THE ANTICOAGULANT ACTIVITY OF SOME CARRAGEENANS

A Thesis presented by

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Summary

Introduction

The biological properties of the carrageenans and of certain other sulphated polysaccharides have been reviewed, and since the object of this work was to study the anticoagulant activity of intravenously-administered carrageenan, current theory of blood coagulation has been outlined. For comparison, the properties and mode of anticoagulant action of heparin have been reviewed.

Experimental

The practical work is reported in three sections.

A preliminary section deals with the anticoagulant activity, in the rabbit, of a number of carrageenans, fractionated where appropriate, into k- and λ-components. Doses fatal to rabbits within 24 hours are also recorded since many of these carrageenans were acutely toxic when administered intravenously. Frequently, lethal doses were close to anticoagulant doses and there seemed, from the preliminary work, reason to believe that the anticoagulant effect of these substances was associated with their toxic manifestations.

In the main section, a more detailed study of the anticoagulant activity of the k- and λ-carrageenans of Chondrus crispus from four habitats, of the carrageenan from Polyides rotundus and of degraded carrageenan from Eucheuma spinosum, is reported, and comparisons are made with heparin. Tests of clotting function were used to localise the anticoagulant activity in the clotting mechanism.

The results of some preliminary experiments to determine the effects of carrageenan on platelets and fibrinogen are reported because it is believed that the toxicity of certain high molecular weight sulphated polysaccharides is caused by fibrinogen precipitation complicated by platelet and blood cell involvement in the precipitated complex, so blocking important vascular beds. These preliminary results give adequate confirmation that the toxic carrageenans behave similarly, while the non-toxic carrageenan does not.

Conclusions

The results of this study confirm the belief that the anti-coagulant activity of λ-carrageenan is greater than that of k-carrageenan and indicate that this holds for Chondrus carrageenans in general, in spite of marked variations in the properties of different carrageenans. They also show that the relationship between activity and ester sulphate, previously believed to be critical, is not a simple one, although the

λ -carrageenan from any seaweed is always higher in sulphate content than the corresponding k-carrageenan. Of all the carrageenans examined, that of Polyides showed greatest activity. Degraded Eucheuma carrageenan was the least toxic of those examined and of lowest molecular weight; but although of high sulphate content, a much higher dose was required to show anticoagulant activity than of other carrageenans.

The anticoagulant activity of the carrageenans can be detected at all stages of the clotting reaction examined, and this supports the view that a non-specific complexing reaction with many plasma proteins is involved, in contrast to the more localised action of heparin.

8th June 1965.

Application by Jessie G. C. Duncan

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I N T R O D U C T I O N

THE CARRAGEENANS

Carrageenan is the name applied to a mixture of water-extractable galactan sulphuric acid esters of certain species of red marine algae (Rhodophyceae), e.g. Chondrus, Gigartina, Iridaea and Eucheuma species, in which they are found as cell-wall constituents. The term "carrageenan" was originally derived from its principal market site, Carrageen, in Ireland and even yet, Chondrus crispus is often referred to as Irish Moss. It has now become clear that carrageenan as a total extract is not one single substance, as has often been assumed in the literature, but is a mixture of sulphated polysaccharides. They are soluble in water, producing liquids of high viscosity and are characterised by their high ester sulphate content. Because of their rheological properties, the substances are frequently employed in the cosmetic and pharmaceutical industry to provide stability to suspensions, emulsions and foams. In the dairy industry, they are useful on account of their ability to alter the aggregation of casein in milk.

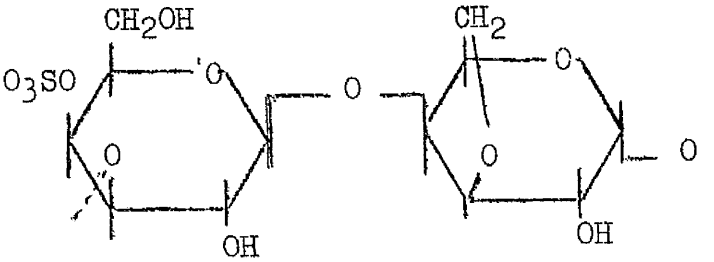
It is now being suggested that the carrageenan derived from a particular seaweed is a mixture of polysaccharides, the yield varying possibly, according to the species, the habitat, age and conditions during growth, the season in which it was harvested, and on the method of extraction.

From carrageenan solutions, two components are evident in the ultracentrifuge in all samples of high viscosity. Fractions corresponding to these two components have been separated by a method based on/

/on the sensitivity of carrageenan to potassium salts.[1]

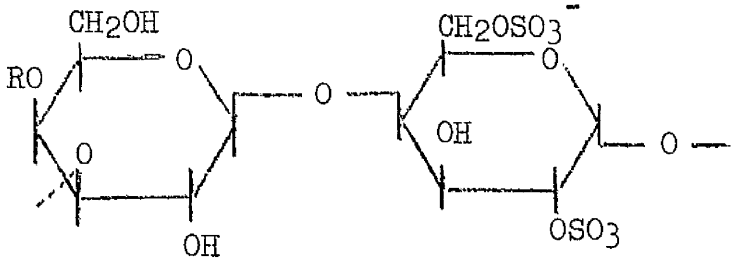
Potassium salts increase the viscosity of carrageenan solutions and enhance their gelling properties. When potassium salts are added to aqueous carrageenan solutions, too dilute to gel, a fraction is precipitated which may be removed by centrifugation. This fraction has been designated the kappa (k) fraction, while the unaffected component remaining in solution has been called lambda (λ) carrageenan. These fractions differ in some chemical and physical properties [2-4] such as potassium sensitivity, sulphate content, optical rotation, X-ray diffraction patterns, intrinsic viscosity and sedimentation rates. The chemical constitution of the carrageenans has been extensively investigated[5] but has not yet been completely elucidated.

The first structural studies showed that galactose units are present, esterified with sulphuric acid at position 4, and combined in glycosidic linkages through position 3. Some branching through position 6 was also shown to occur.[6-9] Later, another sugar component was identified[10] as 3,6-anhydro-D-galactose. The k and λ fractions were separated and, from the results of further experiments, a provisional structure was assigned to the k form. It is thought to exist as an alternating chain of 1,3-linked β-D-galactopyranosyl 4-sulphate units and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl units as shown below.



k - CARRAGEENAN

The λ fraction was further separated into a main component and a polysaccharide containing L-galactose units.[11] A possible structure for the main fraction has been suggested as below.



Thus, the major differences between the two forms are

1) The λ-carrageenan contains 1,4-linked-D-galactopyranose units in place of the 3,6-anhydro-D-galactose which occurs in the k form, and

2) the k-carrageenan contains a higher proportion of 4-sulphated D-galactose units.

Further studies have shown,[12] however, that the sulphate:galactose ratio for the k polysaccharide is ≥1 and the molecule may therefore be more highly sulphated than the above structure would indicate. It is suggested that a sulphate substituent in position 2 of the 3,6-anhydro-galactose unit may exist.

Also, by controlled treatment of λ-carrageenan with alkali, a polysaccharide related to k carrageenan can be prepared. It has therefore been suggested[13] that the main component of λ-carrageenan could be a biological precursor of the k form and this could account for the different percentage yields obtained from batch to batch. Possibly several enzymes are present in the living seaweed, capable of metabolising/

/metabolising the polysaccharides in a manner analogous to that of the alkali.

An enzyme specific for the hydrolysis of k-carrageenan has been extracted[14] from a marine bacterium. Its optimal conditions for activity, as measured by the amount of reducing sugar produced, were found to be a temperature of 40°C and a pH of 7.5. Structural and stereoisomeric differences in the molecules of the k and λ forms probably account for its specificity.

Despite incomplete knowledge of the structure of the carrageenans, the k and λ forms can be readily distinguished by analysis. The λ-fraction has a higher sulphate and galactose content. Also, the 3,6-anhydrogalactose moiety constitutes about 25% of the k fraction while in the "pure" λ carrageenan, it is virtually absent. The infrared absorption spectra of the two fractions differ substantially, due to the different positions of the sulphate groups and to differences in stereoscopic arrangements.

The sulphated polysaccharides, by virtue of their structure, demonstrate a reaction with the basic dye toluidine blue. The colour of the dye, in the presence of carrageenan, changes from its normal (orthochromatic) blue, through purple, to reddish-violet (metachromatic) shades. This phenomenon of metachromasia is not a specific reaction of the carrageenans but is well-recognised for many substances, including heparin, and synthetic sulphated polysaccharides. Investigation has shown that the intensity of metachromasia is directly related to the sulphur/

/sulphur content of the molecule but unrelated to its degree of polymerisation.[15] Although the reaction is apparent with very small amounts of polysaccharide, the non-specificity of the possible reactants limit its usefulness as a conclusive diagnostic test for the presence of carrageenan.

Biological Properties of Carrageenans

Various biological properties of the carrageenans have been reported. Turner and Magnusson have described experiments[16] using the k and λ fractions of carrageenan, extracted from the red alga, Chondrus crispus, as precipitants of two α-glycoproteins. These formed a lipoprotein-polysaccharide complex which could be dissociated by high concentrations of salts, and the two components were separated by centrifugation. This property could be an important factor concerned in the biological actions of the carrageenans.

When injected subcutaneously into rats, a local granulomatous lesion has been produced[17]. This effect has also been observed in the guinea pig[18], described as a deposition of dense connective tissue, of the collagen nature, later to degenerate and be replaced by adipose tissue.

Following numerous reports dealing with the clearing and anti-arteriosclerotic activities of heparin and other sulphated polysaccharides, the effects of intravenous carrageenan on serum lipids and atherosclerosis in cholesterol-treated rabbits has been reported[19]. Serum lipids were suppressed in the carrageenan-treated animals and the/

/the atheromatous lesions were less severe. This antilipaemic action had been previously observed by Houck et al.[20] in dogs, using a number of polysaccharides including extracts of carrageenan from six different species of seaweeds.

The antipeptic activity of sulphated polysaccharides was first reported in 1935,[21,22] using mucoitinsulphuric acid and chondroitin sulphuric acid. This work has been extended to include heparin[23] and degraded carrageenan[24]. Carrageenan will also prevent the occurrence of experimental peptic ulcer[25,26] and it has been suggested[24] that the protection afforded is due to increased mucosal protection. Other work[27] suggests the possibility of a humoral mechanism in the prevention of gastric ulceration.

Carrageenans have also an anticoagulant action. In 1935, Bergström[28] found that polysaccharides with sulphate groupings showed some anticoagulant activity while similar derivatives of mono- and di-saccharides were inactive. A large number of synthetic anticoagulants have been prepared by sulphating polysaccharides such as chondroitin, cellulose, inulin, starch and dextran. These substances all had relatively low anticoagulant activity and at equivalent doses higher toxicity than heparin.

The anticoagulant activity of the polysaccharide laminarin was first described by Jaques and Charles in 1941[29], and this has since been followed up by work described in several papers by Hawkins. Laminarin is a polysaccharide extracted from brown seaweed. It has

a/

/a molecular weight of 4,000-8,000, contains no sulphate in the natural state, but sulphated derivatives may be chemically prepared. O'Neill has demonstrated that these derivatives exhibit anticoagulant activity in vitro[30]. Four derivatives, extracted from Laminaria digitata and subsequently sulphated to different degrees, were tested for anti-coagulant activity in vivo in rats and dogs[31], with heparin as the standard for comparison. Anticoagulant activity was measured by the capillary tube method, the blood samples being taken at intervals after an intravenous injection of the polysaccharide. The results indicated that the activities of the preparations varied from 1/2-1/20 of the potency of heparin.

A more detailed study of the anticoagulant activity of one of these sulphated laminarin preparations was then undertaken[32] and the authors concluded that it acted, like heparin, as an antithrombic agent. In vitro tests indicated that it had 1/5 of the activity of heparin. The tests chosen to determine clotting efficiency were the clotting time, as measured by the Lee and White method, recalcification time, thrombin time and Quick's one-stage prothrombin time. Following an intravenous injection, in the case of both laminarin sulphate and heparin, a peak maximum effect was observed half an hour after the injection.

These authors also reported that laminarin sulphate showed lipaemia-clearing activity in the dog and rat, similar to that demonstrated by heparin.

Hawkins has since extended his work[94] to a study of the carrageenan derived/

/derived from Chondrus crispus. The extract was separated into k and λ fractions according to the usual procedure by potassium precipitation[2]. In both antipeptic and antithrombic activity, the λ fraction was found to be more active than either the k-fraction or the unfractionated extract. These tests were carried out in dogs, anticoagulant activity being indicated by a lengthening of the clotting time and thrombin time. In vitro tests, using human blood, showed that carrageenan has anticoagulant activity but is much less potent than is heparin. Again, an antithrombic activity is suggested for the polysaccharide although tests indicating deficiencies in the early stages of blood coagulation were not carried out. It is suggested that anticoagulant activity of the carrageenan may be associated with sulphate content since the λ-fraction has a higher sulphate content and also a greater anticoagulant activity than the k-form. This corroborates the observation of Anderson and Watt[24] that among degraded carrageenans, antipeptic activity is directly related to sulphate content.

Consequently, in vitro tests using human blood were carried out[95] using the fractionated and unfractionated carrageenan. The results were similar to those obtained with dogs, though quantitatively, lower activity was shown, the most active λ fraction being 1/17 as effective as heparin. It is pointed out that since interest is being shown in the carrageenans in the treatment of peptic ulcer, in which anticoagulant action is contraindicated, the anticoagulant action of carrageenan, although/

/although demonstrable only after intravenous administration, ought to be further examined.

Adams and Thorpe[33] have also conducted experiments which demonstrate the anticoagulant activity of laminarin sulphate. The polysaccharide was extracted from Laminaria cloustoni and four preparations, sulphated to varying degrees, were tested. In rabbits, using the capillary tube method for assessing anticoagulant activity, laminarin sulphate showed 1/3 of the activity of heparin, a result which is in agreement with that of Hawkins using dogs. A slightly more prolonged effect was seen than with heparin and the anticoagulant action could be neutralised by protamine sulphate, a property exhibited also by heparin. These authors, however, report that the toxicity of the polysaccharides tested would preclude their clinical use.

Further anticoagulant studies on carrageenan were reported in 1957 by Houck et al[20]. Dogs were used as the experimental animal and six preparations of carrageenan were tested for anticoagulant activity as demonstrated by the whole blood coagulation time. The seaweed sources were Furcellaria festigiate, Eucheuma spinosum, Gigartina acicularis, G. pistillate, G. radula and Iridaea species, in each case the unfractionated extract being used. Only with one preparation, G. acicularis, was marked activity observed. Contrary to the work of Hawkins et al., a peak anticoagulant effect was observed four hours after the intravenous injection compared with a half-hour peak as occurs with heparin. The effect of the carrageenan was still evident

/24 hours after the injection. Various toxic symptoms were observed, in particular kidney changes reminiscent of glomerulonephritis.

General conclusions reached from the work which has already been carried out are that many sulphated polysaccharides do show some anticoagulant activity in experimental animals, both in vitro and in vivo, the most active ones reported being the extracts from Chondrus crispus, Gigartina acicularis and the sulphated extract from laminarin sulphate. They were all less potent than heparin. The mode of action has been suggested to be an antithrombic one but insufficient investigatory tests have been undertaken to show this conclusively, especially those tests which whould demonstrate deficiencies in the earliest stages of coagulation. Most experimenters have used the whole blood coagulation test as an index of coagulation efficiency and while this does indicate interference in the clotting process, it is an entirely non-specific test, often being the resultant of interference with several clotting factors. Therefore a coagulation time within normal limits does not conclusively indicate that no effect has occurred.

The greatest disadvantage to the possible use of these sulphated polysaccharides as anticoagulant drugs would be their high toxicity following intravenous administration. In chronic toxicity tests in rabbits, loss of tone in ileum and colon has been observed,[33] and diarrhoea or defaecation occurred in many cases. The animals showed lethargy after the injection. Histologically, the most pronounced changes occurred in the kidneys, where degenerative changes in the convoluted/

/convoluted tubules were observed and occasionally, fibrin clots in the glomerular capillaries. These effects were suggested[33] to be possibly due to agglutination of the blood platelets and precipitation of fibrinogen, effects which have already been reported of many synthetic sulphated polysaccharides. [34,93].

In acute toxicity tests, the guinea pig appeared to be most sensitive to the substances, but unlike the toxic action of heparin, the effects did not appear immediately after I/V injection but after a time lapse of 1-2 hours. Pulmonary oedema, bouts of jactitating movements, limpness and finally death were the symptoms described, in some animals causing death within an hour of injection while in other animals, a day or two elapsed before the effects became fatal. These symptoms have been described by Walton and Ricketts [35] following the parenteral adminis-tration of sulphate esters of high molecular weight dextran, and are described as "anaphylactoid" reaction. Superficially, this resembles anaphylaxis but differs in that -

1. it occurred in animals not previously exposed to dextran sulphate
2. it was not accompanied by severe respiratory embarrassment and there was no sign of bronchiolar constriction or pulmonary emphysema
3. the reaction could not be prevented or inhibited by large doses of antihistamine.

Present understanding of the properties and actions of the carrageenans is confused because different authors have used the term "carrageenan" referring to total extracts in some cases, and fractionated extracts in others. The variety of sources of carrageenan has been extensive,/

/extensive, and frequently, the biological effects have been observed in different species of animals. It is therefore difficult to draw direct comparisons of results from different investigations.

The present study has noted and compared the anticoagulant effects of extracts, both fractionated and degraded, from different seaweed sources, because these above discrepancies have become more clearly understood. As it is the anticoagulant properties of the carrageenans which have been studied, it is necessary to consider the present knowledge of blood coagulation.

BLOOD COAGULATION

The transformation of fluid blood to a solid clot of fibrin occurs so quickly and spontaneously that it is difficult to envisage more than a simple scheme of events to bring it about. During the last twenty years, however, many steps in the early stages of clotting have been clarified and the presence of several hitherto unknown factors demonstrated. It appears that blood contains within it, the many clotting factors necessary to initiate and bring about complete coagulation and, at the same time, contains factors which prevent intravascular clotting and dissolve fibrin after it has performed its function. Confusion has arisen, and still exists, in the terminology applied to the various factors by different investigators. Different terms are often used to denote the same entities and in some cases, it is not clear which really are different and which refer to one/

/one and the same factor. In 1947, Owren[36] introduced a Roman numeral for his newly introduced factor V and this convention for all the known clotting factors, was adopted by the International Nomenclature Committee. This system has been adhered to with the newly recognised factors but is still not adopted for the long established terms such as fibrinogin, prothrombin and calcium. A list of factors with their common synonyms is included.

FACTOR	SYNONYMS
I	Fibrinogen
II	Prothrombin
III	Thromboplastin, Thrombokinase
1V	Calcium
V	Proaccelerin, Labile Factor Accelerator Globulin.
Vl	Accelerin
Vll	Proconvertin, Serum prothrombin conversion accelerator (SPCA) Stable factor, Autoprothrombin I
Vlll	Antihaemophilic factor (AHF), Antihaemophilic globulin (AHG), Platelet co-factor II, Autoprothrombin II, Antihaemophilic factor B.
IX /	

IX	Plasma thromboplastin component (PTC), Christmas factor, Platelet co-factor II Autoprothrombin II, Antihaemophilic factor B.
X	Stuart-Prower factor
XI	Plasma thromboplastin antecedent (PTA)
XII	Hageman factor.

