, thus enabling better access of the ionizable groups of the polymer to react and bind with the metal ions, as the strontium ions would then be able to penetrate the acid more readily. For in vivo experiments, proper mixing of the alginate and the metal ions tends to be more continuous throughout the gastrointestinal tract, the less viscous the alginate solution. Partial hydrolysis reduces the viscosity of alginic acid, a consequence of a molecule of a lesser degree of polymerization. It was found that degradation under the proper conditions rendered undegraded alginate with a greater value of G/M ratio compared to the original alginate and of increased effectiveness with respect to biological assays. The greater sequestering power of degraded alginates, with respect to Sr^{89} absorption, coincides with (a) a decrease in the viscosity and (b) a more guluronic acid rich polymer. This implies that the mannuronate units are hydrolysed at a faster rate. The bioassays using rats showed that the hydrolysed products have a greater influence on the inhibition of strontium absorption than the starting material. This does suggest a higher affinity of the guluronate units in alginate to strontium than the corresponding mannuronate parts.

INHIBITION OF CALCIUM ABSORPTION:

At the same time, the inhibition of calcium absorption was tested in comparison to that of strontium. It is shown that the more the sodium alginate is degraded, there is a greater decrease in the inhibitory properties in relation to calcium absorption as opposed to the improved inhibition of strontium absorption from the intestine. It seems therefore, that the alginate preparations maintain the ability to differentiate between Sr^{++} and Ca^{++} ions, irrespective of their comparable nature. The absorption of Ca^{45} is reduced to a slight extent by alginates *in vivo*, however, alginate and its degradation products studied do not disrupt the essential calcium absorption from the intestine or the deposition of this element in the crystalline appatite of the bony tissue, its ultimate metabolic route. Therefore a critical outcome of the reduced strontium absorption and enhanced calcium absorption is related to the uninterrupted maintainance of calcium equilibrium in the body.

DEGREE OF POLYMERIZATION:

Our experiments demonstrate that guluronic acid content of the alginate derivative is a critical factor in determining the effectiveness in binding radiostrontium. However, it should be noted that, *in vivo*, both guluronate and mannuronate polymers present in the guluronate rich preparations, possess the ability to inhibit radiostrontium absorption. Furthermore, as clearly shown by the results in Table IV, some alginates containing only a small percentage of guluronic acid were considerably effective. i.e. the affinity of the mannuronate units toward strontium is similarly great. Some investigators believe that the effectiveness of alginates depends entirely on the concentration of guluronate units. It should be emphasized that partially degraded alginates are composed of both guluronate and mannuronate units, both of which can bind strontium to a different extent. It is not correct to acknowledge the alginate constituents as a mixture of polyguluronate and inert substances.

The preparation of sodium polyguluronate, still containing 20% mannuronic acid, was again hydrolysed under the similar conditions, as described previously. Although the guluronic acid content was definitely increased, a decreased effectiveness of this degraded product was observed in comparison to sodium polyguluronate. As can be observed from Table IV, there is a limit to the amount of degradation which the polymer may undergo in order that it maintains as influential a biological effectiveness in the bio-assay experiments. Although the M/G ratio is indeed important, other determinants such as chain length should be contemplated when considering maximum gel formation with strontium. This suggests that there is an optimum degree of polymerization of the alginate derivative which is appropriate for maximum binding capacity. By partially degrading the polymer, (to reduce the less effective mannuronic acid units and diminish the resulting chain length, to obtain the desired biological effectiveness, it appears that a balance may be achieved which produces maximum binding of strontium ions *in vivo*.

STEREOCHEMISTRY:

There is no direct relationship between the mannuronic acid and the guluronic acid content, and the biological effectiveness. Any irregularity of the relationship of the G/M ratio to the biological activity in the _in vivo_ experiments, may be due in part to biological variations in the response of the experimental animals, but, there are other points of interest to be considered. The exact mechanism of alginates binding remain unknown. In general, because they are polyelectrolytes, it may be stated that they play the role of ion-exchange reagents. The complexities of alginate properties (with respect to chemical reactions of their ion-exchanging carboxyl groups) depend not only on the ionic size of these functional groups, but also on their accessibility to other ions in the environment and their relative reactivity within the uronic acid units themselves.

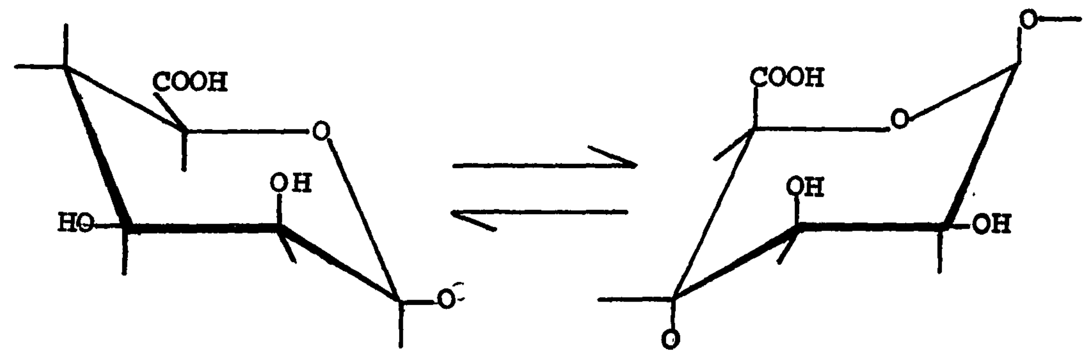
In tertiary structure, there seems to be a preferred configuration of each uronic acid unit within the alginate polymer. β-D-mannuronic acid tends to be more stable in the C 1 conformation (diagram, fig.8) where the carboxyl group is in the equatorial position with respect to the ring structure. However, in the instance of metal ions such as Sr^{++} or Ca^{++} encountering the alginate molecule, there may be less hindrance from neighbouring atoms if the carboxylate ions were in the equatorial position. In the C 1 configuration, the 2 - hydroxyl group bisects the angle of the ring structure between the ring - oxygen and the glycoside - oxygen, causing electrostatic repulsion. Thus, it is apparent that there would be greater potential for ion exchanges between the carboxylate ion of the alginate derivatives and the metal ions if they were to react with the steric configuration of the molecule which is in favour of the 1 C conformation in the diagram.

The stereochemistry at the anomeric center of L-guluronate units in alginate is believed to be in the α - form, based on the structure of the trisaccharide which was isolated from an enzymatic degradation[(74)] product of alginate. Similarly, α-L-guluronic acid favours the 1 C position where the carboxylate ions are in the equatorial plane with respect to the rest of its molecular structure, as opposed to the C 1 arrangement with a closer positioning of the oxygen atoms. This 1 C configuration lessens the possible stearic interference of the other atoms present in the molecule.

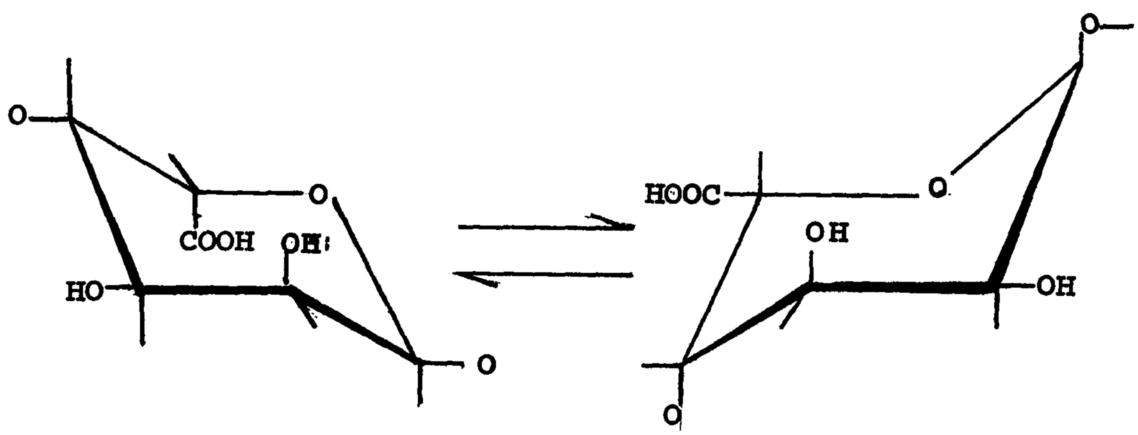
Assuming that the guluronate units in alginate are in the 1 C conformation with the carboxyl group in equatorial position, and as was suggested by Astbury,(79) the mannuronate units of alginate are in the 1 C conformation with the carboxyl group in axial position, it is possible to rationalize the difference in the biological effectiveness of polymannuronate and polyguluronate. Because of a general stearic hindrance in axial position, the strontium ions are unable to approach the carboxylate ion of the mannuronate units as well as that of the guluronate units.