The first theory of blood coagulation, referred to as the classical theory, was postulated in 1905 by Morawitz[37], as follows:-

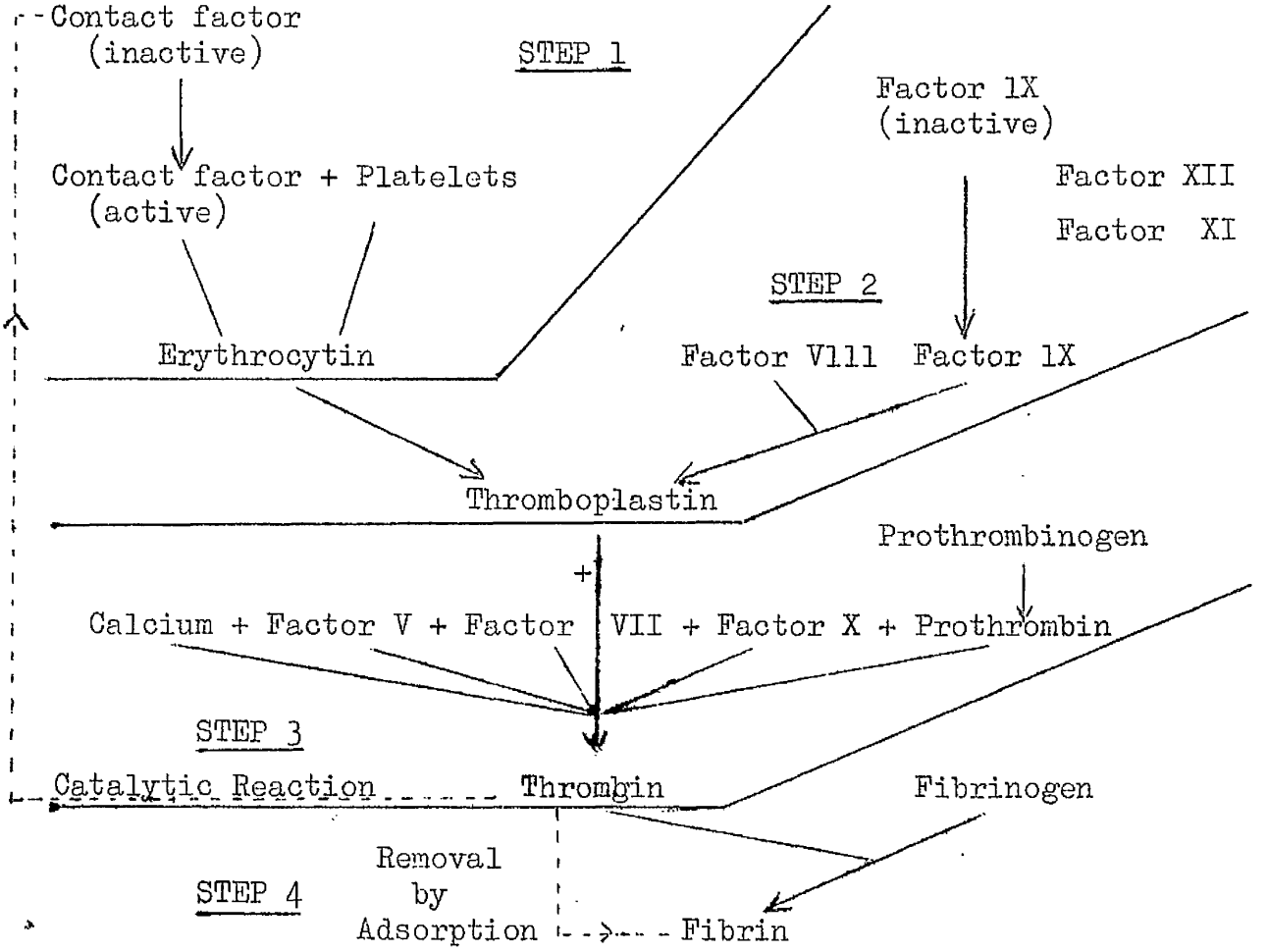
$$\text{PROTHROMBIN} \xrightarrow[\text{CALCIUM}]{\text{TISSUE EXTRACT}} \text{THROMBIN}$$

$$\text{FIBRINOGEN} \xrightarrow{\text{THROMBIN}} \text{FIBRIN}$$

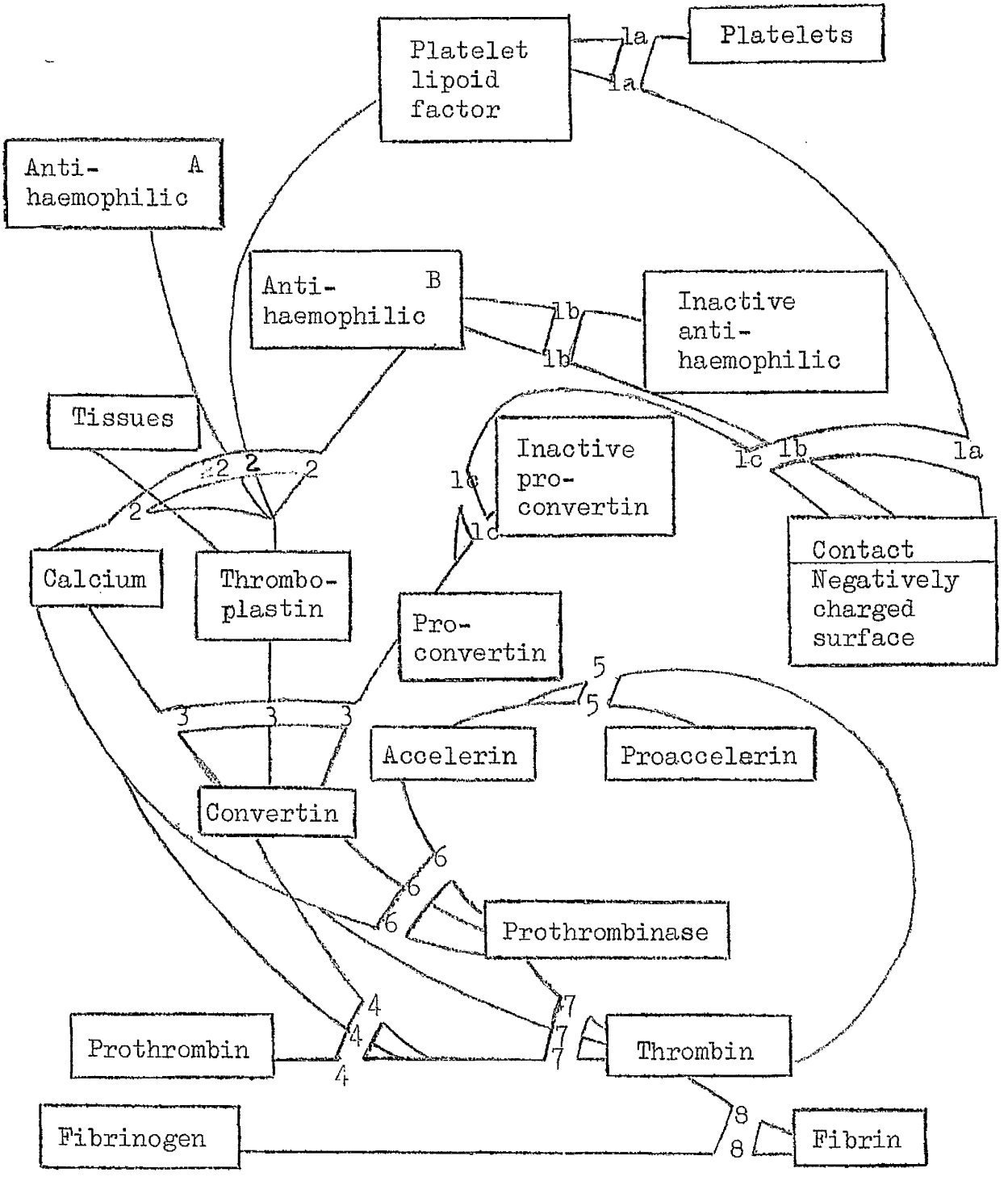
From 1905 until 1940, a great deal of work was carried out, theories introduced, conflicting evidence produced, but with little real progress. The literature concerning work during this period is extensive and, while of interest in the historical and evolutionary sense, is not being reviewed in this account. Concern is now centred on present-day concepts of the blood coagulation process.

Most investigators are agreed upon the main reactions and essential substances for coagulation, but opinions vary as to the links in the chain of reactions and as to where each substance contributes its effect. Owren[38]/

/Owren[38] has outlined his concept of blood clotting (see ovgr) and has given an account of the experiments performed with evidence obtained in support of it. Quick[39] describes his concept of the coagulation process in four stages, similar to Owren's theory except for the initial stages in which he describes a clotting factor erythrocytin, derived from the circulating erythrocytes on interaction with blood platelets.

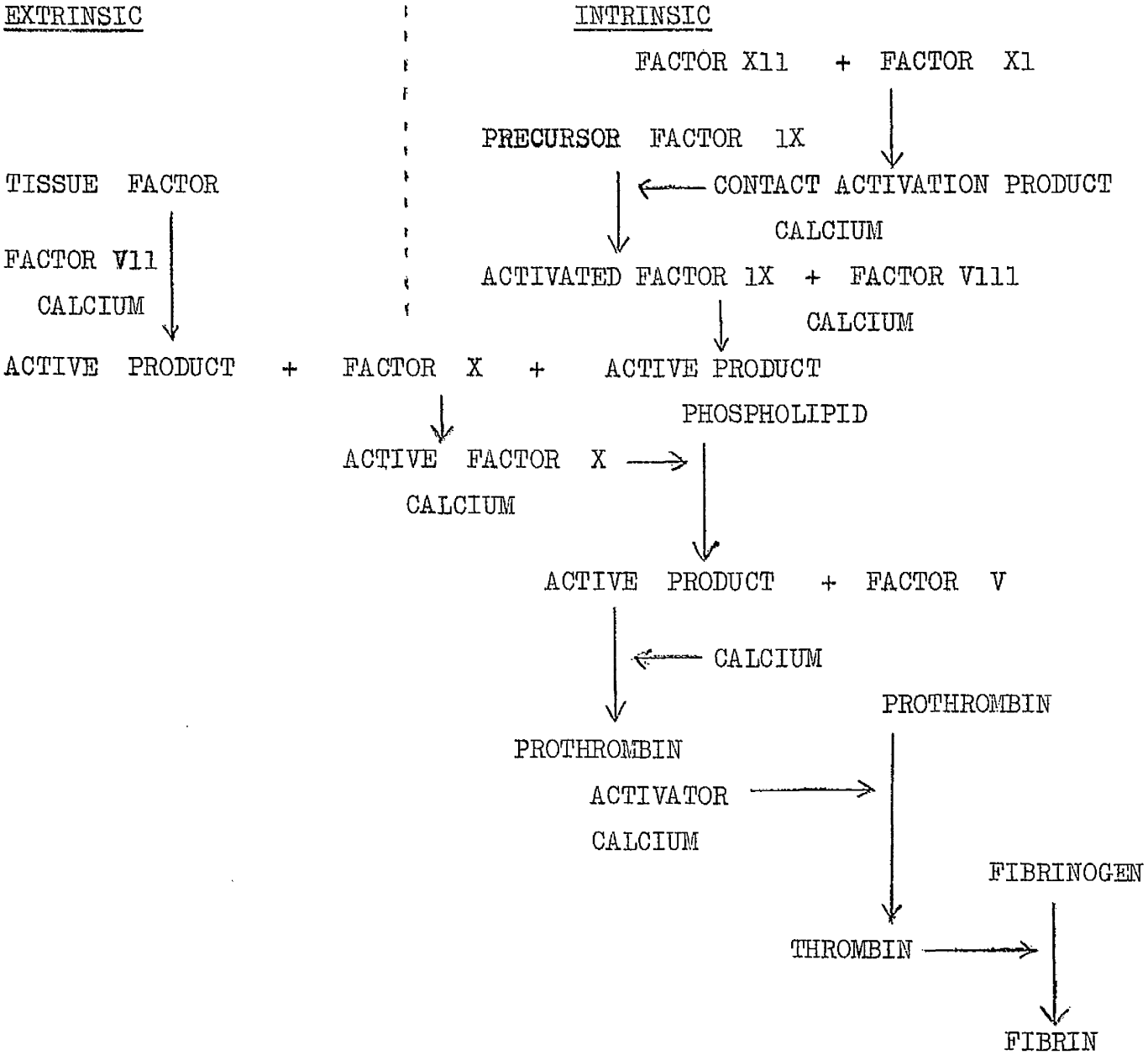


Blood Coagulation Theory Of Quick.



Blood Coagulation Theory Of Owren.

The hypothesis suggested by Biggs and Macfarlane[40], very clearly demonstrates possible extrinsic and intrinsic prothrombin activator chains of reactions. Factor X is the focal point of the process, for both systems, and it is suggested that the active product formed on each route, which acts with factor X, could possibly be the same substance.



BLOOD COAGULATION THEORY OF BIGGS AND MACFARLANE

Blood Coagulation Factors

Briefly, we may consider the properties, important for the understanding of the clotting tests, of the various factors involved, as far as present knowledge allows.

Fibrin. This is an insoluble protein produced in the coagulation of blood. A network of protein strands of fibrin is laid down and in the interstices of this network, large amounts of liquid may be held, though easily extruded on slight tension or pressure. The fibrin clot has considerable strength and is strongly adhesive to surfaces such as body tissues or glass. Its molecular weight is probably similar to that of fibrinogen though it is thought to have a shorter molecular length.

Fibrinogen. Fibrinogen is a globulin, normally present in the circulating plasma from which it can be readily precipitated by salt solutions e.g. 0.25 saturated ammonium sulphate. Estimates of its molecular weight agree at about 340,000 [41], and its shape is considered elongate and nodular. By the action of thrombin, fibrinogen is converted quantitatively into fibrin.

Thrombin. Thrombin is a specific proteolytic enzyme, produced in the process of coagulation and which acts upon fibrinogen, probably disrupting the arginyl-glycine bonds in its molecule. These so-formed "fibrin monomers" polymerise, in a reaction which is independent of thrombin, to form the protein strands of fibrin polymer. Thrombin is/

/is not normally present in plasma, but is formed from its precursor prothrombin.

Prothrombin. This is an α-globulin with a molecular weight of 68,000 and an elongate shape. It is synthesised in the liver, in the presence of vitamin k, a vitamin important also in the synthesis of factors VII and IX[42]. It appears that in the conversion of prothrombin to thrombin, the prothrombin molecule undergoes proteolysis, and derivatives, which show thrombic activity, have been isolated, of about half its molecular weight.

Prothrombin in plasma can be activated by the addition of tissue extract in the presence of factors V, VII, X and calcium. It may also be activated in the absence of tissue damage, by contact with a "foreign" surface, along with factors V, VIII, IX, X, platelets and calcium. The former is called the extrinsic system of activation while the latter, the intrinsic system.

Extrinsic System of Prothrombin Activation. Tissue extracts, usually prepared from brain, lung or placenta, exhibit the property of accelerating blood coagulation and as this was originally thought to be the sole accelerating effect on the prothrombin-thrombin conversion, in the presence of calcium, the term "thrombokinase", followed by thromboplastin, was applied to the active principle. Now, this term has been used both to denote the activity of tissue extract and of tissue extract in addition to various plasma factors. To avoid confusion/

/confusion, Straub and Duckert[43] have suggested the use of the term extrinsic prothrombin activator for the prothrombin converting system derived from plasma and tissue factors.

The active constituent of brain extract is considered as being a lipo-protein of large molecular weight which can be centrifuged at 10,000 r.p.m. There appears to be an optimal concentration of tissue extract indicating the presence of an inhibitor which has been extracted from brain tissue. Tissue extract and calcium alone may convert prothrombin to thrombin but the reaction is exceedingly slow in the absence of the factors V, VII and X.

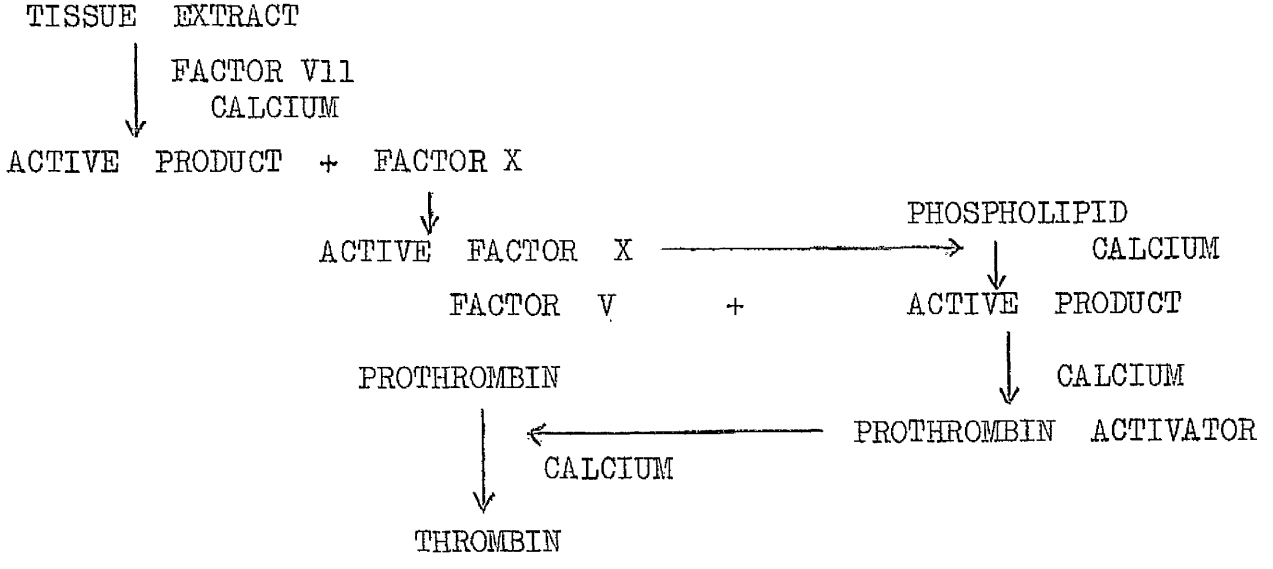
Factor V is a constituent of the globulin fraction of blood plasma but has not yet been isolated or prepared in a pure form. It is unstable, deteriorating rapidly on storage, is destroyed by heating and is not adsorbed, like prothrombin, by inorganic precipitants. Factor V is present in plasma but is reduced or absent in normal serum, showing that it is consumed during the clotting process. It has been shown that human blood contains a lower level of factor V than the blood from other mammals[44].

Factor VII is a β-globulin found in normal plasma and serum. It is difficult to separate from prothrombin since in most fractionation processes, they are adsorbed or precipitated together, but a chromatographic method has been developed which effects a separation. It acts, like factor V, as a prothrombin conversion accelerator, and is/

/is present in man and other mammals to a similar extent.

Factor X has been recognised more recently[45] since a patient suffering from lack of factor VII could provide this factor and so return the blood picture of a patient suffering from lack of factor X to normal. It has also been called the Stuart-Prower factor, named after the first patients to show its defect. It is an α-globulin, present in both plasma and serum, is adsorbed by alumina and similar adsorbents, is destroyed by heating but is stable on storage. Its concentration is reduced by liver disease, by dicoumarol or by vitamin K deficiency.

Experimental evidence[44] has indicated that tissue extract reacts initially with factor VII and calcium, then factor X and calcium. This resulting intermediate product could react with factor V to give a potent prothrombin activator.



Intrinsic System of Prothrombin Activation. It appears that normal plasma itself contains all the factors necessary to promote normal clotting without access to tissue extract from damaged cells. A number of clotting factors appear to be necessary. Factors V and X take part in the activation of prothrombin in the intrinsic system while factor VII plays no part.