Alteration in the chain length of the polymer concerned, (which is simultaneously accompanied by a decrease in viscosity) and in the configuration of the structural units, as a result of acid degradation, would

Fig. 8



β - D - mannuronic acid



α - L - guluronic acid

necessarily play a part in the rate, extent and direction of the ion exchanges. Therefore, it can be said that the strontium binding capacity *in vivo* of both alginates and oliguronides of shorter chain length is dependent on the presence of a high concentration of guluronic acid.

CHAPTER VII

CONCLUDING COMMENTS AND SUMMARY

1. With the realization that alginic acid is of considerable importance, not only with respect to preventing metal poisoning, but as it is a means of eliminating undesirable substances therapeutically, it seemed beneficial to consider the composition of the polysaccharide and its derivatives in relation to their biological activity. In accordance with Haug and Smisrød,[78] experiments on the alginate, rich in guluronic acid and those rich in mannuronic acid, demonstrate that the guluronic acid content of the alginate derivative is the crucial factor in determining the effectiveness in binding radiostrontium. Nevertheless, both guluronate and mannuronate polymers are capable of binding strontium among guluronate rich preparations.

There is no apparent correlation between biological effectiveness of alginates and their chemical composition, therefore, other factors, such as viscosity and chain length, were examined.

2. Due to the high viscosity of water - soluble alginates, it was thought that their effectiveness may be enhanced if the viscosities were lowered, since strontium would then be able to penetrate the acid more readily. Partial hydrolysis reduced the viscosity of alginic acid. It was found that degradation under various conditions render the undegraded alginate with a greater value of guluronic/mannuronic acid ratio compared to the original alginate, and of increased effectiveness with respect to biological assays. This implies that the mannuronate units are hydrolysed at a faster rate, and, using rats, bioassays of the hydrolysed alginate products exhibit a greater influence on the inhibition of intestinal absorption of radiostrontium than the undegraded starting material. This indicates a greater affinity of the

guluronate units in alginate to strontium than the corresponding mannuronate components.

3. In order to further examination of the variation in biological activity of the two uronic acids, polymannuronate and polyguluronate were obtained in a similar manner to Haug,(67) with the exception that only one hydrolysis in the procedure was performed to avoid the possibility of losing valuable material. The non-dialysable degraded sodium alginate was fractionated and the two acids thus obtained were converted into their sodium salts and assayed for inhibitory activity. Greater effectiveness was observed with sodium polyguluronate than with sodium polymannuronate; nevertheless, the latter was comparable in effectiveness to the undegraded parent polymer.

4. Calcium and strontium are chemically very similar, however, alginate displays a markedly greater affinity towards strontium. The degradation products and their parent alginates were tested for their capacity to bind with radiocalcium and no differences were recorded

in their binding properties; calcium absorption from the intestine is not suppressed.

5. Due to the persistent presence of mannuronic acid in the polyguluronate fraction, (20%), it was again hydrolysed under the same conditions and repeated fractionation in accordance with the foregoing procedure was performed on the degraded polyguluronate. This additional hydrolysis of polyguluronate produced a purer derivative, but one which possesses a lower inhibitory activity than sodium polyguluronate. Fractional precipitation of this polymer into its component uronic acids resulted in a polyguluronate and polymannuronate, which, while displaying similar biological activity, are very different in chemical composition, i.e. G/M ratio.

6, There seems to be an optimum degree of polymerization which is suitable for maximum binding capacity. It appears, therefore, that the maximal conditions for binding can be achieved by balancing two factors:- guluronate rich preparations which are most effective binders of strontium and size of molecule. Partial degradation in order to minimize the amount of less effective mannuronate units is necessary, at the same time maintaining a polymer of desired (i.e. biologically effective) chain length.