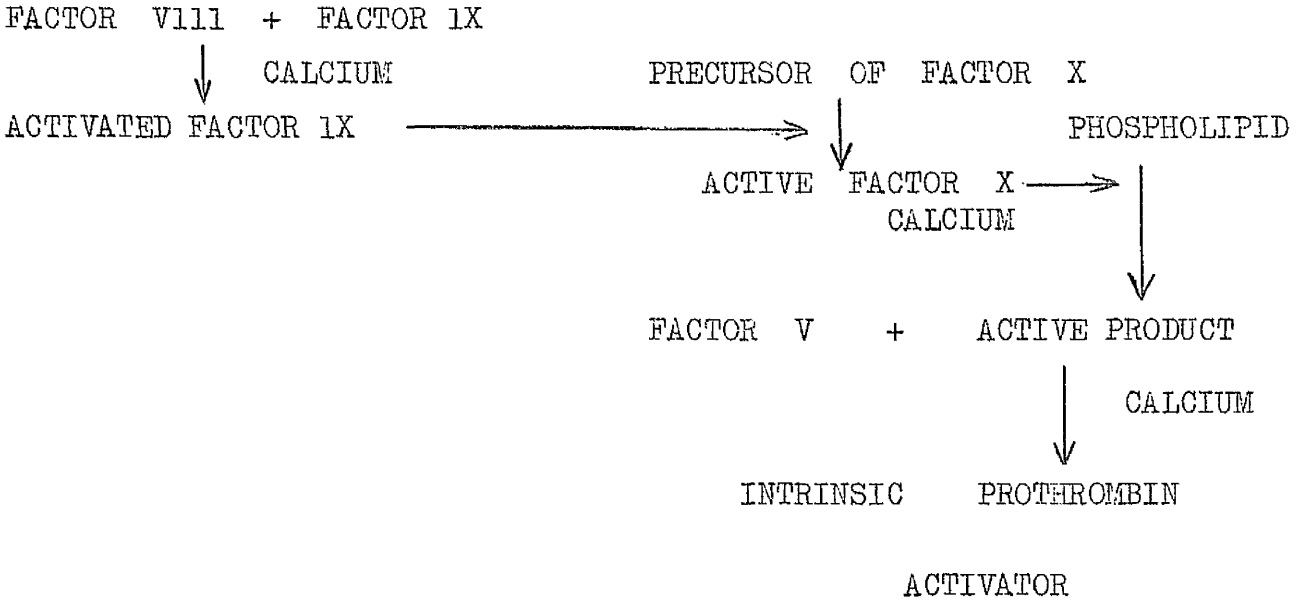
Factor VIII, or antihaemophilic globulin, is required for the normal clotting process and is the substance lacking in the blood of haemophilic patients. It is a β-globulin, separates with the fibrinogen fraction on salt precipitation, and its activity is lost rapidly after withdrawal of blood and storage. Since it is present in plasma but not in serum, it must therefore be consumed during clotting. Factor VIII has been shown to occur in the blood of the rabbit [44].

Factor IX is also commonly referred to as Christmas factor, the name of the first patient recognised as to be suffering from its defect. This haemorrhagic condition closely resembles haemophilia and it is only recently that the two have been differentiated. It is a β-globulin, easily destroyed by heat but relatively stable on storage. Serum appears to contain more factor IX than plasma and this has led to the conclusion that it exists in plasma in an inactive form but is activated on contact with a foreign surface.

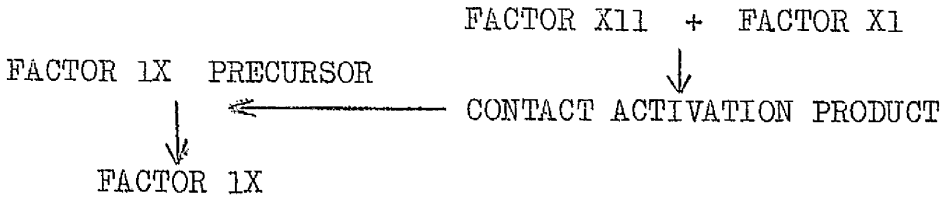
The platelets are thought to release, on contact with a foreign surface, a phospholipid or phospholipo-protein substance which takes part/

/part in the production of the prothrombin activator. Phospholipids from sources other than platelets e.g. a chloroform extract of brain tissue, can show similar activity and may be used as platelet substitute in the thromboplastin generation test[47]. The actual active substance has not yet been identified from any source.

The hypothesis suggested for the intrinsic activator system by Biggs and Macfarlane is as follows, though experimental observations are more confusing than conclusive.



The activation of factor IX has been a subject of interest and conjecture in recent years, with the establishment of two new factors, factor XII (the Hageman factor) and factor XI (plasma thromboplastin antecedent). Patients suffering from a deficiency of these factors show abnormal blood reactions in vitro but show only slight or no haemostatic defect. In some way the factors influence the reaction of the plasma on a glass surface, so their activity is not well-marked if silicone-coated apparatus is used. Their mode of action is not clear but they are thought to act together forming a contact activation product which is able to activate factor IX.



They may be associated with either the β or γ-globulin fraction of plasma proteins, are resistant to heat, but changes in clotting efficiency occur on storage.

Calcium. In 1890, Arthus and Pages[48] established that calcium is necessary for the formation of thrombin. Since the activity of calcium is lost on the addition of oxalate or citrate, it was concluded that ionised calcium was important for its accelerating action but in 1940, Quick[49] suggested that bound, rather than ionised, calcium was required. By/

/By adding sodium oxalate to blood, which would immediately remove ionic calcium and inhibit clotting at once, a slow loss of coagulability is observed. Quick believes that this slow action results from gradual removal of bound calcium, possibly from a clotting factor.

Quick[50] has given evidence which suggests that calcium acts stoichiometrically in the prothrombin-thrombin conversion. However, Lovelock and Porterfield[51] suggest that calcium is used in the coagulation process in the adsorbed state and that its chief function is to maintain the surface charge necessary for the interaction of various plasma colloids.

It is difficult to study the role of calcium in individual clotting reactions, since these cannot be carried out in pure solutions. The activation of factors XII and XI, followed by factors VII and IX can occur without calcium, but are enhanced in its presence. The reaction between thrombin and fibrinogen can also occur in the absence of calcium but, while an excess of calcium acts as an inhibitor, an optimal concentration is an accelerator. It is probable that calcium is necessary for the activation of prothrombin in both the extrinsic and intrinsic activator systems. A complex involving calcium in addition to other factors necessary for the activation of factor IX has been suggested[52].

However, the precise role of calcium, although necessary for several stages, and acting as an accelerator in others, remains obscure.

This account relates the present state of knowledge regarding blood/

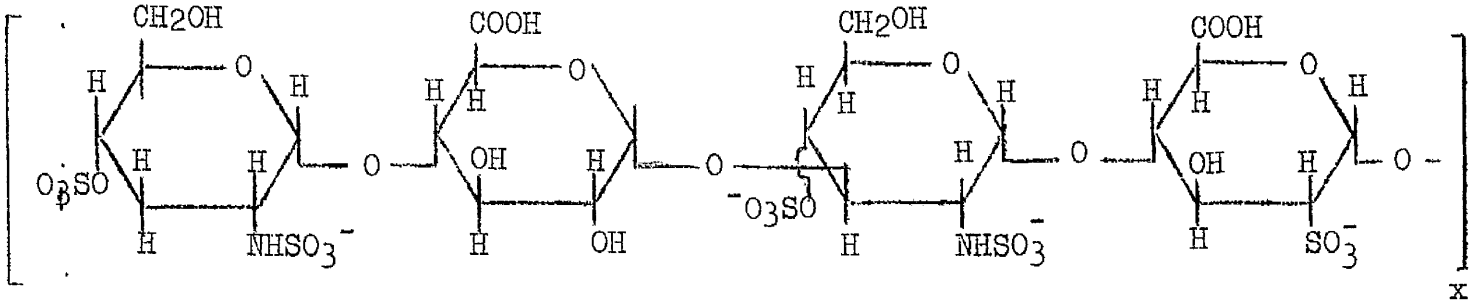
/blood coagulation and includes hypotheses which may be outdated in a few years time. In anticoagulant studies, our conclusions of the possible mode of action of drugs are founded on the results of clotting efficiency tests based on the above theory. These tests may in the future be proved faulty but at the moment they provide us with the most satisfactory methods of investigation available.

HEPARIN

Chemical and General Properties

In many anticoagulant studies, and in the consideration of possible new anticoagulants, heparin is taken as a quantitative and qualitative standard. It was the first therapeutically useful anticoagulant, is still used in most, acute thrombotic episodes, and is in fact regarded as a physiological, and therefore desirable anticoagulant substance. Heparin was discovered in 1916 by M^{c}Lean [53] while he was extracting cephalin thromboplastin from tissues. The work was continued by Howell and Holt [54], who described the action of the extract in retarding blood coagulation and, because it was first extracted from liver, in which it occurs in fairly high concentration, it was named heparin. The substance was soon identified as a polysaccharide but despite much chemical investigation, its constitution is still not known. It is believed to be a mucoitin sulphuric acid, consisting of equal parts of hexuronic acid and sulphated glucosamine units. The sulphur content, of considerable significance for its activity,/

/activity, is estimated, on average, to be 11·5%. The degree of polymerisation and branching which exists is not known but the molecular weight has been estimated at approximately 20,000.[55] A possible structure for the tetrasaccharide unit of heparin is shown below, though it is appreciated that heparin is probably not a single, well-defined chemical compound.



TETRASACCHARIDE UNIT OF HEPARIN

The chemical structure may be compared with those of the carrageenans shown on pages 2 and 3.

Heparin is a strong, naturally-occurring acid, a principal characteristic of the molecule being its electronegative charge, which is suggested[56] as being a possible basis for its anticoagulant action. The negative charge may influence the proteins concerned in blood clotting and in some way prevent their participation in the clotting reactions.

Heparin, effective both in vitro and in vivo by intravenous route, is non-toxic in therapeutic doses. It exerts its peak effect after about 15 minutes, this short latent period being possibly required to allow full combination or complexing with the plasma proteins./

/proteins.

Up to 50% of an injected dose of heparin can be recovered in the urine[57] in various species. The remainder is believed to be inactivated by the enzyme "heparinase", found in the liver, and by its action the effectiveness of heparin passes within a few hours. This enzymic activity appears to be non-specific and is possibly due to sulphatases known to be present in liver[58].

Correlation between chemical structure and biological activity of heparin has always been sought in an attempt to discover a synthetic substitute for clinical use because heparin is expensive to prepare, is difficult to purify in large quantity, must be given parenterally, has a short duration of action and is therefore not ideal for prolonged therapy. The importance of the sulphate groups was recognised when it was shown[59] that anticoagulant activity appeared to rise with increase in sulphate content. There followed many reports[56,59] of work on polysaccharides, sulphated to varying extents and with varying N-sulphate: O-sulphate ratios but a definite conclusion regarding the structures essential for the activity of heparin cannot yet be reached. The sulphamino group, unique in biological substances, electronegativity and suggestions such as S-bridges, all appear to be involved. Furthermore, when heparins from different species are compared[60], the correspondence between activity and sulphur content is less clear, due probably to variations in other molecular characteristics.

Mode of Anticoagulant Action./

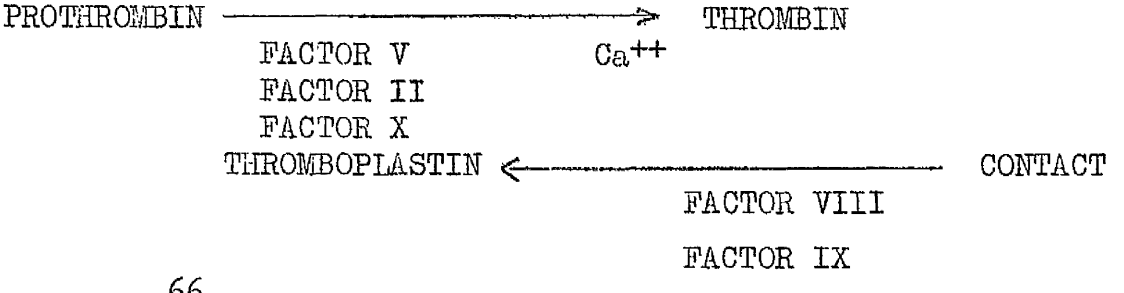
/Mode of Anticoagulant Action.

Although the antithrombic activity of heparin had been accepted for some time, increasing attention has more recently been focussed on the way in which it interferes with earlier stages of blood coagulation, and the apparent inconsistencies in the literature regarding its action probably arise, at least in part, from the numerous variables, not always readily controlled, in experimental procedure. In addition to this, different concentrations of heparin are now believed to affect the various stages in the clotting sequence to predominantly different extents.

The idea that heparin might affect the early stages of clotting is not new and was introduced by Howell[61] in 1917, his experiments suggesting that heparin acted as an antiprothrombin. However, Quick[62] supposed that heparin did not influence the action of thromboplastin and concluded that its activity could be wholly explained by an antithrombic action, but in the following year, Brinkhous et al.[63] appeared to contradict this conclusion with their work which suggested antiprothrombin or antithromboplastin activity.

The precise action of heparin still remains obscure. It has been shown[64] that when purified thrombin was allowed to interact with prepared fibrinogen, in the absence of plasma, no clotting occurred in the presence of heparin. It appears that heparin requires the presence of a co-factor, belonging to the albumin fraction of plasma, to exert its action. This co-factor, as yet, has escaped isolation and identification./

/identification. It has been suggested[65] that in vivo, heparin is complexed with a lipo-protein and that during the extraction process, the two components become separated, but heparin co-factor is not believed[87] to be identical to naturally-occurring plasma antithrombin. The anti-thrombin action of heparin is thought to be two-fold: firstly to promote the adsorption of thrombin by fibrin, and secondly, to prevent the enzymic action of thrombin on fibrinogen. It appears that this antithrombic action is more pronounced with the larger doses of heparin than the smaller amounts, and is probably a relatively unimportant factor with small, or physiological concentrations of heparin. More important with smaller doses, are the effects of heparin on the earlier stages. Although the formation of thrombin is certainly delayed there is increasing evidence to suggest that several factors are affected in this conversion.



Douglas[66] has shown that in the presence of heparin, factors V and VII are not utilised and, coupled with defective consumption of prothrombin, he concluded interference in the prothrombin to thrombin conversion. O'Brien suggested[67] that heparin combines with, and therefore inactivates, factor IX, thereby delaying the formation of thromboplastin./

/thromboplastin. Normally, heparin is bound to plasma proteins and shows preferential affinity in the following order:- affinity for /3 lipoprotein < thrombin clotting system < factor IX < platelet protein < protamine sulphate. Thus, when bound with/3 -lipoprotein, heparin is still available for delaying the thrombin clotting system. Bound to factor IX, it cannot influence the thrombin system and, on the addition of platelet protein, factor IX has been liberated[67]. The affinity of heparin for protamine sulphate is well-established, and this property is employed clinically for neutralising the action of heparin. The action is due to the presence of free basic groups in the protein molecule which combine with the acidic groups of heparin. It is appreciated, however, that the basic substance shows, in itself, anticoagulant activity and if present in excess of the amount required to neutralise heparin, there may follow an increase in coagulation time rather than the desired reduction.

M^{c}Millan and Brown[68] report that although normal serum does not contain factor VIII or factor V, they are present in the serum of patients treated with heparin, so indicating faulty thromboplastin generation. In the thromboplastin generation test, heparin delayed the formation, and decreased the amount, of thromboplastin produced. Large amounts of heparin actually destroyed preformed thromboplastin. Their results indicate that in small doses, the major effect of heparin is on the formation of thromboplastin and this would explain why the one-stage/

/one-stage prothrombin test is either unaffected or very slightly lengthened in the presence of heparin, the test itself providing a thromboplastic source.

It has now been shown that heparin is physiologically present in the circulating plasma[69] and it probably exerts a prophylactic action to prevent spontaneous thrombosis by inhibiting thromboplastin generation.

AIMS OF THE PRESENT STUDY

The present study was undertaken because of the difficulty in deciding whether the various reports regarding the anticoagulant activity of carrageenans from red algae referred to the same or even comparable substances. A number of purified carrageenans were therefore prepared from different species of seaweeds from different habitats. It was considered of interest to establish whether or not these preparations showed anticoagulant activity of the same order as the few carrageenans already studied, and to investigate and compare the activities of k and λ fractions from samples obtained from different sources of Chondrus crispus (5 samples), Gigartina (2 species), in addition to the carrageenan from Polyides rotundus, two degraded carrageenans, and one highly purified λ carrageenan from Chondrus crispus.

As shown in TABLE A, the k and λ fractions differ in sulphate, 3,6-anhydrogalactose, optical rotation and in inherent viscosity. The structure of pure λ carrageenan is thought not to contain 3,6-anhydrogalactose but fractionation of all seaweed extracts does not always give/

/give a sharp separation into the k and λ components, the λ component often containing up to 10% 3,6-anhydrogalactose. The anticoagulant activity and general toxicity, in rabbits, of the k and λ fractions have been compared.

From these preliminary tests, four k and λ fractions from different sources of C. crispus were selected for further investigation. The carrageenan from Polyides and the degraded carrageenan from Eucheuma spinosum were also selected for further study. Effects on blood clotting were studied in vivo, using rabbits as the experimental animal, in an attempt to gain some knowledge as to whether the various λ's and k's had similar activity and whether they interfered with the clotting process at the same stages. Tests were chosen which would demonstrate abnormalities both in the early stages of clotting and in the later stages. For comparison, the same tests were carried out using heparin as the anticoagulant, in doses which gave equivalent whole blood clotting time results.

Some in vitro tests were carried out to investigate the effects of carrageenans on blood platelets and on fibrinogen since interaction with these seemed possible causes of the observed acute toxicity of many carrageenans.

For the main part of the work, two methods of approach were considered. Several tests of clotting function, and a larger number of substances could be examined using a relatively small number of animals; or a larger number of animals could have been/

/employed but fewer carrageenans would have been tested. The former approach was chosen, taking results from groups of four animals. The consistency of these results have indicated a trend in the action of the various carrageenans and would allow subsequent construction of statistically designed experiments if quantitative differences were to be sought.

E X P E R I M E N T A L

PART 1.

PRELIMINARY EXPERIMENTS TO DETERMINE SUITABLE DOSAGE.

In order to determine a dosage level suitable for subsequent administration in coagulation studies, whole blood coagulation tests and toxicity observations were carried out in rabbits, using a number of carrageenans. The preparations investigated are listed in TABLE A.

Each substance was dissolved in 0·1M sodium chloride to give a solution of 2·5mg./ml. Many of the carrageenans were slowly soluble and were therefore allowed to hydrate for up to 18 hours at 4°C after which dissolution had occurred or was effected rapidly with shaking at room temperature. Solutions were injected intravenously into rabbits, at the following dose levels (mg./kg) : 1,3,5 and 10.

The rabbits used were of mixed breed, male and female, and weighed between 1·75 and 3·75 kg. For each substance injected, two rabbits received the 1mg. dose, two rabbits the 3mg. dose, one rabbit the 5mg. dose and one rabbit the 10mg. dose.

The whole blood coagulation time was performed according to the method of Lee and White[70]. The fur growing over the marginal vein of the ear was removed by means of a sharp scalpel blade, and slight vasodilatation was produced by warming the carbon tetrachloride-cleaned ear with a lamp for a few minutes. A 3-5mm. incision was made in the vein with a new Hagedorn needle and the blood, freely-flowing,/

/freely-flowing, was collected in tubes held under the ear. The first tube (1ml.) was discarded to minimise tissue fluid contamination, and further 1ml. volumes were collected in each of four tubes, starting a stop-watch as the blood first flowed into each tube. The tubes were then placed in a water bath maintained at 37°C and each tube was tilted at half minute intervals until it could be completely inverted without dislodging the clot. The mean clotting time for the four tubes was recorded.

In the rabbit, the venous pressure in the ear is insufficient to make possible the convenient use of the syringe for repeated blood collection; and while blood could be obtained once in this way from the central artery, the resulting damage and general ischaemia in the ear made subsequent sampling impracticable.

Initially, glass tubes 10 x 75mm. were coated with a 5% solution of silicone fluid MS 1107 in carbon tetrachloride, and baked at 150°C for one hour. This increased the normal coagulation time from an average of 5 minutes to 12-20 minutes and so small differences in coagulation time might then have become more obvious. It was found, however, that the range of times within the four tube samples was very much wider by this method and more consistent results were obtained with the uncoated glass tubes. The technique finally used was fully standardised and gave reproducible results, variation in times within the four tubes being in the range ± 1 of the mean.

In/

/In the preliminary tests, coagulation times were estimated prior to the injection but due to the uniformity of the results and to avoid excessive blood loss with repeated bleedings, a normal coagulation time of 5-6 minutes was assumed; any rabbit having an abnormal coagulation time could be detected by failure of the clotting time to return to within normal limits at the end of the test. In results from 100 rabbits, the mean coagulation time was found to be 5·4 minutes with a standard deviation of 0·9.

Preliminary experiments showed that maximum anticoagulant effect occurred 2 hours after injection (see TABLE 3) and that after 5 hours the coagulation time was returning to normal. For comparative purposes the results are tabulated as coagulation times, 2 hours after injection. (Results are in TABLE 1.)

At the higher dose levels, most of the substances were acutely toxic to the rabbits, causing death between 5 and 24 hours after the injection. TABLE 2 gives an indication of the corresponding doses of carrageenan which proved lethal to the rabbits within 24 hours.

Even with the lower doses, difficulty in obtaining blood was often experienced as the peripheral blood vessels, despite warming, remained flaccid with slow blood flow. After the injection, the rabbits often appeared listless and dyspnoeic with nasal discharge. The blood pressure appeared low, satisfactory bleeding was difficult to obtain and in some cases, diarrhoea, and more often convulsions, preceded death. Post-mortem,/

/Post-mortem, the heart consistently appeared normal, but the kidneys and lungs were frequently congested.

To ensure that the results were not influenced by the withdrawal and loss of blood, coagulation times were carried out on control rabbits, bleeding on four occasions at two-hourly intervals. No difficulty in obtaining blood was experienced and the coagulation times were consistent. It therefore appeared that the loss of tone and low blood pressure, so often causing difficulty in obtaining blood samples, was due to carrageenan.

PART 2.

THE ANTICOAGULANT ACTION OF VARIOUS CARRAGEENANS.

The following substances were studied:-

	Code	Dose
k- and λ-carrageenan from Chondrus crispus	CNS	3mg./kg. and 5mg./kg.
k- and λ-carrageenan from Chondrus crispus	CMI	3mg./kg. and 5mg./kg
k- and λ-carrageenan from Chondrus crispus	CSE	3mg./kg. and 5mg./kg.
k- and λ-carrageenan from Chondrus crispus	CY	3mg./kg and 5mg./kg.
carrageenan from Polyides rotundus		3mg./kg.
degraded carrageenan from Eucheuma spinosum		200mg./kg.
Heparin Sodium		75i.u./kg. and 200i.u./kg

Because of its acute toxicity λ-CNS at the 5mg./kg. dose level had to be omitted from the study.

Four rabbits were used at each dose level of each substance. Male, albino, rabbits were chosen, weighing between 2·0 kg. and 3·75kg., no rabbit being used on more than one occasion. Food was withheld for 18 hours prior to the tests being carried out, but water was allowed ad lib.

Blood was collected from the marginal vein of the ear by the method previously described (Part 1.), 4 x 1ml. volumes being collected for the whole blood coagulation test. 2ml. of blood were added to a centrifuge tube containing 0·2ml. of 0·85% sodium chloride solution for the thrombin generation test, and 6·3ml. of blood added to another centrifuge tube containing/

/containing 0·7ml. of a 3·13% solution of trisodium citrate, mixed thoroughly to avoid clotting and the citrated plasma was separated by centrifuging for 10 minutes at 1400 rpm.

A total of eight tests of clotting function were carried out on the blood withdrawn before administration of the carrageenan solution. The rabbit was then given on intravenous injection of the carrageenan, the dose being calculated on the basis of body weight. The volume was made up to 8ml. with saline to avoid injecting very viscous solutions and to standardise the injection procedure. Two hours after the injection, the rabbit was bled from the marginal vein of the opposite ear. Again, the eight tests of clotting function were carried out. Thus, in each case, a comparison of the results of the tests before and after the injection could be made directly, on the same rabbit.

Occasionally, due to the action of the carrageenan, blood flow in the ear was reduced and it was difficult to obtain the required volume of blood sufficiently free-flowing. In these cases, an injection of Nembutal Sod., 15mg./kg. body weight was given. This produced peripheral vasodilatation and the blood flow became satisfactory for rapid collection. To ascertain whether the Nembutal itself would interfere with the blood clotting tests, the tests were carried out on four rabbits prior to, and immediately following, an injection of 25mg./kg. body weight of Nembutal.

Results are shown in the appropriate tables.

PREPARATION OF REAGENTS

SODIUM CITRATE SOLUTION.

A solution containing 3·13% trisodium citrate was prepared in freshly distilled water. 1 oz. bottles containing approximately 10ml. of this solution were sterilised by autoclaving, and one bottle was used on each day of the tests. Without sterilisation, the solution may rapidly become contaminated by moulds and bacterial growth.

CALCIUM CHLORIDE SOLUTION.

A 4% solution of hydrous calcium chloride was prepared in freshly distilled water and standardised by titration with sodium ethylene diamine tetraacetate using solochrome black as indicator. This solution was diluted appropriately to give M/40 and M/20 solutions for use in the tests. Fresh solutions were prepared each week, to ensure consistent, optimal activity.

SODIUM CHLORIDE SOLUTION.

A 0.85% solution of sodium chloride was prepared. Where saline is referred to in this thesis, this solution was used.

OWREN'S VERONAL BUFFER.[71]

1·175G of sodium diethylbarbiturate and 1·467G of sodium chloride were dissolved in a mixture of 157ml. distilled water and 43ml. of 0.1N HCL. The pH of the final solution was checked or adjusted to 7·35.

THROMBIN SOLUTION.

Maw's/

/Maw's Thrombin Topical was used. It was supplied as a cream-coloured freeze-dried preparation, each ampoule containing 50 units of thrombin. This was dissolved in saline to give a solution of 20 units/ml. for use in the tests. A fresh solution was prepared on alternate days as, in solution, the clotting activity decreases rapidly.

BRAIN THROMBOPLASTIN.

Fresh rabbit brain was chosen as the source of thromboplastin since it is more easily freed from blood than is lung tissue, and, due to this, the resulting preparation has been found to have a lower Factor V11 content.[72] The method of extraction was developed from that described by Owren[73].

Acetone-dried brain (thromboplastin source).

The brains were macerated with acetone in a glass mortar and maintained at a temperature of 4°C for 48 hours. During this time, the acetone was renewed on several occasions, with mixing and grinding of the tissue before and after each change. The brain tissue, in coarse powder form with some fibrous tissue, was transferred to a MSE tissue homogeniser, with fresh acetone, and ground for 5 minutes. This finer powder was filtered through a suction filter and dried at 37°C for 30 minutes.

A 3-4 weeks' supply of acetone-dried thromboplastin was prepared at a time, and stored at 4°C. It was found that, contrary to ... reports[36,74], this thromboplastin preparation did not maintain its full potency/

/potency for longer periods, even when stored in sealed ampoules.

Saline suspension of acetone-dried brain.

For use each day, 0·5G of the powder was weighed into a glass tube, 10ml. of 0·85% saline added, and the suspension incubated at 37°C for 15 minutes, during which occasional mixing ensured dispersion of the powder in the saline. The supernatant which formed on standing was the thromboplastin preparation used in the tests.

Preliminary extraction experiments.

Tests were carried out using various methods of extraction. Frozen brain, stored for one month at -20°C before the extraction process was prepared as above but the resulting suspension was less satisfactory than that from fresh brain material, as indicated by activity measurements.

In some cases, the dried material was mixed with saline and after thorough mixing, the suspension was stored frozen at -20°C until required for use when it was reconstituted as before by incubating at 37°C for 15 minutes. No advantage over the standard method was gained by this method.

When the thromboplastin suspension was centrifuged and the supernatant milky fluid removed for use, it was found to be slightly less active than the standard preparation which contained small particles in suspension.

Grinding in a small agate mortar produced a finely divided, even suspension/

/suspension but its clotting activity varied throughout the day and results of the one-stage prothrombin test using it were inconsistent.

These methods were therefore discarded in favour of the one previously described which gave results satisfactory both in activity and in consistency throughout the day.

PLATELET SUBSTITUTE.

For use in the thromboplastin generation test.

This preparation was used as a substitute for platelet suspension and was prepared according to the method described by Bell and Alton[47] and used by Hicks and Pitney[75]. Acetone-dried, brain thromboplastin powder, as prepared above, was tested for absence of cholesterol as judged by a negative Liebermann-Burchardt reaction[96]. 1G. of the powder was extracted at room temperature for 2 hours with 50ml chloroform. The mixture was filtered, the filtrate evaporated and the gummy residue, scraped from the evaporating dish, was homogenised (MSE tissue) in 50ml. of saline for 10 minutes to give the final suspension.

Dilutions of 1 in 50, 1 in 100 and 1 in 200 were made of this suspension in saline and tested by the screening test of Hicks and Pitney[75], to select the dilution which gave the shortest clotting times of substrate plasma. In this test, plasma was diluted with Owren's veronal buffer in the proportion of one part of plasma to nine parts of buffer. 0·5ml. of diluted plasma and 0·5ml. of the platelet substitute were mixed together in a test-tube in a water bath at 37°C. 0·5 ml./

/0·5 ml. of M/40 calcium chloride solution at 37°C was added rapidly and a stop-watch started as the solutions mixed. At 1 minute intervals, successive 0·1 ml. samples were removed from the mixture and added, together with 0·1 ml. of M/40 calcium chloride solution, into one of a series of tubes containing 0·1 ml. of substrate plasma previously placed in the water-bath. The clotting times of the substrate plasma were recorded. The results of the screening test are shown in TABLE 4 and from these, a dilution of 1 in 100 was selected for use in the thromboplastin generation test.

The concentrated suspension was kept frozen at -20 C and each day, it was thawed to be diluted for use. Bell and Alton[47] report that the concentrated preparation retains its potency for 12 months, stored at -20 C and being repeatedly frozen and thawed. It was found, however, that after 3 months, the activity suddenly decreased and it was necessary to prepare a further batch. When the second preparation was screened, similar clotting times to those of the first batch were obtained with a 1 in 20 dilution. (see TABLE 4), which was then used.

FIBRINOGEN.

Fibrinogen was prepared according to the method described by Biggs and Macfarlane[76].

Preparation of Alumina Suspension[77].

100 ml. of ammonia solution (Sp. Gr. 0.88 diluted 1 in 2) was poured into 600 ml. of water at 63°C containing 22G. of ammonium sulphate/

/sulphate and the temperature brought to 58°C. The mixture was stirred and poured, in one lot, into a solution of 76·7G. of ammonium alum at 58°C in 1 litre of distilled water. The mixture was stirred for 10 minutes maintaining the temperature at 58°C and the precipitate was separated by centrifuging. The precipitate was washed five times with 1·5 litres of distilled water, the precipitate being separated by centrifuging on each occasion. To the first washing water, 0·22 ml. of Sp. Gr. 0·88 ammonia was added and to the second, 0·44 ml. The precipitate was suspended in water, making up a total volume of 700 mls. to give a gelatinous suspension which could be pipetted.

Preparation of Phosphate Buffer.

A 2M phosphate buffer at pH 6·6 was prepared by dissolving 817G. of anhydrous potassium dihydrogen phosphate in 1 litre of distilled water, to which had been added 750 ml. of 4N potassium hydroxide. After dissolving by heating, the volume was made up to 3 litres and the solution was filtered. M and M/4 solutions were prepared from this stock solution.

Fibrinogen.

Fresh bovine blood was collected from the slaughter house in bottles containing 5% potassium oxalate solution in the proportion one part of oxalate to nine parts of blood. The blood was centrifuged and the oxalated plasma separated.

To 1 litre of plasma, 50 ml. of aluminium hydroxide suspension was added and the mixture stirred at room temperature for 15 minutes.

The/

/The mixture was centrifuged and the sediment discarded. The supernatant plasma was tested by the one-stage prothrombin time method, and the clotting time was found to be greater than 1 minute, so ensuring a low prothrombin content. The plasma was then cooled to 12-14°C and an equal volume of 2M phosphate buffer at 2-4°C was added. The mixture was allowed to stand for 15 minutes to allow the formation of a precipitate. The mixture was centrifuged for 10 minutes at 2000 rpm. and the supernatant discarded. The precipitate was washed with 1 litre of M phosphate buffer, the precipitate recovered by centrifuging and then dissolved in 500 ml. of M/4 phosphate buffer. The volume was measured and the fibrinogen reprecipitated by adding an equal volume of 2M phosphate buffer. This precipitate was sedimented by centrifuging, and washed with 500 ml. of M phosphate buffer. Precipitation and washing were repeated once more and the final precipitate dissolved in 100 - 150 ml. of citrate saline (nine parts of saline and one part of 3·8% trisodium citrate). The solution was dialysed in the cold overnight against 5 litres of citrate saline, the dialysing fluid being stirred during this time. The product was centrifuged to remove any precipitate which may have formed and freeze dried. Drying was completed in a vacuum desiccator over phosphorus pentoxide.

For use in the tests, a solution containing 1mg./ml. of fibrinogen in saline was used. This concentration was found by considerable trial and error, using thrombin solutions, and in the prothrombin consumption/

/consumption test (see later), to give minimum clotting times and yet to produce a solid clot rather than wispy flakes of fibrin.

The dried fibrinogen product was kept at 4°C and maintained its potency for at least six weeks, a fresh solution being prepared each day. The first batch was used for eight weeks but between nine and twelve weeks, the activity gradually decreased and occasionally, when the solution was constituted for the day, a wispy precipitate appeared spontaneously two to three hours after preparation of the solution. Results during this period were discarded and the fibrinogen was consequently prepared from fresh blood at intervals of six weeks.

HEPARIN

Heparin Sodium, Evans (150 units per mg.) was used to prepare a saline solution containing 100 units/ml.

PENTOBARBITONE.

Nembutal solution (Abbott) was used intravenously; 1 ml. of this solution contains 60 mg. of Pentobarbitone Sodium.

GLASSWARE.

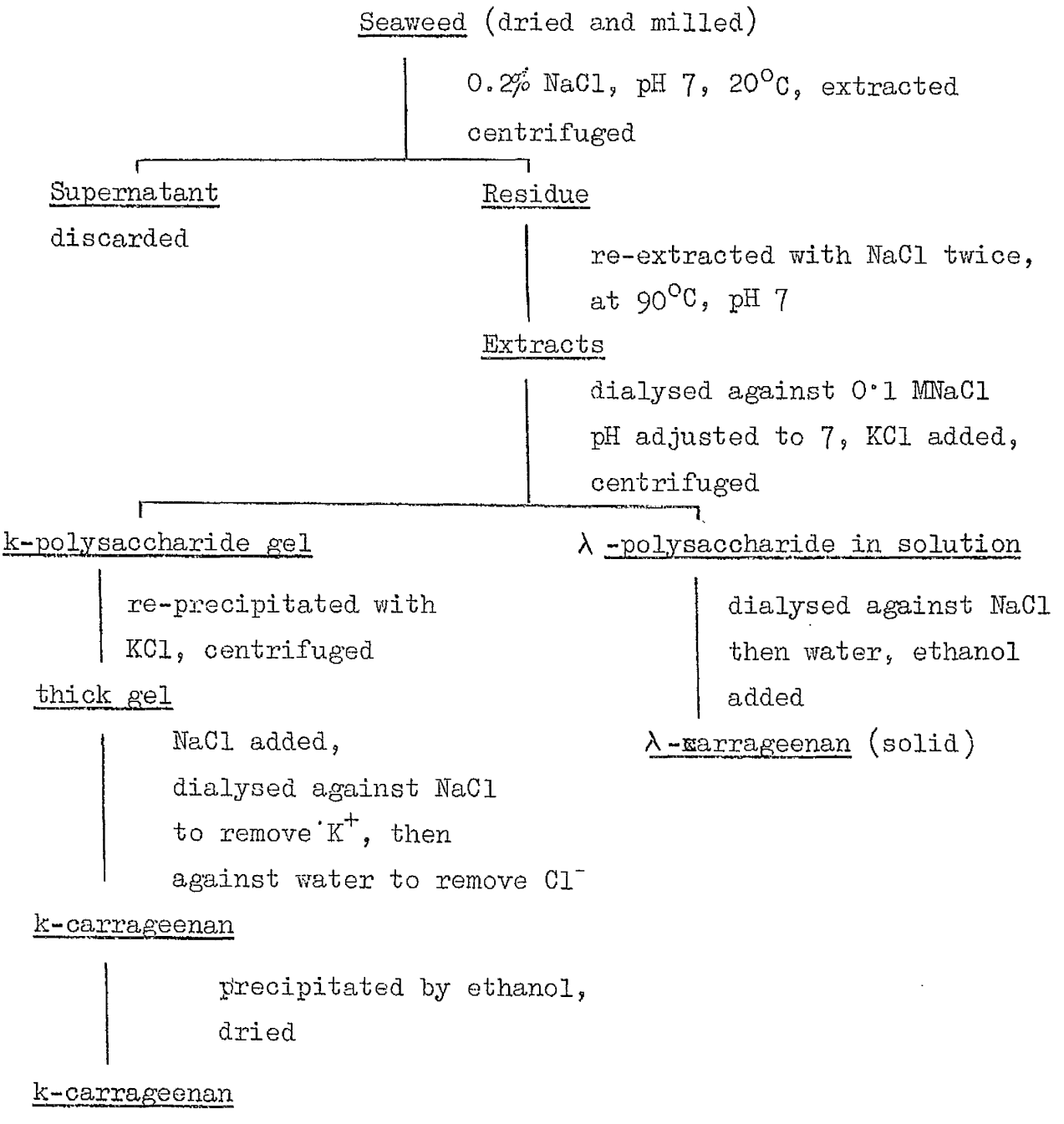
Pyrex glass tubes, 10 x 75 mm. were used in all tests. Pyrex, graduated centrifuge tubes of 10 ml. capacity were used for the collection of blood. All glassware was cleaned by removal of the clots, scrubbed in warm water with a small quantity of detergent, thoroughly rinsed several times in warm water and finally in cold distilled water. The tubes were then dried in the hot air oven.

PREPARATION OF CARRAGEENANS.

Chondrus carrageenans.

Method of Extraction and Fractionation.

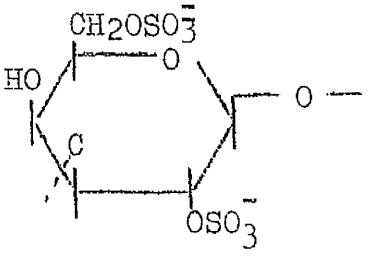
The extraction procedure was carried out by Dewar et al.[12] using a method modified from that described by Smith[2]. The principal stages in the process may be followed from the scheme outlined below.



Polyides rotundus.

The extraction process was similar to the above but no fractionation was possible since no gelling occurs on the addition of potassium chloride. The sodium salt of the carrageenan was prepared by dialysis against sodium chloride, followed by precipitation with ethanol.

The seaweed gave a low yield of a low viscosity carrageenan. Experimental evidence suggests that the carrageenan is similar to that of a λ-component from Chondrus crispus, with a high sulphate, and a low 3,6-anhydrogalactose content. It is believed[12] that the 1,4-linked D-galactose 2,6-disulphate radicle present in λ-carrageenan is not the major structural unit of Polyides carrageenan but may be replaced by a 1,3-linked D-galactopyranose unit.



Carrageenan from Eucheuma spinosum.

Eucheuma was extracted[12] similarly to Chondrus but again, no fractionation was possible by potassium precipitation. When potassium chloride was added, to a concentration of 0·25M, the solution thickened considerably but centrifugation produced no separation of the gel, even when the concentration of potassium chloride was increased. Experimental evidence suggests that Eucheuma carrageenan contains no

λ-component/

/λ-component comparable to that of Chondrus. The polysaccharide is similar in structure to k-carrageenan but has a much higher sulphate content. This seaweed gave a high yield of the polysaccharide, of low viscosity.