7. The inhibition of calcium absorption was tested and compared to that of strontium. It was shown that the more the sodium alginate is hydrolysed, the greater is the decrease in the inhibitory characteristics concerning calcium absorption as opposed to the enhanced inhibition of strontium absorption in the intestinal tract. A critical outcome of the reduced strontium absorption and enhanced calcium absorption is the uninterrupted maintenance of calcium equilibrium in the body.

8. In vitro studies were carried out to confirm the binding properties of the in vivo studies by dialysing alginate solutions against water containing strontium and calcium and aspirating the final solutions via atomic absorption. The results supported the above evidence.

REFERENCES

1. McLean, F.C., and Budy, A.M.
Radiation, Isotopes, and Bone
Academic Press, New York and London, (1964)

2. Paul, T.M., Waldron-Edward, D., and Skoryna, S.C.
Can. Med. Ass. J., 91: 553, (1964)

3. Skoryna, S.C., Paul, T.M., and Waldron-Edward, D.
Can. Med. Ass. J. 93: 404, (1965)

4. Waldron-Edward, D., Paul, T.M., and Skoryna, S.C.
Can. Med. Ass. J., 91: 285, (1964)

5. Skoryna, S.C., Paul, T.M., and Waldron-Edward, D.
Can. Med. Ass. J., 91: 285, (1964)

6. Moore, W., and Elder, R.L.
Nature, London, 206, 841, (1965)

7. Harrison, J., McNeil, K.G., and Janiga, A.
Can. Med. Ass. J., 95, 532, (1966)

8. Paul, T.M., Skoryna, S.C., and Waldron-Edward, D.
Studies on the Inhibition of Intestinal Absorption of Radioactive Strontium: V The Effect of Administration of Calcium Alginate.
Can. Med. Assoc. J. 95: 957-60, (1966)

9. Pizzarello, D.J., and Witcofski, R.C.
Basic Radiation Biology
Lea and Febiger, Philadelphia, (1967)

10. Libby, W.F., Gerger, R., Mead, J., Alexander, G., & Ross, J.F.
Replacement rates of carbon in tissues from bomb radiocarbon.
Science 146: 1179, (1964)

11. Cohn, S.H., Rinehart, R.W., Gong, J.K., Robertson, J.S., Milne, W.L., Bond, V.P., and Cronkite, E.P.
Internal Deposition of radionuclides in human beings and animals. Chapter 5: Some Effects of Ionizing Radiation On Human Beings. U.S. AEC, July, (1956)

12. Blahd, W.H.
Nuclear Medicine: Biologically important radiations
McGraw-Hill Book Co., New York, (1965)

13. Shilling, C.W.
Atomic Energy Encyclopedia in the Life Sciences:
Strontium-90.
W.B. Saunders Co., Philadelphia, London, P. 409
(1964)

14. Chart of the Nuclides
General Electric Co., (1956)

15. Shilling, C.W.
Atomic Energy Encyclopedia in the Life Sciences:
Food Contamination.
W.B. Saunders Co., Philadelphia, London, P. 169
(1964)

16. Spiers, F.W.
Radioisotopes in the Human Body: Physical and
Biological Aspects.
Academic Press, New York, London, (1968)

17. Engstrom, A., Bjornerstedt, R., Clemedson, C.-J.,
Nelson, A.
Bone and Radiostrontium
Wiley and Sons, Inc., New York. P. 16, (1958)

18. Shilling, C.W.
Atomic Energy Encyclopedia in the Life Sciences:
Discrimination Factor
W.B. Saunders , Philadelphia, London, P. 125. (1964)

19. Kulp, J.L., Eckelman, W.R., and Schulert, A.R.
Strontium - 90 in man
Science 125; 219, (1957)

20. Bowen, H.J.M.
Trace Elements in Biochemistry
Academic Press, London and New York.

21. Winton, F.R., Bayliss, L.E.
Human Physiology
J. & A. Churchill Ltd. (1962)

22. Wasserman, R.H.,
Metabolic basis of calcium and strontium discrimination: Studies with surviving intestinal segments.
Proc. Soc. Exptl. Biol. Med. 104, 92-95 (1960)

23. Dumont, P.A., Curran, P.F. and Solomon, A.K.
Calcium and strontium in rat small intestine, their fluxes and their effect on Na flux.
J. Gen. Physiol. 43, 1119-36 (1960)

24. Volf, Vladimir
"Intestinal Absorption of Strontium"
Intestinal Absorption of Metal Ions - edited by Skoryna, S.C. To Be Published.