For testing in experimental animals, a degraded product of the carrageenan was prepared[12], of low viscosity yet with a sulphate content similar to that of the undegraded substance.

Degraded λ-carrageenan

Pure λ-carrageenan and Polyides carrageenan are difficult to degrade satisfactorily by acid, presumably because of the absence of the acid labile 3,6-anhydro link in the polymer chain and the drastic conditions which would therefore be necessary would almost certainly remove a significant proportion of the sulphate ester groupings.

A method was developed[12] for degrading λ-type carrageenans satisfactorily by periodate oxidation, and was accomplished for the λ-fraction of C. crispus (CNS). The viscosity was thus reduced from 21·7 to 1·3 dl/g.

Rees' pure λ-carrageenan.

Rees [78,79] has extracted and purified λ-carrageenan from Chondrus crispus obtained from the coast of Maine, USA. Its characteristics are shown in TABLE A.

It has been observed that the yield of carrageenan varied from different samples, the results being indicative of seasonal variation. With/

/With C. crispus (CNS), lower carrageenan yields were obtained in the autumn than in the summer samples. Also, the k/λ ratio varied widely in different samples and with the season of the year in which the seaweed was harvested. It is suggested that the k-polysaccharide accumulates in the plant throughout the winter months.

The seaweeds investigated, their sources, time of harvesting, sulphate content, 3,6-anhydrogalactose content, galactose content, k/λ ratio, optical rotation and inherent viscosity* are shown in TABLE A.

TESTS OF CLOTTING FUNCTION.

1. Whole Blood Coagulation Time.

This test was carried out according to the method of Lee and White[70], already described. The results are tabulated in TABLE 5.

2. Thrombin Generation Test.

Macfarlane and Biggs[81], in 1953, described the method which was used. A row of test-tubes containing 0·4 ml. of fibrinogen solution was set up in the water bath. 2 ml. of blood was collected in a glass centrifuge tube to which had been added 0·2 ml. of 0·85% saline solution mixed, and the tube held in the water bath at 37°C. At one minute intervals from collection of the blood, 0·1 ml. of the blood was removed and added to a tube containing the fibrinogen solution, in each case the clotting times of the blood-fibrinogen mixtures being recorded. As sampling continued, a fibrin clot appeared in the blood and was held to/

* Viscosity is considered a relative indication of molecular weight of the carrageenan.

/to one side of the centrifuge tube by a wooden swab stick while fluid samples continued to be taken. Sampling was continued until either the clotting time exceeded 3 minutes or until no blood remained, whichever occurred first. From the clotting times of each tube, the concentration of thrombin in the blood at the time of sampling was obtained from the thrombin-dilution curve (see below). By plotting thrombin concentration against time, a curve showing thrombin generation and destruction in the blood was constructed. The graphs for the blood of each rabbit, comparing normal with abnormal, are shown in APPENDIX I.

Thrombin - Fibrinogen Dilution Curve.

Maw's Thrombin Topical was diluted with saline to give solutions containing 20, 15, 10, 8, 5, 4, 3, 2, 1 and 0·5 units of thrombin per ml. A series of test-tubes containing 0·4 ml. of fibrinogen solution were placed in the water-bath at 37°C and to these 0·1 ml. of the thrombin solutions were added, the clotting time in each case being recorded. For each thrombin solution prepared, duplicate additions to fibrinogen were made and an average clotting time obtained. The clotting times were plotted against thrombin concentration and a curve obtained, as shown in APPENDIX I. This curve was used in converting clotting times to thrombin units in the thrombin generation test and also in the two-stage prothrombin test. While these thrombin units have no absolute significance, they are useful in comparing amounts of thrombin formed in these two tests.

3. Thrombin Time

The method used was that described by Hawkins and Leonard[32]. 0·2 ml. of citrated plasma was mixed with 0·1 ml. of 0·85% saline in a test-tube maintained at 37°C in the water bath. 0·1 ml. of thrombin solution, containing 20 units/ml., was added and the tube tilted frequently until a firm clot appeared. The time from the addition of the thrombin solution until the clot formed was recorded, and an average of three determinations calculated. A thrombin index has been calculated for the blood of each rabbit as follows:-

$$\text{THROMBIN INDEX} = \frac{\text{NORMAL CLOTTING TIME}}{\text{ABNORMAL CLOTTING TIME}} \times 100$$

and the results shown in TABLE 6.

4. Recalcification Time[80].

To a tube in the water bath at 37°C, 0·1 ml. of citrated plasma and 0·1 ml. of saline were added and mixed. 0·1 ml. of M/40 calcium chloride solution was added and a stop-watch started simultaneously. The mixture was examined by gentle tilting at frequent intervals until a firm clot appeared. The time taken for coagulation to occur was recorded. This test was carried out in triplicate and the average clotting time calculated. For each rabbit, a recalcification index has been calculated as follows:-

$$\text{RECALCIFICATION INDEX} = \frac{\text{NORMAL CLOTTING TIME}}{\text{ABNORMAL CLOTTING TIME}} \times 100$$

The results are shown in TABLE 7.

5. One - stage Prothrombin Test[80, 4, 71].

0·1 ml. of thromboplastin suspension was added to a test-tube in the water bath at 37°C, containing 0·1 ml. of citrated plasma and the solutions mixed. 0.1 ml. of M/40 calcium chloride solution was added and the stop-watch begun. The tube was tilted at frequent intervals until a firm clot appeared and the time recorded. The test was carried out in triplicate and an average result calculated. The results were expressed as:-

$$\text{PROTHROMBIN INDEX} = \frac{\text{NORMAL CLOTTING TIME}}{\text{ABNORMAL CLOTTING TIME}} \times 100$$

These are compared in TABLE 8.

6. Two - stage Prothrombin Test.

Since it is now realised that the one - stage prothrombin test does not measure any one specific substance in blood but may be influenced by the absence of Factors V, Vll, and X as well as a very low prothrombin concentration, a more specific two - stage test has been described[82]. The modification suggested by Biggs and Macfarlane[83] was adopted i.e. the thromboplastin suspension was diluted with saline approximately 1 in 20, so as to give a one - stage prothrombin time of between 25 and 30 seconds. The use of this diluted brain suspension slows the formation of thrombin and so makes the test technically simpler to carry out.

A/

/A row of test-tubes containing 0·4 ml. of fibrinogen solution was set up in the water bath at 37°C. 0·4 ml. of citrated plasma was mixed with 0·4 ml. of diluted brain suspension in another test-tube and 0·4 ml. of M/40 calcium chloride added. A stop-watch was started as the calcium solution was added, and at minute intervals 0·1 ml. samples were removed from the incubation mixture and added to the fibrinogen tubes. The clotting time for each tube was recorded. During the first or second minute in a normal test, a clot appeared in the incubation mixture and this was removed by winding it on to a wooden swab stick and holding this to the side of the tube, so enabling further liquid samples to be obtained. Sampling was continued until the fibrinogen clotting time exceeded 3 minutes.

From the thrombin - fibrinogen dilution curve, the thrombin concentration at each minute, equivalent to the clotting times obtained, was read. From these figures, a curve of thrombin generation and its disappearance was drawn for the blood of each rabbit before and after the injection of carrageenan. The areas under the curves were computed with a planimeter and in each case, the area obtained for the abnormal was expressed as a percentage of the normal. (see TABLE 9).

7. Prothrombin Consumption Test.

The method used was the one described by Biggs and Macfarlane [84] and discussed by Merskey[85]. It measures the amount of prothrombin present in the original plasma and the amount remaining in the serum one/

/one hour after the blood has clotted.

When coagulation had occurred in the four clotting tubes for the Lee and White method, the tubes were allowed to stand in the water bath at 37°C for 50 minutes from the time of coagulation. The clots were then freed from the sides of the tubes with a wooden swab stick and centrifuged for two minutes at 1400 r.p.m. The serum was collected and at one hour after coagulation had occurred, the prothrombin consumption test was carried out.

0·4 ml. of fibrinogen solution was added to four test-tubes in the water bath at 37°C. Another tube was placed in the water bath and 0·2 ml. of the serum added to it. To this, 0·2 ml. of 0·85% sodium chloride solution and 0·2 ml. of M/20 calcium chloride solution was added immediately and a stop-watch started. The contents of the tube were mixed and at 30 seconds from the addition of the calcium solution, 0·1 ml. of the mixture was removed and added to one of the fibrinogen tubes. The clotting time of this mixture was recorded. At 60 seconds from the calcium addition, a further 0·1 ml. of the mixture was transferred to a fibrinogen tube and again, the clotting time recorded.

The second part of the test was carried out in exactly the same way using 0·2 ml. of citrated plasma in place of the serum. In this case, the mixture coagulated during the first period of 30 seconds. because the plasma contained fibrinogen so this clot had to be removed on a wooden swab stick before the 30 second sample was taken.

The/

/The results show the minimum clotting time for the plasma expressed as a percentage of the minimum clotting time for the serum.

$$\text{PROTHROMBIN CONSUMPTION INDEX} = \frac{\text{Min. clotting time of plasma}}{\text{Min. clotting time of serum}} \times 100$$

The results of this test are shown in TABLE 10.

8. Thromboplastin Generation Test

The thromboplastin generation test described by Biggs and Douglas[86] is sensitive in detecting abnormalities affecting the early stages of blood coagulation. The simplified modification of this test by Hicks and Pitney[75] was found to be useful for screening tests.

For this test, 12 rabbits were kept as controls to provide normal plasma. One was bled each day so that the blood loss over a period of time was not severe.

9 ml. of blood was collected from the control rabbit in 1·0 ml. of citrate solution, and centrifuged for 10 minutes at 1400 r.p.m. A series of test - tubes each containing 0·1 ml. of this citrated plasma was set up in the water - bath at 37°C.

The plasma to be tested was diluted one part of plasma to nine parts of veronal buffer. 0·5 ml. of the diluted plasma and 0·5 ml. of platelet substitute were mixed together in a test-tube in the water bath at 37°C. 0·5 ml. of M/40 calcium chloride solution, previously warmed/

/warmed to 37°C, was added, mixed and a stop-watch started. At one minute intervals, 0·1 ml. samples of the mixture were removed and added, simultaneously with 0·1 ml. of M/40 calcium chloride solution, to the tubes containing 0·1 ml. of control plasma. The clotting times of the plasma were recorded in each case over a period of eight minutes. The clot which usually formed in the incubation mixture during the first two minutes of incubation, was removed by means of a wooden swab stick to allow further sampling. The results of clotting times of normal and abnormal blood are shown in TABLE 11.

PART 3.

IN VITRO PLATELET COUNTS AND FIBRINOGEN PRECIPITATION TESTS.

In view of the acute toxicity exhibited by some of the carrageenans on intravenous administration, some in vitro tests were carried out to examine possible causes of these toxic effects.

PLATELET COUNTING.

One Chondrus crispus carrageenan, CY, fractionated into its k- and λ-components, Polyides and degraded Eucheuma carrageenans were selected for platelet count tests and their actions compared with those of heparin.

The method used for platelet counting was that described by Brecher and Cronkite[91], modified by the use of citrate as anticoagulant and to diminish the agglutination of platelets inherent in the technique 1ml. of blood was collected from the rabbit's ear into a tube containing 0·1 ml. of 3·5% sodium citrate solution and the contents mixed thoroughly to avoid coagulation. 0·9ml. of a carrageenan solution, of concentrations ranging from 1% to 0·01%, was added to the tube, mixed thoroughly and allowed to stand for 30 minutes, so allowing any reaction between carrageenan and blood constituents to proceed. This mixture was then drawn up to the 1 mark of a white cell diluting pipette and the pipette was filled to the 11 mark with a 1% solution of ammonium oxalate. The pipette was shaken for 3 minutes; thereafter, a Thoma counting chamber was filled, after having discarded the first/

/first few drops of liquid from the pipette. The chamber was left for 20 minutes to allow complete haemolysis of the red corpuscles and to allow the formed elements remaining to settle. The free platelets in 5 groups of 16 small squares were then counted. Thus, if x is the total number of platelets counted, each square represents a volume of 1/4000 cu.mm. and the blood is diluted, in all, 20 times, then the number of platelets in the blood sample is

$$\frac{x}{80} \times \frac{4000}{1} \times \frac{20}{1} = x \times 1000/\text{cu.mm.blood}$$

Table 12 shows the platelet counts at each concentration of the five substances tested, with the counts obtained for controls which were always carried out concurrently. No agglutination of the platelets was evident in any of the controls examined. With k-CY, λ-CY and Polyides carrageenan, agglutination of the platelets was obvious in every case, being most marked in the ones showing the lowest counts. Degraded Eucheuma carrageenan showed some small agglutinates of platelets but, as may be seen from the counts obtained, many free platelets remained. There was no noticeable platelet agglutination in any of the heparin tests as may also be observed from the counts tabulated.

Approximate physiological concentration effects.

Attempting to correlate these in vitro tests with possible in vivo effects of the carrageenans, a calculation based on a 4mg./kg. dose of Chondrus and Polyides carrageenan, a 200mg./kg. dose of degraded/

/degraded Eucheuma carrageenan and a dose of 75-200 I.U./kg. of heparin was made assuming the blood volume of a 2·5 kg. rabbit to be approximately 200 ml. If the carrageenan injected intravenously were diluted evenly in this blood volume, then additions, in vitro, of a 0·01% solution represents the physiological concentration for k-CY λ-CY, and for Polyides, 0·5% is comparable for degraded Eucheuma and 0·001% and 0·003% for heparin. Table 12A shows the platelet counts, at these concentrations, compared.

Platelet counts (in vivo)

Intravenous doses of k-CY, λ-CY (5mg./kg.) and degraded Eucheuma carrageenan 3mg./kg. were administered to rabbits. Two hours after the injection, whole blood coagulation times and platelets counts were carried out. Table 12B compares the results which were obtained, with relevant comments.

REACTION OF CARRAGEENAN WITH FIBRINOGEN SOLUTIONS.

1ml. of a 0·4% solution of fibrinogen was added to 1ml. of carrageenan solutions, ranging in concentration from 0·625 mg./ml. to 0·005mg./ml. The concentration range for degraded Eucheuma carrageenan was five times as great, from 3·13 to 0·025 mg./ml. The opacity of the mixture in each tube was then compared with that of a control containing 1ml. of fibrinogen solution and 1ml. of distilled water. The 0·4% solution of fibrinogen was in itself, opalescent and three types of reaction were observed;

1./

/1. the opalescence was removed by some carrageenan solutions

2. no observable effect was detected

3. a precipitate became obvious.

Table 13 indicates the reactions which occurred with each solution of carrageenan, and with heparin solutions.

D I S C U S S I O N

PART 1.

PRELIMINARY EXPERIMENTS - DOSAGE AND TOXICITY.

The results show that the carrageenans examined interfere with the normal coagulation process of blood.

In Part 1 of the Experimental section, the k and λ fractions extracted from seven sources of seaweed were compared by means of the whole blood coagulation time. In five of these pairs, the λ fraction consistently showed greater anticoagulant activity than the k-component. With the other two seaweed sources, Chondrus crispus (CBC) and Gigartina radula, the actions of the k and λ fractions could not be distinguished.

The λ degraded fraction of Chondrus crispus (CNS) did not show activity which differed from the corresponding undegraded fraction but it was less toxic. This difference in toxicity may well be a reflection on their different molecular weights.

In most cases, very little, or no, anticoagulant activity was apparent at the lowest dose level (1mg./kg.), activity appearing at the 3mg./kg. dose and increasing to 10mg./kg. when acute toxicity appeared. Thus, all the carrageenans showed anticoagulant activity but this was accompanied by acute toxicity, the two properties appearing to run parallel. As may be seen from TABLE 2, in each case for which the λ-fraction showed greater anticoagulant activity than its k counterpart, it also showed greater toxicity. The other two k and

λ pairs/

/λ pairs showed equivalent toxicity as well as anticoagulant activity. Degraded Eucheuma carrageenan produced no toxic symptoms at a dose level as high as 1G./kg. possibly due to its much lower molecular weight.

A detailed study of these acute toxic effects, which appeared similar to the anaphylactoid reaction described by Walton and Ricketts[35] should be carried out and would probably provide useful information regarding the actions of intravenous carrageenan.. On superficial examination, post-mortem observations have suggested kidney damage as a toxic effect but the possibility of emboli obstructing vital structures cannot be ignored. The carrageenans are generally of high molecular weight and so produce solutions of high viscosity. Degraded Eucheuma carrageenan, however, is non-toxic and gives a solution of relatively low viscosity.

Although the k and λ fractions obviously differ in their anticoagulant and toxic actions, the λ fractions from different sources vary only very slightly in their activity. In comparing the k fractions from each seaweed source, there appears to be slight differences in their activities as indicated by the whole blood coagulation test, those from Chondrus crispus (CY) and from Gigartina radula showing greater activity than the others.

PART 2.

BLOOD COAGULATION TESTS.

The results obtained in the experiments of Part 1 suggested that the carrageenans of the Gigartina species showed no additional features of special interest. It was therefore decided to confine the more detailed study to the Chondrus carrageenans, the degraded Eucheuma, and the Polyides, carrageenans. The latter two are of special interest because of their more marked anticoagulant activity, their lower molecular weights, high sulphate content and, in the case of Eucheuma, remarkably low toxicity.

The more detailed study with these selected carrageenans was undertaken with the object of localising their action in the coagulation process and of comparing their actions with those of heparin. Clotting tests designed to indicate deficiencies in both the early and later stages of coagulation were used.

1. Whole blood coagulation test. This is a useful but non-specific test. A prolonged coagulation time indicates interference in the coagulation system at one or more points but gives no indication of whether an effect is seen in the early or late stages of the process. However, a negative result i.e. where the coagulation time is still within normal limits, does not necessarily indicate no interference in the mechanism. The initial phases of coagulation are generally believed to occupy the greater part of the time, perhaps 3-4 minutes, and/

/and if this phase is prolonged, the coagulation time may be increased substantially. The last phase occurs rapidly, perhaps in 15 seconds, and therefore even a ten-fold increase at this stage would be scarcely detectable in the whole process.

Confirming the results obtained in Part 1, in the four pairs of k and λ carrageenans chosen, the λ fraction was consistently more active, at both dose levels, than the corresponding k fraction, in prolonging coagulation time. The results also indicate a definite difference between the activities of the carrageenans from different habitats. CY and CNS showed greatest activity in both a k-comparison and λ-comparison. The fractions extracted from CMI showed least activity and its k fraction gave results which could be considered within normal limits. The lower dose of heparin (75 I.U./kg.) showed activity within the range of activity of the Chondrus carrageenans. Heparin (200I.U./kg) was equivalent to Polyides carrageenan, at 3mg./kg., and degraded Eucheuma carrageenan, at 200mg./kg., showed only slightly less activity.

2. Thrombin generation test. Measuring the amounts of thrombin formed at intervals of time after the collection of blood, this test is also non-specific and may indicate defects in the coagulation system at any stage. Nevertheless, it gives more information than the whole blood coagulation test, is more sensitive to minor defects, and gives a good picture of the general pattern of thrombin formation. There is no suitable numerical method of expressing the results of this/

/this test but they can be described graphically showing generation and disappearance of thrombin.

It is apparent from the graphs (Appendix 1) that the Chondrus carrageenans, at 5mg./kg., reduced and delayed the amount of thrombin formed. With CY, there was no apparent difference between the k and λ fractions, both being effective, whereas in the other three pairs of carrageenans, the λ fraction exerted a more marked effect.

Of the four k fractions, k-CY and k-CNS were more active, but the four λ fractions did not appear to differ significantly amongst themselves.

At the 3mg./kg. dose level, there was slight delay and decrease in the amount of thrombin formed in most cases. Differences between k and λ fractions were less marked than at the higher dose level.

Polyides and degraded carrageenan showed a most marked delay in the generation of thrombin; and, of the thrombin formed, very little or none was detectable in the test.

The low dose of heparin gave a slight delay in the generation of thrombin, similar to the results of the Chondrus carrageenans. With the high dose, no thrombin was detected in the test, in three out of the four rabbits. In the fourth, there was a delayed and very small amount detected

3. Thrombin time. This test consists of adding standard amounts of thrombin solution to citrated plasma, thereby testing the efficiency of the/

/the last stage in the coagulation process, the fibrinogen-fibrin reaction. A prolonged thrombin time could result from either a severely depleted free fibrinogen content of the blood, which could follow reaction of the carrageenan with fibrinogen, or to an antienzymic action on the thrombin added in the test, by substrate competition.

At the 5mg./kg. dose, all the λ's were considerably more active than the corresponding k fractions. Of the four k's, CY, CNS and CSE were similar in activity and more effective than CMI which was normal. In the case of the λ fractions, CSE was most potent, followed by CY, CNS and CMI.

At the lower dose, λ-CY and λ-CSE were more active than their corresponding k, while the k's and λ's of CNS and CMI showed similar activities.

Polyides and degraded Eucheuma carrageenans, although equivalent to heparin (200 I.U./kg.) in the whole blood coagulation test, were very much less active than this dose of heparin in this test and even less effective than some of the λ Chondrus carrageenans. The smaller dose of heparin showed a marked action in this test while the higher dose had such a prolonged inhibitory action that clotting times could not be measured.

4. Recalcification time. Again, a non-specific test since calcium is probably involved in a great many of the stages in coagulation. The/

/The number of platelets present can affect the result of this test[88], hence centrifugation conditions are important and must be standardised. If platelet clumping occurred then the clotting time in the test would be markedly prolonged. A prolonged recalcification time often indicates that the clotting process is interrupted at a stage prior to the conversion of prothrombin-thrombin.

In all cases, the recalcification time was prolonged. At the higher dose level of three carrageenans, recalcification time was prolonged more by the λ's than by the k's. In the fourth, CY, the k, on average, was slightly more active than the λ. At 3mg./kg., similar results were obtained although the CMI pair did not influence the result of the test significantly. k-CSE appeared more active than the λ-fraction. Generally, CY and CNS showed greatest activity.

Polyides carrageenan prolonged the test to a degree equivalent to the low dose of heparin which was greater than any of the above effects. Degraded Eucheuma carrageenan prolonged the time to a considerably greater extent, equivalent to the effect of the large dose of heparin.

5. <u>One-stage prothrombin test</u>. In this test, thromboplastin and calcium are added in excess and, since it was formerly believed that clotting then depends only on prothrombin concentration, a prolonged clotting time would indicate lack or absence of prothrombin. However, it is now widely accepted that in the conversion of prothrombin to thrombin,/

/thrombin, factors V, Vll, X and calcium are necessary in addition to prothrombin. The test is therefore not specific for prothrombin and is, in fact, very insensitive to hypoprothrombinaemia. Nevertheless, it remains a useful guide, is simple to carry out and provides information on the efficiency of the reactions involving all these factors.

With the Chondrus carrageenans, at both dose levels, the λ-fraction was consistently more effective in prolonging the clotting time than the corresponding k fraction. A definite effect was shown in this test in most cases although low doses of the least active k's gave only slight prolongation.

At both dose levels, differences were not marked among the k fractions, k-CSE and k-CNS showing slightly greater activity.

Little difference could be detected between the λ fractions from different habitats, λ-CNS possibly showing greater potency.

Polyides carrageenan gave a prothrombin index of 58 and degraded Eucheuma carrageenan of 69. By this test therefore, these are more active than even the high dose of heparin which gave an average value of 86, equivalent to many of the k carrageenan samples. The lower dose of heparin (75 I.U./kg.) showed no effect whatever on this test.

6. Two-stage prothrombin test. This test provides a more specific estimate of the amount of prothrombin available for conversion to thrombin than the one-stage method. Thromboplastin and calcium are added to the plasma and at intervals of time following the addition of/

/of calcium, samples are removed from the mixture and added to fibrinogen. The clotting times of the fibrinogen solutions are noted and graphs can be constructed showing the formation of thrombin with time. A peak, showing when the maximum quantity of thrombin is freely available is seen but very rapidly falls off because the thrombin is being neutralised by antithrombin. This neutralisation is probably occurring from the start of the formation of thrombin and the peak thus represents the point at which the rate of formation of thrombin equals its neutralisation. The height of the peak may be used as a measure of prothrombin but this depends on the rate of the reactions which are proceeding and the efficiency of the thrombin neutralisation process. It is now generally agreed that a better measure of total available prothrombin can be obtained by measurement of the area under the curve, the actual time and position of the peak bearing little weight in estimating total prothrombin concentration. The area under the abnormal curve is expressed as a percentage of the normal area, for the results from each rabbit.

Differences between the results for corresponding λ and k fractions were in many cases not marked due to great variation within the results from the four rabbits. At the lower dose level, the λ fraction of CNS and CMI indicated a more marked effect. In all cases, however the amount of prothrombin available for conversion to thrombin was lower after 1/V injection of the carrageenan than before.

When/

/When comparison is made between the k fractions, CY, CNS and CSE are of similar activity. CMI showed lower activity. In general, CY and CNS show slightly greater activity amongst the λ fractions.

Polyides carrageenan does not exert a marked action in this test being less active than the other carrageenans. Degraded Eucheuma does, however, markedly reduce the prothrombin available. Heparin, although interfering very slightly in the one-stage test, shows marked action presumably at least partly due to its antithrombic action. The low dose of heparin is comparable with degraded Eucheuma carrageenan.

7. Prothrombin Consumption Test. The next two tests which were carried out indicate any abnormality occurring early in the clotting mechanism i.e. in the first stages, in the formation of thromboplastin.

When whole blood clots in a tube, practically all the prothrombin is converted to thrombin, the conversion depending upon the amount of thromboplastin formed. If there is any gross abnormality in thromboplastin formation, then the conversion of prothrombin to thrombin is delayed or absent. The test is therefore an indirect determination of the amount of thromboplastin produced and the results should be in parallel with those of the thromboplastin generation test.

The test itself consists of comparing the prothrombin, remaining in serum one hour after coagulation has occurred, with that in plasma. The prothrombin consumption index, recorded as the plasma clotting time/

/time as a percentage of serum clotting time is usually low, below 20% due to the reduction of prothrombin in serum. Sometimes, however, the index of the blood tested after the administration of the carrageenan exceeded 100%. That is, the serum appeared to contain more prothombin than the plasma and this, one would think, is most unlikely. The reason for this finding cannot be explained but has already been noted in many cases[89,90], in both thrombocytopenia and haemophilia. It appears that whenever the platelets are removed, an index of $\geq$100% is obtained.

The results of the test show that in every case in which a carrageenan had been administered, there followed an increase in the index, so showing a less efficient consumption of prothrombin in the serum. The degree of effectiveness was extremely variable in the four pairs and no general trend was obvious when the k and λ fractions were compared, the four k fraction, and the four λ fractions.

With Polyides carrageenan, a marked increase was shown, giving an average index of about 150%, while Eucheuma showed slightly less activity.

Heparin (75I.U./Kg.) produced no effect whatever on the result of this test while the 200 I.U./Kg. dose of heparin increased the index markedly to an average of 335%

8. Thromboplastin Generation Test. The screening test as carried out was found[75] in a wide range of coagulation disorders to give as accurate/

/accurate and as sensitive results as the test of Biggs and Macfarlane[86] yet was very much simpler and quicker to perform. Its clinical limitation is that it will not distinguish between haemophilia and Christmas disease, but is a non-specific test for disorders of thromboplastin generation. It was originally devised to study the intrinsic prothrombin activator system.

The test consists of recalcifying diluted plasma in the presence of a platelet substitute and the thromboplastin which is generated is tested by adding samples of this mixture along with excess calcium, to normal citrated plasma. If there is defective thromboplastin generation, then the amount of thrombin formed is low and coagulation times of the normal plasma will be abnormally prolonged. It therefore determines whether a deficiency exists in the early stages of blood coagulation.

At both dose levels of the Chondrus carrageenans, the λ fraction in each case was more active than the k fraction. In all cases, there was an increase in clotting times and delay in formation of thrombin, so indicating a slow formation of a small amount of thromboplastin.

Between the k components from different sources, there was no detectable difference, all increasing the final time by a factor of between one and two.

Comparing the λ fractions, the average increase over normal values lay between 4 and 6 times normal, CMI being less effective than the other/

/other three.

Polyides carrageenan did not produce a marked change, but one similar to the k fractions above.

Degraded Eucheuma carrageenan was potent in its inhibitory thromboplastic activity, producing a seven-fold increase in the normal values, a greater increase than any of the above fractions.

Heparin, at 75 I.U./kg. showed only slight activity in this test causing an increase in clotting time of twice the original time, an effect similar to that of the k carrageenan fractions. The higher heparin dose gave results comparable with those of degraded Eucheuma.

Pentobarbitone. The results of the tests in control rabbits given an injection of Nembutal are reported in the appropriate tables. There was no effect in six of the tests. In the recalcification test, a slight increase in index was obtained and a slight reduction of prothrombin was indicated in the two-stage prothrombin test. These tests were carried out in rabbits given almost twice the intravenous dose of Nembutal required to produce vasodilatation and to aid rapid bleeding from the ear, in a few carrageenan-tested rabbits. It is therefore assumed that in these cases, the injection of Nembutal did not influence the results of the blood clotting tests in carrageenan-treated rabbits.

GENERAL DISCUSSION.

Anticoagulant Activity.

Previous reports have dealt with the anticoagulant properties of seaweeds: Houck et al.[20] reported that one of their extracts, of Gigartina acicularis, showed marked activity while five other seaweed extracts did not, but none was fractionated. It is therefore possible that further differentiation of the carrageenan extracts could have revealed activity which has currently been overlooked. Rees has suggested[13] that λ-carrageenan may be a biological precursor of the k-fraction, and it is possible that a seaweed be harvested containing a high proportion of the relatively inactive k polysaccharide. Hawkins and Leonard,[94] on the other hand, have used an extract fractionated into its k and λ components and have shown λ to be more active biologically. The results presented here confirm this finding and show the extent to which their conclusions can be carried to other carrageenans. The λ fractions studied had a consistently higher anticoagulant activity coupled with a consistently higher sulphate content than the corresponding k fractions, but while activity appears to be correlated with sulphate content, highest sulphate content does not ensure highest activity, λ-CY having a higher sulphate but lower activity than Polyides carrageenan. This may, however, indicate an optimum sulphate content for activity, though undoubtedly other factors are significant.

The/

/The k and λ-carrageenans differ widely in their 3;6 anhydrogalactose content but no relationship between content of this grouping and lack of anticoagulant activity can be seen. Degraded Eucheuma carrageenan has still a high 3,6-anhydrogalactose content but retains anticoagulant activity, although not at a similar dose level. Degradation yields a much smaller molecule, with toxicity so reduced that anticoagulant activity can be demonstrated with safe intravenous doses.

As with heparin, it is unlikely that any one property of the sulphated polysaccharide structure is wholly responsible for anticoagulant activity but more likely, a combination of several factors confers optimal activity. A certain sulphate content is obviously important, and maximum activity is seen when this is between 30 and 35%, but a high molecular weight undoubtedly confers acute toxicity. However, molecular weight is not alone responsible for toxicity since a degraded λ-carrageenan differs markedly in toxicity from a degraded k-carrageenan, of similar sulphate and molecular weights. Molecular configuration is also of relevance and must be extremely specific for the polysaccharide molecules which are large yet similar to each other in many chemical respects.

Localisation of interference in blood coagulation.

Since all the tests show a positive result to some extent, indicating some interference in most of the stages of coagulation, it appears that/

/that carrageenan affects many clotting factors. Probably, the effect is most pronounced on the earliest stages of coagulation since the thrombin time, which measures interference with the fibrinogen-fibrin conversion, is affected only slightly, and certainly to a very much smaller extent than it is with heparin. On the other hand, the thromboplastin generation test and prothrombin consumption test are affected in all cases and these tests specifically indicate a slow and defective production of thromboplastin, (or prothrombin activator). The mode of action of the various carrageenans appears to be similar since the same tests are affected though to varying degrees. Thus, they possibly act in qualitatively similar ways but differ from heparin since a peak effectiveness is reached within half an hour after intravenous heparin but two hours is necessary to show peak effectiveness for the carrageenans. Hawkins[32] reports a peak effect for laminarin sulphate in dogs after half an hour whereas Houck et al.[49] found maximum effect after 4 hours with carrageenan extracts, in dogs, and found that anticoagulant activity was still evident 24 hours after the injection, a finding which this work has confirmed. This latent period for effectiveness cannot be explained at the present time but may possibly be due to interaction of the carrageenan with plasma proteins e.g. fibrinogen, or by causing clumping of the platelets. It would be of interest to determine whether the administration of heparin and carrageenan together,/

/together, would influence the action of the former or whether they would act independently to produce an additive effect. At the low dose of heparin, the results of the one-stage prothrombin test and of the prothrombin consumption test were unaffected while with equivalent doses of carrageenan, as measured by the whole blood coagulation time, these tests were affected, so indicating a difference in mode of action. With heparin, the thrombin time was most markedly prolonged, again a distinct difference in mode of action being suggested. Thus, the carrageenans have shown differences, both qualitative and quantitative as regards anticoagulant actions, from those of heparin. Some clearer understanding of these findings might be forthcoming from the use of antagonists such as protamine sulphate, or toluidine blue, in conjunction with the carrageenans. With heparin, the actions of these antagonists are well-established.

Since many of the carrageenans are acutely toxic, it was initially thought that the demonstrated anticoagulant action might be due to a release of endogenous heparin in greater than normal physiological amounts, as is believed to occur in conditions of shock. However, the marked differences which have been pointed out above eliminate this explanation. It is possible, nevertheless, that the effects seen in tests using acutely toxic doses of carrageenan may be complex, being a combination of true carrageenan action and of heparin release.

Further work could be extended to in vitro testing, using rabbit blood/

/blood followed by tests on human blood. Results from such experiments would be interesting, especially on consideration of the lag phase necessary for optimal anticoagulant action in vivo. Using rabbit blood, results would be directly comparable with those already obtained in vivo. These in vitro results could then be compared with advantage to those on human blood.

Intravenous toxicity of carrageenans.

As already described in Experimental, Part 111, platelet counts and fibrinogen precipitation tests were carried out on the carrageenan solutions, in vitro. Walton[34] has reported that large molecular weight dextran sulphates, (i.e. above 40,000), formed complexes with fibrinogen which were insoluble and led to instability of the formed elements of the blood, causing platelets and white cells to agglutinate Lower molecular weight dextran sulphates formed soluble complexes and were not found to cause agglutination of the formed elements. Walton[9] has also shown the presence of emboli in various organs of mice and rats given acutely toxic doses of high molecular weight dextran sulphate.

Work has therefore been initiated to determine in vitro effects of carrageenan on blood platelets and on fibrinogen both of which are concerned in blood coagulation. Results from preliminary experiments are interesting and suggest further, more complete, investigation. The free platelet count was markedly reduced in the presence of Chondrus/

/Chondrus and Polyides carrageenan while with degraded Eucheuma and heparin, no effect was apparent. It is of interest that as the concentration of carrageenan was reduced so was the platelet count and platelet clumping became much more obvious. A fuller investigation of this effect, which is presumed from these experiments to reach a maximum at around physiological levels, is indicated, and should include other Chondrus carrageenans. The in vivo tests, although few, showed a marked difference in effect between the k and λ carrageenan. The k carrageenan had caused platelet agglutination which was evident on the haemocytometer but the λ treated blood showed no agglutination although the number of free platelets was small. These findings suggest that toxicity may well be related to the formation of emboli or precipitates to which the platelets become attached, which are then deposited in pulmonary vessels or other vital structures causing respiratory embarrassment and rapid fatality. Although these effects might be related to toxicity, they may also be of importance as a factor in the anticoagulant action since a thrombocytopenia might reduce the thromboplastin readily available in vitro and so affect the clotting tests.

The results of the reactions of carrageenans with fibrinogen may be seen in TABLE 13. Instructive interpretation of these preliminary tests is difficult at this stage since care has to be taken to avoid making erroneous assumptions. A calculation based on a carrageenan/

/carrageenan dose being diluted in 200 mls. of rabbit blood, evenly, gives an indication of approximate in vivo concentrations of carrageenans and the results at these show no positive precipitation with fibrinogen. However, it may well be the case that in vivo, precipitation or complexing of carrageenan with fibrinogen or other plasma proteins occurs rapidly and before sufficient time has passed to ensure thorough admixture with the entire blood volume. Even if complexing with fibrinogen does occur under these in vitro conditions, a reaction with other plasma proteins will most likely also occur in whole blood and possibly this non-specific protein reaction involves many of the blood clotting factors in plasma. The toxic effects apparent on intravenous administration of carrageenan could be due to insoluble protein-carrageenan complexing with adherence of the blood platelets so forming obstructive emboli which become attached to endothelial surfaces and eventually block circulation in vital structures leading to rapid fatalities. This must form the basis for further investigation to establish whether or not these assumptions are borne out in vivo and complete histological examination of post-mortem tissues would provide useful information.

With the higher concentrations of carrageenan, a clearing of the opalescent fibrinogen solution occurred, indicating soluble complex formation. An interpretation of this reaction and of its possible importance depends upon the exact nature of the substances causing the opalescence. If traces of insoluble fibrin are being solubilised by/

/by the carrageenan, this might be of obvious importance as a fibrinolytic effect. A much fuller investigation, however, is necessary.

While the carrageenans from Chondrus crispus already tested show anticoagulant activity, their clinical use would appear to be precluded on account of their general toxicity, and further blood clotting investigation would thus be of academic, though not of clinical, value. Further investigation into the toxicity, mode of action and possible clinical use of carrageenan from P. rotundus and degraded E. spinosum is suggested as being worthwhile from both the academic and possible clinical viewpoint. The greatest activity, as indicated by the whole blood coagulation time, was obtained with Polyides carrageenan which showed just less than 1/2 the activity of heparin, weight for weight. Degraded E. spinosum showed 1/150 the activity of heparin but these comparative values are only significant when considered in relation to their toxicity. The carrageenans from C. crispus showed an average activity of 1/6 that of heparin but were, at that dose level, extremely toxic.

Interest is focused on the carrageenans not only for their anticoagulant action, but on account of their antipeptic and anti-ulcer activity. Possible anticoagulant action which they exhibit might then be of importance not as the direct clinical action but as an important side effect.

R E S U L T S

TABLE A

PROPERTIES OF CARRAGEENANS

SEAWEED	SOURCE	TIME OF HARVESTING	SO_3Na	3,6 ANHYDRO-GAL.	OPT. ROT.^{N}	INH. VISC.* dl/g	k:λ RATIO	GALACTO
CHONDRUS CRISPUS (CNS)	NORTHUMBERLAND STRAIT NOVA SCOTIA	AUTUMN 1962						
k FRACTION			28·4	25·3	75	20·8	1·8	35·9
λ FRACTION			34·9	4·1	30	21·7		45·8
CHONDRUS CRISPUS (CMI)	MUD ISLANDS NOVA SCOTIA	JUNE 1960						
k FRACTION			27·0	24·8	67	8·6	1·9	28·7
λ FRACTION			32·2	9·8	32	-		36·4
CHONDRUS CRISPUS (CSE)	SEBASCO ESTATES MAINE, U.S.A.	JUNE 1963						
k FRACTION			29·8	25·2	37	14·3	3·4	28·3
λ FRACTION			32·3	9·1	20	13·8		38·6
CHONDRUS CRISPUS (CY)	YARMOUTH NOVA SCOTIA	AUGUST 1962						
k FRACTION			28.2	29.2		13.7	1·2	31.7
λ FRACTION			37·3	3.5		16.2		38·5
CHONDRUS CRISPUS (CBC)	BURRIN CO. CLARE IRELAND	JUNE/JULY 1962						
k FRACTION			29·6	22·9	50	11·6	1·3	28·6
λ FRACTION			34·6	4·7	38	14·4		39·4

* Inherent viscosity $(\eta)_{inh.} = 1/c \ \ln(\eta_{soln.}/\eta_{solv.})$ dl/g. where c = g. of solute in 100 ml. solution.