25. Dolphin, G.W. and Eve, I.S.,
Phys. Med. Biol. 8 (2): 193 (1963)

26. Giese, W. and Comar, C.L.
Existence of Non-exchangeable Calcium Compartments in Plasma Nature, 202, 31 - 33, (1964)

27. Balabuha, V.C. and Razumovsky, N.O.
Perspective in Excretion of Radioactive Strontium from Organism
Meditsinskaia Radiologica, 8: 71-76 (1963)

28. O. van der Borght, J., Colard, S. Van Puymbroedk and R. Kirchman.
Abst. Internat. Symp. Radioecol. Concentration Process Stockholm (1966)

29. Stara, J.F.
Abst. Symp. Nuc. Med.,
Omaha, Nebraska, (1965)

30. Hodgkinson, A., Nordin, B.E.C., Hambleton, S., and Oxby, C.B.
Can. Med. Ass. J., 97: 1139, (1967)

31. Sutton, A.
Nature (London), 216, 1005, (1967)

32. Hesp, R. and Ramsbottom, B.
Nature (London), 208, 1341, (1965)

33. Stara, J.F., and Waldron-Edward, D.
Repressive action of sodium alginate on absorption of radioactive strontium and calcium in cats.
Paper presented at the Sumposium on Diagnosis and Treatment of Deposited Radionuclides, Richland, Washington. (1967)

34. Harrison, G.E.
Symposium on Diagnosis and Treatment of Deposited Radionuclides
Richland, Washington. (1967)

35. Paul, T.M., Skoryna, S.C., and Waldron-Edward, D.
Can. Med. Ass. J. 95, 957 (1966)

36. Waldron-Edward, D., Paul, T.M., and Skoryna, S.C.
Can. Med. Ass. J., 91, 1006 (1964)

37. Stanford, E.C.C.
Brit. Patent, 142 /(1881)

38. Atsuki, K., and Tomoda, Y.
J. Soc. Chem. Ind. Japan, 29, 509, (1926)

39. Schnidt, E., and Vocke, F
Chem. Ber. 59: 1585, (1926)

40. Nelson, W.L., and Cretcher, L.H.
J. Am. Chem. Soc. 51, 1914, (1929)
52, 2130, (1930)
54, 3409, (1932)

41. Bird, G.M. and Haas, P.
Biochem. J., 25: 403 (1931)

42. Miwa, T.
J. Chem. Soc. Japan, 51: 738 (1930)

43. Hirst, E.L., Jones, J.K.N. and Jones, W.O.
J. Chem. Soc. P. 1880 (1939)

44. Kringstad, H. and Lunde, G.
Kolloid Z. 83: 202 (1938)

45. Lucas, H.J., and Stewart, W.T.
J. Am. Chem. Soc. 62: 1070 (1940)

46. Chanda, S.K., Hirst, E.L., Percival, E.G.V. and Ross, A.G.
J. Chem. Soc. P. 1833 (1952)

47. Fischer F.G. and Dörfel, H.
Z. Physiol. Chem. 302: 186 (1955)

48. Whistler, R.L. and Kirby, K.W.
Z. Physiol. Chem. 314: 46 (1959)

49. Vincent, D.L.
Chem. Ind. (London), P.1109 (1960)

50. Hirst, E.L., Percival, E. and Wold, J.K.
Chem. Ind. (London), P.257 (1963)

51. Hirst, E.L., Percival, E. and Wold, J.K.
J. Chem. Soc. P. 1493 (1964)

52. Haug, A.
Acta Chem. Scand., 13: 601 (1959)

53. Haug, A.
Report No. 30, Norwegian Institute of Seaweed Research, N.T.H., Trondheim (1964)

54. Haug, A. and Larnsen, B.
A Study on the Constitution of Alginic Acid by Partial Acid Hydrolysis.
Prodeedings of the Fifth International Seaweed Symposium, Halifax, Canada.
Edited by E. Gordon Young and J.L. McLachlan
Pergamon Press.