TABLE A (Contd.)

SEAWEED	SOURCE	TIME OF HARVESTING	SO_3Na	3,6-ANHYDRO-GAL	OPT.ROT.^{N}	INH.VISC.$^\gamma$ dl/g.	k :λ RATIO	GALACTOSE
GIGARTINA RADULA	SOUTH AFRICA	-						
k FRACTION			31·0	23·2	110	15·7	3·5	29·1
λ FRACTION			35·6	9·6	44	15·7		37·0
GIGARTINA STELLATA (GM)	MILLPORT SCOTLAND	AUGUST 1962						
k FRACTION			30·1	22·6	76	15·0	1·3	34·8
λ FRACTION			28·3	15·9	28	13·6		41·5
POLYIDES ROTUNDUS	MOOSE HEAD HALIFAX NOVA SCOTIA	JULY 1963	35·0	2·3	+30		-	25·4
EUCHEUMA SPINOSUM (DEGRADED)	SOUTH EAST ASIA	-	30·7	19·0	+35	0·2	-	30·3
C. CRISPUS CNS (λ-DEGRADED)	AS ABOVE	AS ABOVE	30·7	-	-	1·3	-	-
REES' λ ("PURE")	C. CRISPUS MARINE COLLOIDS INC MAINE U.S.A	-	25·6	1·6	81	-	-	44·5

TABLE I

AVERAGE COAGULATION TIMES (IN MINS) 2 HOURS AFTER I.V. INJECTION

CARRAGEENAN	1 mg/kg	3 mg/kg	5 mg/kg	10 mg/kg
CHONDRUS CRISPUS CNS				
k FRACTION	6	6	11	12
λ FRACTION	8	12	TOXIC	-
CHONDRUS CRISPUS CMI				
k FRACTION	6	7	7	12
λ FRACTION	7	10	12	15
CHONDRUS CRISPUS CSE				
k FRACTION	6	6	7	13
λ FRACTION	7	10	13	20
CHONDRUS CRISPUS CY				
k FRACTION	7	9	10	TOXIC
λ FRACTION	9	10	13	TOXIC
CHONDRUS CRISPUS CBC				
k FRACTION	6	8	7	8
λ FRACTION	6	7	8	9
GIGARTINA STELLATA GM				
k FRACTION	5	6	8	9
λ FRACTION	6	9	10	12
GIGARTINA RADULA				
k FRACTION	8	11	12	TOXIC
λ FRACTION	8	11	TOXIC	-
POLYIDES ROTUNDUS	5	20	TOXIC	-
CHONDRUS CRISPUS CNS (λ FRACTION, DEGRADED)	7	9	8	10
PURE λ -CARRAGEENAN	6	6	10	26

DEGRADED EUCHEUMA CARRAGEENAN	10 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
	5	8	19	15

TABLE 2

CARRAGEENAN			SMALLEST DOSE PROVING FATAL WITHIN 24 HOURS (mg/kg) k-FRACTION	λ-FRACTION
CHONDRUS	CRISPUS	CNS	5	3
"	"	CMI	>10	3
"	"	CSE	5	1
"	"	CY	3	1
"	"	CBC	3	3
GIGARTINA	RADULA		1	3
"	STELLATA	GM	50	20
CHONDRUS CRISPUS (λ-FRACTION, DEGRADED)		CNS	10	
PURE λ-CARRAGEENAN			5	
POLYIDES ROTUNDUS			5	
DEGRADED EUCHEUMA CARRAGEENAN			>1000	

TABLE 3

MAXIMUM ANTICOAGULANT ACTION

CARRAGEENAN CHONDRUS CRISPUS	DOSE mg/kg	WHOLE BLOOD COAGULATION TIME (MINS) AFTER INJECTION 0·5 HOUR	2 HOURS	5 HOURS
k- CNS	5	6	10	TOXIC
k- CNS	10	7	12	TOXIC
λ- CNS	3	5	13	6
λ- CNS	3	5	11	TOXIC
λ- CNS	1	5	7	TOXIC
k- CMI	1	5	13	TOXIC
k- CMI	3	6	15	12
k- CMI	5	6	21	19
λ- CMI	1	6	11	7
λ- CMI	3	7	13	TOXIC
λ- CY	1	6	13	10
λ- CY	2	7	13	TOXIC
k- CSE	2	5	8	6

TABLE 4

SCREENING TEST FOR PLATELET SUBSTITUTE SUSPENSION

DILUTION

		1 in 50	1 in 100	1 in 200
CLOTTING TIMES (IN SECS)	1.	28	15	70
	2.	12	6·4	22
	3.	6·5	6·0	14
	4.	7·4	5·6	14
	5.	6·0	5·4	13
	6.	6·0	5·6	15
	7.	5·8	6·0	14
	8.	7·0	5·8	14

SECOND PREPARATION

	1 in 100	1 in 50	1 in 20
MINIMUM CLOTTING TIMES (IN SECS)	11	8	6

TABLE 5

WHOLE BLOOD COAGULATION TEST

DOSE	CARRAGEENAN	COAGULATION TIME (MINS.) k- FRACTION		λ - FRACTION	
		INITIAL	2 HOURS AFTER INJECTION	INITIAL	2 HOURS AFTER INJECTION
5 mg/kg.	CHONDRUS CRISPUS CY	5	9	5½	11
		6	11	5½	11
		5½	14	5	19
		5½	9	6½	-
	AVERAGE	5½	11	5½	14
5 mg/kg.	CHONDRUS CRISPUS CMI	5	6	5½	8
		5	5	5	7½
		4½	6	5½	8½
		5	5	6	16
	AVERAGE	5	5½	5½	10
5 mg/kg.	CHONDRUS CRISPUS CSE	5	6	5	5½
		5½	7	4½	8½
		5	7	5	-
		4½	6	4½	11
	AVERAGE	5	6½	5	8
5 mg/kg.	CHONDRUS CRISPUS CNS	5½	7½	5	8½
		5½	8½	TOXIC	TOXIC
		5	7½	"	"
		4½	7½	"	"
	AVERAGE	5	8		

TABLE 5 (CONTD.)

WHOLE BLOOD COAGULATION TEST

DOSE	CARRAGEENAN	COAGULATION TIME (MINS.)			
		k- FRACTION		λ - FRACTION	
		INITIAL	2 HOURS AFTER INJECTION	INITIAL	2 HOURS AFTER INJECTION
3 mg/kg.	CHONDRUS CRISPUS CY	5	8	5	-
		5	5½	4½	10½
		5	6½	5	11
		5	5	4½	9½
	AVERAGE	5	6	5	10
3 mg/kg.	CHONDRUS CRISPUS CMI	4	5	5½	8
		4	4½	5	8
		5	5½	6	10
		5	5½	5	10
	AVERAGE	4½	5	5½	9
3 mg/kg.	CHONDRUS CRISPUS CSE	5	5½	5	7½
		5½	6½	4½	8
		5	6	5	8
		5½	7½	5	6
	AVERAGE	5½	6½	5	7½
3 mg/kg.	CHONDRUS CRISPUS CNS	5½	8½	4½	14
		4½	8	4	13
		5½	9½	5	-
		5	10½	4½	12
	AVERAGE	5	9	4½	13

TABLE 5 (Contd.)

	COAGULATION TIME (MINS.) INITIAL	2 HOURS AFTER INJECTION		COAGULATION TIME (MINS.) INITIAL	2 HOURS AFTER INJECTION
P. ROTUNDUS	4	28	DEGRADED	5½	9
3 mg./kg.	3	17½	EUCHEUMA	4½	21
	3	19	CARRAGEENAN	6	13
	4½	14	200 mg./kg.	5½	25
AVERAGE	3½	19½		5½	17
		0.5 HOUR AFTER INJECTION			0.5 HOUR AFTER INJECTION
HEPARIN	4½	7½	HEPARIN	4	15½
75 I.U/kg.	4½	8½	200 I.U/kg.	5½	20
	5	7		5	30
	4½	9		4½	16
AVERAGE	4½	8		5	20
		IMMEDIATELY AFTER INJECTION			
NEMBUTAL (CONTROL)	5½	5			
25mg./kg.	4½	4½			
	6	6½			
	5½	5			
AVERAGE	5½	5½			

TABLE 6

THROMBIN TIME

CHONDRUS CARRAGEENANS. THROMBIN INDEX.

DOSE	CODE	k	λ	DOSE	CODE	k	λ
5mg./kg.	CY	83·0	61·0	5mg./kg.	CMI	110·0	68·1
		90·7	64·0			90·5	63·0
		86·5	53·8			99·0	71·0
		71·8	94·1			108·5	66·7
	AVERAGE	83·0	68·2		AVERAGE	102·0	67·2
5mg./kg.	CSE	77·8	46·0	5mg./kg.	CNS	82·4	74·1
		82·4	51·2			85·9	-
		80·7	60·0			87·2	-
		107·0	61·1			82·6	-
	AVERAGE	87·0	54·6		AVERAGE	84·5	-
3mg./kg.	CY	92·4	70·4	3mg./kg.	CMI	102·6	97·9
		69·5	82·0			97·3	90·7
		100·0	80·9			92·7	97·1
		101·3	70·8			93·5	90·7
	AVERAGE	90·8	76·0		AVERAGE	96·5	94·1
3mg./kg.	CSE	84·2	71·4	3mg./kg	CNS	86·9	72·9
		72·6	79·4			87·5	92·9
		90·7	80·9			82·2	91·2
		79·6	65·9			84·4	89·0
	AVERAGE	81·8	74·4		AVERAGE	85·3	86·5

TABLE 6 (Contd.)

	THROMBIN INDEX		THROMBIN INDEX
P. ROTUNDUS	65·9	DEGRADED EUCHEUMA	57·3
3mg./kg.	58·8	200 mg./kg.	58·9
	66·7		48·7
	94·6		66·7
AVERAGE	71·5	AVERAGE	59·9
HEPARIN	7·2	HEPARIN	$\leq$0·55
75 1.U/kg.	2·7	200 1.U/kg.	$\leq$0·55
	13·3		$\leq$0·55
	8·6		$\leq$0·55
AVERAGE	8·0	AVERAGE	$<$ 0·55
NEMBUTAL	118·7		
(Control)	95·4		
25 mg./kg.	108·3		
	101·4		
AVERAGE	105·9		

TABLE 7

RECALCIFICATION TIME

CHONDRUS CARRAGEENANS - RECALCIFICATION INDEX

DOSE	CODE	k	λ	DOSE	CODE	k	λ
5mg./kg	CY	46·0	81·0	5mg./kg.	CMI	81·4	32·2
		69·3	48·3			85·3	69·9
		106·7	89·4			62·3	39·3
		58·9	110·0			109·3	61·4
	AVERAGE	70·0	82·8		AVERAGE	84·6	50·7
5mg./kg	CSE	80·8	38·3	5mg./kg.	CNS	73·0	15·7
		57·1	56·0			36·8	-
		42·3	61·6			48·4	-
		100·0	47·1			116·9	-
	AVERAGE	70·0	50·7			68·8	-
3mg./kg	CY	51·6	44·4	3mg./kg.	CMI	114·8	41·3
		69·6	42·2			74·5	54·7
		28·5	70·2			117·9	49·0
		51·4	61·9			93·5	66·7
	AVERAGE	50·3	54·6		AVERAGE	100·2	52·9
3mg./kg.	CSE	47·1	58·4	3mg./kg.	CNS	59·1	45·5
		82·7	47·2			71·2	32·5
		96·9	71·5			86·8	81·5
		81·6	56·9			59·0	53·2
	AVERAGE	77·1	58·5		AVERAGE	69·0	53·2

TABLE 7 (Contd.)

	RECALCIFICATION INDEX		RECALCIFICATION INDEX
P. ROTUNDUS 3mg./kg	36·7	DEGRADED EUCHEUMA 200mg./kg	22·4
	23·1		19·1
	45·5		20·9
	58·5		20·3
AVERAGE	40·9	AVERAGE	20·7
HEPARIN 75 i.u/kg.	21·3	HEPARIN 200 i.u/kg.	20·6
	36·7		42·0
	56·8		18·7
	49·0		18·7
AVERAGE	40·9	AVERAGE	25·0
NEMBUTAL (Control)	112·6		
	111·7		
	127·0		
	122·2		
AVERAGE	118·4		

TABLE 8

ONE-STAGE PROTHROMBIN TEST

DOSE	CODE	PROTHROMBIN INDEX K	PROTHROMBIN INDEX λ	DOSE	CODE	PROTHROMBIN INDEX k	PROTHROMBIN INDEX λ
5mg./kg	CY	97·2	66·7	5mg./kg.	CMI	98·0	74·2
		69·0	57·6			76·6	66·3
		71·8	57·8			98·4	50·3
		75·2	51·2			95·7	62·9
	AVERAGE	78·3	58·3		AVERAGE	92·2	63·4
5mg./kg.	CSE	92·6	78·0	5mg./kg	CNS	81·7	73·1
		64·2	64·9			74·1	-
		73·4	56·6			94·4	-
		84·5	48·0			85·4	-
	AVERAGE	78·7	61·9		AVERAGE	83·9	-
3mg./kg.	CY	85·3	63·8	3mg./kg.	CMI	93·7	58·2
		92·3	78·7			100·9	73·9
		111·1	53·4			76·2	78·5
		90·3	80·9			75·4	68·3
	AVERAGE	94·7	69·2		AVERAGE	86·5	69·7
3mg./kg.	CSE	74·6	64·7	3mg./kg.	CNS	83·8	45·2
		82·4	71·0			69·9	54·0
		84·8	80·8			95·1	47·5
		69·3	83·1			74·5	50·5
	AVERAGE	77·8	74·9		AVERAGE	80·8	49·2

TABLE 8 (Contd.)

	PROTHROMBIN INDEX.		PROTHROMBIN INDEX.
P. ROTUNDUS 3 mg./kg.	50·6 57·7 63·4 60·8	DEGRADED EUCHEUMA 200 mg/kg.	58·1 52·6 84·6 80·6
AVERAGE	58·1	AVERAGE	68·9
HEPARIN 75 i.u/kg.	94·8 100·0 111·4 95·5	HEPARIN 200 i.u/kg.	85·4 98·6 78·1 81·1
AVERAGE	100·4	AVERAGE	85·8
NEMBUTAL (Control) 25mg./kg.	100·8 129·1 99·3 95·5		
AVERAGE	106.1		

TABLE 9

TWO-STAGE PROTHROMBIN TEST

DOSE	CODE	RATIO OF AREAS (%) k	λ	DOSE	CODE	RATIO OF AREAS (%) k	λ
5mg./kg.	CY	70·6	38·3	5mg./kg.	CMI	61·0	64:5
		39·0	38·9			93·0	66·7
		34·7	46·7			42·1	88·9
		74·1	28·6			88·9	51·9
	AVERAGE	54·6	38·1		AVERAGE	71·3	68·0
5mg./kg.	CSE	93·9	57·1	5mg./kg.	CNS	64·5	61·6
		66·7	85·7			64·0	-
		58·3	41·0			70·8	-
		45·0	44·4			52·6	-
	AVERAGE	66:0	57·1		AVERAGE	63·0	-
3mg./kg.	CY	66·7	63·3	3mg./kg.	CMI	45·7	66·7
		51·9	56·0			107·4	56·2
		68·4	33·3			93·0	33·3
		56·9	48·7			80·0	68·8
	AVERAGE	61·0	50·3		AVERAGE	81·5	56·3
3mg./kg.	CSE	42·6	61·3	3mg./kg.	CNS	50·0	30·0
		73·3	48·3			63·2	32·4
		47·2	54·5			46·6	31·9
		71·4	70·0			64·3	50·0
	AVERAGE	58·6	58·6		AVERAGE	56·0	36·1

TABLE 9 (Contd.)

TWO-STAGE PROTHROMBIN TEST

	RATIO OF AREAS (%)		RATIO OF AREAS (%)
P. ROTUNDUS 3mg./kg.	29·4 31·6 35·0 77·3	DEGRADED EUCHEUMA. 200mg./kg.	50·0 9·1 33·3 21·4
AVERAGE	43·3	AVERAGE	17·2
HEPARIN. 75 i.u./kg.	10·7 18·8 31·8 35·4	HEPARIN 200 i.u./kg.	23·5 8.3 0.0 2·8
AVERAGE	24.2	AVERAGE	8·7
NEMBUTAL (Control) 25mg./kg.	120.0 55·6 78·0 80·0		
AVERAGE	83·4		

TABLE 10.

PROTHROMBIN CONSUMPTION TEST

DOSE	CODE	PROTHROMBIN CONSUMPTION INDEX			
		k- CARRAGEENAN		λ - CARRAGEENAN	
		INITIAL	2 HOURS AFTER INJECTION	INITIAL	2 HOURS AFTER INJECTION
5mg./kg.	CY	4·5	35·1	17·5	53·8
		5·7	15·0	3·4	22·9
		2·3	43·0	19·2	36·5
		9·7	31·1	4·1	56·5
	AVERAGE	5·5	31·1	11·1	42·4
5mg./kg.	CMI	1·5	11·9	3·5	42·7
		2·8	17·0	4·1	48·7
		4·2	30·3	8·8	26·0
		3·8	16·0	10·7	147·0
	AVERAGE	3·1	18·8	6·8	66·1
5mg./kg.	CSE	5·5	21·5	39·3	75·0
		6·9	33·3	32·8	156·5
		3·5	16·7	20·7	105·0
		4·0,	94·7	4·4	124·3
	AVERAGE	5·0	41·6	24·3	115·2
5mg./kg.	CNS	2·5	30·0	3·9	20·0
		3·1	26·3	-	-
		11·9	67·7	-	-
		9·4	61.5	-	-
	AVERAGE	6·7	46·4	-	-

TABLE 10. (Contd.)

DOSE	CODE	PROTHROMBIN OONSUMPTION INDEX			
		k- CARRAGEENAN		λ - CARRAGEENAN	
		INITIAL	2 HOURS AFTER INJECTION	INITIAL	2 HOURS AFTER INJECTION
3mg./kg.	CY	3·9	31·3	6·3	13·8
		7·0	57·1	7·3	95·7
		30·0	22·1	5·5	38·2
		1·7	10·6	2·6	50·0
	AVERAGE	10·7	30·4	5·4	49·4
3mg./kg.	CMI	18·3	35·3	24·0	10·9
		10·3	42·6	6·4	97·2
		4·6	23·2	11·5	77·9
		3·8	13·3	14·3	38·2
	AVERAGE	9·3	28·6	14·1	56·1
3mg./kg.	CSE	5·3	31·6	4·7	22·7
		14·9	28·6	2·7	15·5
		28·6	7·1	2·7	18·1
		3·3	20·8	4·3	60·7
	AVERAGE	12·8	22·0	3·6	29·3
3mg./kg.	CNS	13·8	11·5	3·2	83·3
		6·2	100·0	6·6	90·0
		6·7	109·0	5·5	12·4
		7·7	68·4	8·4	22·7
	AVERAGE	8·6	72·2	5·9	52·1

TABLE 10. (Contd.)