55. Haug, A., Larsen, B. and Baardseth, E.
Comparison of the constitution of Alginates from different sources.
Proceedings of the Sixth International Seaweed Symposium, Santiago de compostela,
Edited by Ramón Margalef, Sept. 9-13 (1968)

56. Thiele, H. and Andersen, G.
Kolloid Z. (a) 140: 76, (1955)
(b) 142: 5, (1955)
(c) 143: 21, (1955)

57. Thiele, H. and Hallich, K.
Kolloid Z. 151: 1 (1957)

58. Thiele, H. and Schacht, E.
Kolloid Z. 161: 120, (1958)

59. Harrison, G.E., Humphreys, E.R., Sutton, A., and Shepherd, H.
Science, N.Y., 152: 655, (1966)

60. Patrick, G., Carr, T.E.F. and Humphreys, E.R.
Inhibition by alginates of strontium absorption studied *in vivo* and *in vitro*.
Int. J. Radiat. Biol., Vol. 12, No. 5, 427-434, (1967)

61. Tanaka, Y., Skoryna, S.C. and Waldron-Edward, D.
Studies on the Inhibition of Intestinal Absorption of Radioactive Strontium: VI Alginate Degradation products as potent *in vivo* Sequestering Agents of Radioactive Strontium.
Presented in part at the meeting of the Canadian Federation of Biological Sciences, Montreal, July (1967)

62. Haug, A. and Smisrød, O.
Strontium - Calcium Selectivity of Alginate
Nature, 215: P. 757 (1967)

63. Carr, T.E.F., Nolan, J., and Durakovic, A.
Effect of Alginate on the Absorption and Excretion
of ^{203}Pb in rats fed Milk and Normal Diets.
Nature, Vol. 224: P. 1115, Dec. 13 (1969)

64. Harrison, G.E., Carr, T.E.F., Sutton, Alice
and Humphreys, E.R.
Effect of Alginate on the Absorption of Lead in Man.
Nature, Vol. 224: P. 1115-6 Dec. 13 (1969)

65. Stanford, E.C.C.
Chem. News, 47: 254, 262 (1883)

66. Wassermann, A.
Ann. Bot., 12: 137, (1948)

67. Haug. A., and Smisrød, O.
Acta Chem. Scand., 20: 183, (1966)

68. Experimental Immunochemistry
Revised and Enlarged by Kabat, E.A. and Mayer, M.M.
Publisher: Charles C. Thomas. Springfield, Illonois,
U.S.A. 2nd Edition (1961)
Carbohydrate Estimation by Orcinol Method.

69. Paul, T.M., Waldron-Edward, D., and Skoryna, S.C.
Can. Med. Ass. J., 95: 957, (1966)

70. Paul, T.M., Waldron-Edward, D., and Skoryna, S.C.
Nature (London) 205: 1117, (1965)

71. Waldron-Edward, D., Paul, T.M.,and Skoryna, S.C.
Inhibition of Absorption of radioactive strontium
by alginic acid derivatives. In: Strontium
metabolism: Proceedings of the International
Symposium on some aspects of Strontium Metabolism,
Held at Chapelcross, Glassgow, and Strontian.
May 5-7 (1966).
Edited by J.M.A. Lenichan, J.F. Loutit and J.H. Martin,
Academic Press Inc. Ltd., London (1967) P. 329.

72. Harrison, G.E.
Absorption of Strontium in rats on alginate supplemented diet.
Paper presented at the Symposium of Diagnosis and Treatment of Deposited Radionuclides, Richland, Washington, May 15-17 (1967)

73. Mongar, I.L.
J. Chem. Soc., 492, (1952)

74. Yoshikawa, M. and Kiyohara, T.
Hyogo Noka Daigaku Kenkyu Hokoku, 6: 51, (1964)

75. Linker A., and Jones, R.S.
J. Biol. Chem. 241: 3845, (1966)

76. Haug. A.
Acta Chem. Scand. 15: 950, (1961)

77. Haug, A. and Smisrød, O.
Acta Chem. Scand. 19: 341, (1965)

78. Haug, A., Smisrød, O., and Larsen, B.
A study of the constitution of alginic acid by acid hydrolysis.
Acta Chem. Scand. 20: 183-90, (1966)

79. Astbury, W.T.
Nature, London, 155, 667. (1945)

80. Dickinson, Carola I.
British Seaweeds, The Kew Series. P.83
Eyre and Scottiswoode. (1963)