	PROTHROMBIN CONSUMPTION INDEX			PROTHROMBIN CONSUMPTION INDEX	
	INITIAL	2 HOURS AFTER INJECTION		INITIAL	2 HOURS AFTER INJECTION
P. ROTUNDUS	12.0	90.3	DEGRADED	8.2	142.0
3mg./kg.	8.9	104.0	EUCHEUMA	15.2	82.1
	15.8	235.4	200mg/kg.	10.1	79.8
	14.0	154.5		19.0	111.0
AVERAGE	12.7	146.1		10.6	104.7
		0.5 HOUR AFTER INJECTION			0.5 HOUR AFTER INJECTION
HEPARIN	5.1	4.4	HEPARIN	3.6	51.5
75i.u./kg.	7.7	5.1	200 i.u./kg	4.2	303.0
	4.0	6.9		10.4	447.3
	2.2	3.4		3.0	536.2
AVERAGE	4.8	5.0		5.3	334.5
NEMBUTAL	3.6	4.1			
(Control)	7.3	5.3			
25mg./kg.	9.0	1.5			
	20.0	12.0			
AVERAGE	10.0	5.7			

TABLE 11

THROMBOPLASTIN GENERATION TEST

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS).							
k - CY 5 mg./kg.	Initial 2 Hours after Injection	1.	43,	23,	12,	12,	12,	12,	12,	12.
			47,	55,	52,	41,	51,	32,	21,	18.
"	"	2.	33,	38,	23,	15,	15,	15,	15,	15.
			65,	52,	42,	46,	32,	20,	16,	16.
"	"	3.	86,	34,	14,	12,	10,	11,	10,	10.
			65,	55,	55,	53,	47,	40,	33,	26.
"	"	4.	43,	9,	6,	5,	4,	4,	5,	5.
			52,	53,	43,	28,	17,	15,	12,	13.
λ - CY 5 mg./kg.	Initial 2 Hours after Injection	1.	60,	17,	9,	8,	6,	6,	6,	6.
			69,	79,	92,	81,	112,	74,	45,	40.
"	"	2.	100,	35,	9,	8,	6,	6,	6,	6.
			122,	110,	120,	110,	85,	94,	65,	36.
"	"	3.	68,	60,	25,	16,	14,	14,	15,	16.
			60,	70,	65,	74,	58,	61,	55,	61.
		4.	45;	6,	5,	5,	5,	5,	4,	5.
			57,	52,	35,	33,	26,	24,	23,	23.
k - CM1 5mg./kg.	Initial 2 Hours after Injection	1.	25,	6,	5,	4,	5,	4,	4,	5.
			40,	9,	7,	6,	5,	5,	4,	5.
"	"	2.	17,	5,	5,	4,	4,	5,	4,	4.
			27,	12,	10,	8,	8,	7,	7,	8.
"	"	3.	12,	5,	4,	4,	4,	4,	4,	4.
			34,	26,	10,	10,	9,	8,	8,	9.
"	"	4.	11,	6,	4,	4,	4,	4,	3,	4.
			47,	32,	13,	10,	9,	8,	9,	8.

TABLE 11 (Contd.)

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS.)							
λ- CM1 5 mg./kg.	Initial 2 Hours after Injection	1.	11,	6,	4,	3,	4,	4,	3,	4.
			58,	60,	46,	26,	24,	17,	16,	16.
"	"	2.	31,	5,	5,	4,	5,	4,	4,	4.
			48,	51,	48,	53,	32,	21,	18,	22.
"	"	3.	13,	7,	5,	4,	5,	4,	4,	4.
			46,	50,	35,	21,	16,	14,	14,	11.
"	"	4.	23,	11,	9,	8,	8,	8,	8,	7.
			70,	50,	45,	54,	55,	54,	48,	51.
k - CSE 5 mg./kg.	Initial 2 Hours after Injection	1.	45,	13,	7,	6,	6,	5,	6,	5.
			50,	27,	18,	12,	10,	10,	10,	9.
"	"	2.	20,	5,	4,	4,	4,	5,	5,	4.
			29,	24,	15,	10,	9,	9,	8,	9.
"	"	3.	18,	6,	6,	4,	5,	5,	5,	5.
			50,	60,	55,	50,	37,	27,	26,	22.
"	"	4.	7,	5,	5,	5,	5,	5,	5,	5.
			32,	30,	19,	15,	12,	14,	15,	12.
Soln. λ - CSE 5 mg./kg.	Initial 2 Hours after Injection	1.	12,	6,	5,	5,	5,	5,	5,	6.
			87,	92,	79,	80,	73,	92,	85,	70.
"	"	2.	27,	8,	8,	7,	6,	7,	7,	7.
			98,	38,	25,	30,	28,	23,	28,	28.
"	"	3.	55,	17,	7,	6,	6,	6,	6,	6.
			90,	64,	80,	67,	67,	64,	54,	43.
"	"	4.	30,	9,	6,	6,	6,	5,	5,	5.
			97,	75,	55,	63,	54,	56,	48,	30.

TABLE 11 (Contd.)

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS.)							
k - CNS 5 mg./kg.	Initial 2 Hours after Injection	1.	25,	6,	5,	4,	4,	3,	4,	4.
			37,	38,	17,	12,	9,	10,	10,	10.
"	"	2.	27,	6,	5,	4,	4,	4,	4,	4.
			37,	35,	25,	22,	14,	14,	14,	15.
"	"	3.	30,	7,	7,	7,	7,	6,	6,	6.
			46,	11,	8,	6,	6,	6,	6,	6.
"	"	4.	34,	7,	5,	4,	5,	4,	4,	4.
			48,	28,	14,	13,	12,	11,	11,	12.
λ - CNS 5 mg./kg. (1 result only)	Initial 2 Hours after Injection		33,	9,	8,	6,	6,	6,	5,	6.
			75,	60,	55,	31,	30,	25,	24,	28.
k - CY 3 mg./kg.	Initial 2 Hours after Injection	1.	15,	7,	6,	5,	5,	5,	4,	5.
			34,	24,	26,	15,	12,	12,	12,	13.
"	"	2.	35,	7,	6,	6,	5,	5,	5,	5.
			40.	27,	15,	11,	12,	11,	11,	11.
"	"	3.	34,	12,	10,	9,	8,	7,	7,	7.
			24,	15,	9,	10,	9,	9,	8,	8.
"	"	4.	28,	7,	5,	4,	4,	5,	4,	4.
			44,	35,	16,	10,	12,	11,	12,	12.
λ - CY 3 mg/kg.	Initial 2 Hours after Injection	1.	7,	6,	5,	4,	4,	4,	4,	4.
			40,	41,	28,	22,	19,	17,	18,	19.
"	"	2.	16,	7,	5,	5,	4,	4,	4,	4.
			39,	34,	23,	19,	15,	16,	15,	16.
"	"	3.	36,	20,	9,	7,	6,	6,	5,	5.
			39,	51,	52,	47,	36,	22,	16,	13.
"	"	4.	30,	12,	8,	6,	5,	5,	4,	5.
			73,	65,	62,	65,	43,	33,	20,	18.

TABLE 11 (Contd.)

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS.)							
k - CM1 3 mg./kg.	Initial 2 Hours after Injection.	1.	16,	8,	7,	6,	7,	8,	9,	9.
			32,	20,	15,	15,	15,	14,	14,	15.
"	"	2.	36,	15,	7,	8,	8,	8,	7,	8.
			46,	23,	11,	8,	9,	8,	9,	8.
"	"	3.	38,	6,	6,	7,	7,	7,	7,	7.
			42,	13,	8,	8,	9,	8,	8,	9.
"	"	4.	8,	6,	6,	5,	6,	6,	6,	6.
			13,	8,	8,	9,	8,	8,	8,	8.
λ- CM1 3 mg./kg.	Initial 2 Hours after Injection	1.	18,	7,	7,	7,	7,	8,	7,	7.
			70,	51,	19,	23,	16,	20,	16,	17.
"	"	2.	31,	12,	10,	9,	9,	8,	8,	9.
			65,	60,	60,	47,	29,	23,	17,	17.
"	"	3.	40,	9,	8,	7,	7,	7,	7,	7.
			59,	55,	23,	19,	15,	16,	15,	16.
"	"	4.	21,	5,	6,	6,	5,	6,	6,	6.
			40,	40,	15,	13,	13,	12,	11,	12.
Soln. k - CSE 3 mg./kg.	Initial 2 Hours after Injection.	1.	7,	3,	3,	4,	3,	3,	4,	4.
			44,	40,	15,	10,	10,	9,	9,	10.
"	"	2.	6,	6,	4,	4,	4,	5,	4,	4.
			48,	40,	38,	20,	15,	20,	15,	16.
"	"	3.	45,	20,	10,	9,	7,	6,	6,	6.
			48,	21,	8,	9,	7,	7,	6,	8.
"	"	4.	32,	8,	5,	4,	4,	4,	3,	4.
			84,	54,	21,	15,	11,	11,	11,	10.

TABLE 11 (Contd.)

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS.)							
Soln. λ- CSE 3 mg./kg.	Initial 2 Hour after Injection	1.	37,	10,	7,	7,	6,	6,	6,	6.
			66,	49,	60,	62,	41,	40,	26,	26.
"	"	2.	49,	23,	10,	8,	9,	8,	7,	8.
			65,	91,	60,	48,	68,	56,	45,	31.
"	"	3.	36,	8,	3,	4,	3,	3,	4,	4.
			29,	9,	6,	7,	7,	7,	7,	7.
"	"	4,	26,	6,	5,	5,	5,	4,	4,	5.
			45,	53,	45,	35,	20,	15,	16,	15.
k - CNS 3 mg./kg.	Initial 2 Hours after Injection.	1.	36,	8,	7,	7,	6,	6,	6,	7.
			70,	78,	30,	16,	16,	16,	15,	17.
"	"	2.	8,	5,	8,	7,	7,	7,	7,	7.
			13,	8,	8,	9,	8,	8,	9,	9.
"	"	3.	6,	6,	6,	5,	5,	6,	6,	6.
			33,	24,	17,	17,	17,	17,	18,	19.
"	"	4.	71,	7,	6,	6,	6,	6,	6,	6.
			66,	60,	35,	16,	15,	14,	17,	15.
λ- CNS 3 mg./kg.	Initial 2 Hours after Injection	1.	11,	6,	5,	4,	4,	4,	5,	4.
			37,	40,	31,	32,	27,	22,	20,	24.
"	"	2.	19,	11,	9,	9,	7,	8,	6,	8.
			32,	30,	35,	35,	38,	34,	31,	28.
"	"	3.	15,	12,	6,	5,	5,	4,	5,	4.
			36,	21,	17,	16,	14,	16,	15,	15.
"	"	4.	18,	6,	5,	5,	4,	4,	5,	4.
			36,	32,	22,	16,	16,	16,	17,	16.

TABLE 11 (Contd.)

CODE AND DOSE			SUBSTRATE CLOTTING TIMES (IN SECS.)							
P. ROTUNDUS 3 mg./kg.	Initial 2 Hours after Injection	1.	60,	15,	8,	7,	7,	7,	7,	7.
			80,	50,	36,	21,	18,	18,	16,	18.
"	"	2.	14,	7,	7,	6,	6,	7,	7,	7.
			77,	55,	50,	51,	18,	16,	16,	15.
"	"	3.	34,	9,	6,	6,	6,	6,	6,	6.
			97,	39,	13,	14,	11,	13,	13,	14.
"	"	4.	7,	3,	6,	4,	6,	6,	5,	5.
			60,	9,	9,	9,	9,	10,	10,	10.
DEGRADED EUCHEUMA CARRAGEENAN. 200 mg./kg.	Initial 2 Hours after Injection	1.	42,	58,	22,	11,	10,	12,	12,	12.
			89,	83,	68,	104,	76,	89,	99,	85.
"	"	2.	56,	55,	32,	7,	6,	6,	6,	7.
			130,	-	87,	92,	88,	98,	90,	96.
"	"	3.	40,	42,	23,	12,	12,	12,	13,	13.
			50,	55,	50,	46,	90,	50,	51,	50.
"	"	4.	69,	31,	20,	17,	19,	22,	23,	26.
			70,	87,	110,	90,	91,	97,	71,	84.
HEPARIN 75 1.U./kg.	Initial ½ Hour after Injection	1.	28,	6,	4,	4,	5,	5,	5,	4.
			45,	29,	21,	13,	12,	12,	11,	11.
"	"	2.	43,	8,	5,	5,	4,	4,	5,	5.
			44,	28,	13,	9,	9,	8,	8,	8.
"	"	3.	21,	5,	4,	3,	3,	3,	4,	3.
			23,	11,	6,	5,	5,	5,	5,	5.
"	"	4.	37,	5,	4,	4,	4,	3,	3,	4.
			29,	19,	10,	8,	7,	6,	6,	6.

TABLE 11 (Contd.)

CODE AND DOSE		SUBSTRATE CLOTTING TIMES (IN SECS.)							
HEPARIN 200 I.U./kg.	Initial $\frac{1}{2}$ Hour after Injection	1.	31,	7,	5,	5,	5,	5,	4,	4.
			49,	50,	45,	42,	44,	48,	42,	50.
"	"	2.	65,	44,	14,	9,	9,	7,	8,	7.
			32,	32,	49,	36,	39,	36,	39,	32.
"	"	3.	25,	6,	5,	5,	4,	4,	4,	4.
			40,	42,	41,	33,	35,	29,	36,	29.
"	"	4.	30,	10,	6,	5,	5,	4,	4,	5.
			33,	25,	32,	30,	27,	37,	37,	32.
NEMBUTAL (Control)	Initial after Injection.	1.	20,	6,	4,	3,	4,	4,	3,	4.
			10,	5,	4,	1,	4,	4,	4,	4.
"	"	2.	60,	9,	7,	7,	7,	7,	6,	6,
			16,	7,	6,	6,	6,	6,	6,	6.
"	"	3.	35,	9,	6,	5,	5,	5,	4,	4.
			30,	11,	7,	5,	5,	5,	4,	4.
"	"	4.	31,	6,	5,	5,	4,	4,	4,	4.
			18,	6,	4,	4,	4,	3,	4,	4.

TABLE 12

PLATELET COUNTS (IN VITRO)

IN THOUSANDS/cu.mm. BLOOD.

	CONCENTRATION OF CARRAGEENAN				
	1%	0·5%	0·1%	0·05%	0·01%
k - CY	109	122	135	83	65
λ - CY	223	142	186	95	80
POLYIDES ROTUNDUS	108	184	166	217	113
DEGRADED EUCHEUMA	370	352	347	359	299
HEPARIN	431	348	433	473	383

CONTROL PLATELET COUNTS

Rabbit No.	Count
120B	373, 421, 394, 397, 294, 380.
122B	310, 250, 410, 394.
128B	380.
130B	444

TABLE 12A

COMPARISON AT APPROXIMATE PHYSIOLOGICAL CONCENTRATIONS

SUBSTANCE	CONCENTRATION	PLATELET COUNT
k - CY	0·01%	65
λ - CY	0·01%	80
POLYIDES ROTUNDUS	0·01%	113
DEGRADED EUCHEUMA	0·5%	352
HEPARIN	0 01%	383

TABLE 12B

PLATELET COUNTS (IN VIVO)

SUBSTANCE	COAGn. TIME	PLATELET COUNT	COMMENTS
k - CY	10 mins.	210	Considerable agglutination No severe toxic symptoms apparent. Survived.
λ - CY	14 mins.	60	No agglutination observed. Few free platelets. Rabbit severely dyspnoeic, died overnight.
DEGRADED EUCHEUMA (4 Rabbits)	15-20 mins.	300-350	No agglutination observed. Normal free platelets. Non-toxic.

TABLE 13

FIBRINOGEN - CARRAGEENAN REACTIONS

CARRAGEENAN	CONCENTRATION OF CARRAGEENAN (mg/ml)							
	0·625	0·313	0·157	0·079	0·04	0·02	0·01	0·005
CY λ-CARRAGEENAN	-	-	-	-	+	+	++	+++
CNS "	-	-	-	-	0	0	++	+++
CSE "	-	-	-	-	-	0	+	++
CM1 "	-	-	-	-	-	-	0	0
POLYIDES ROTUNDUS	-	-	-	-	-	0	+	++
CY k-CARRAGEENAN	-	-	-	0	+	+	+	+
CNS "	-	0	0	0	+	+	+	+
CSE "	-	-	-	-	-	-	0	0
CM1 "	-	-	-	-	-	-	0	0
HEPARIN "	-	-	-	-	-	-	0	0
	3·13	1·57	0·79	0·40	0·20	0·10	0·05	0·025
DEGRADED EUCHEUMA CARRAGEENAN	-	-	-	-	-	-	-	-

\- clearing of opalescent solution

0 no observable effect

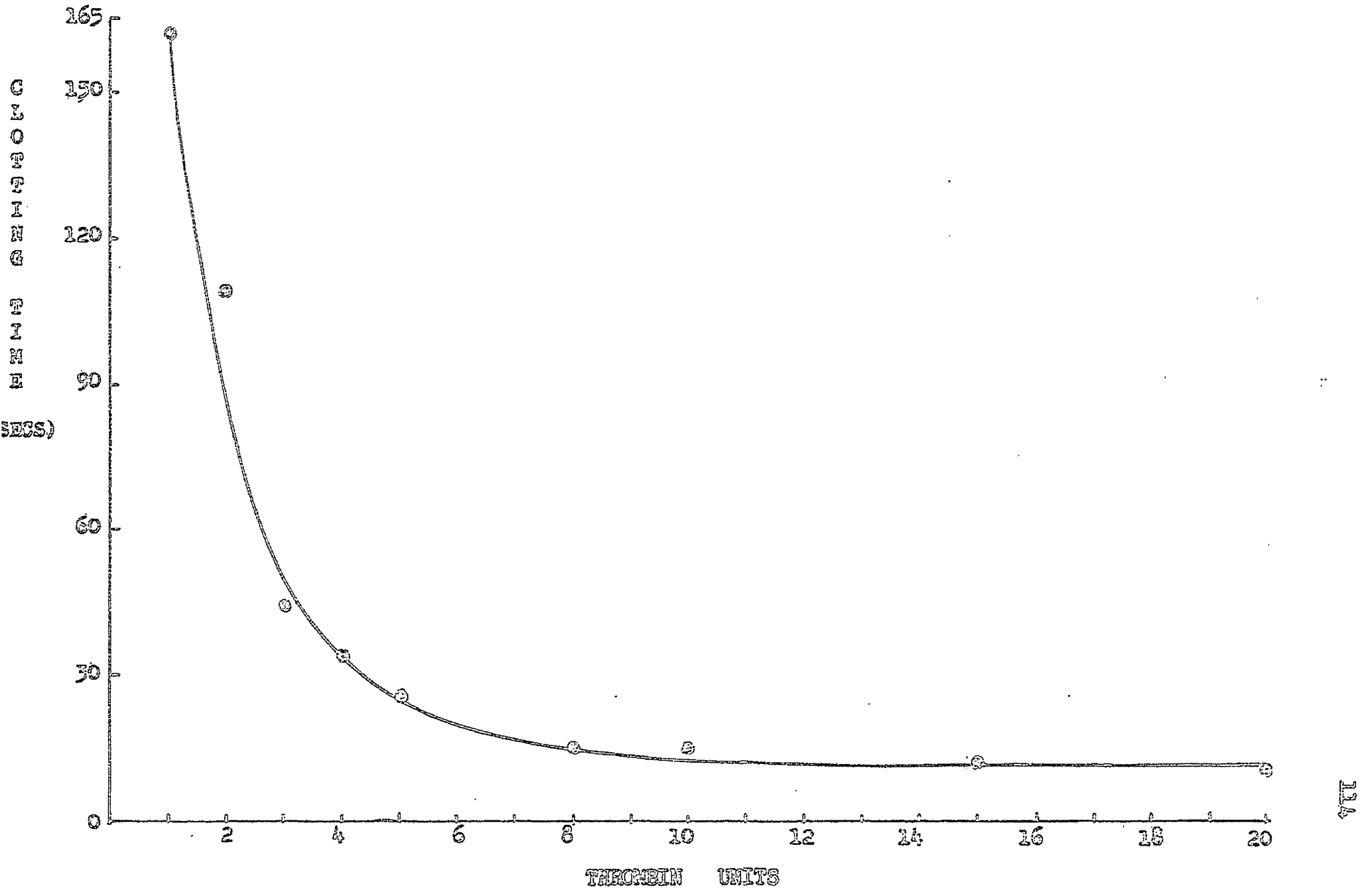
\+ precipitate formed

+++ marked precipitation

λ - carrageenans and Polyides gave granular precipitates

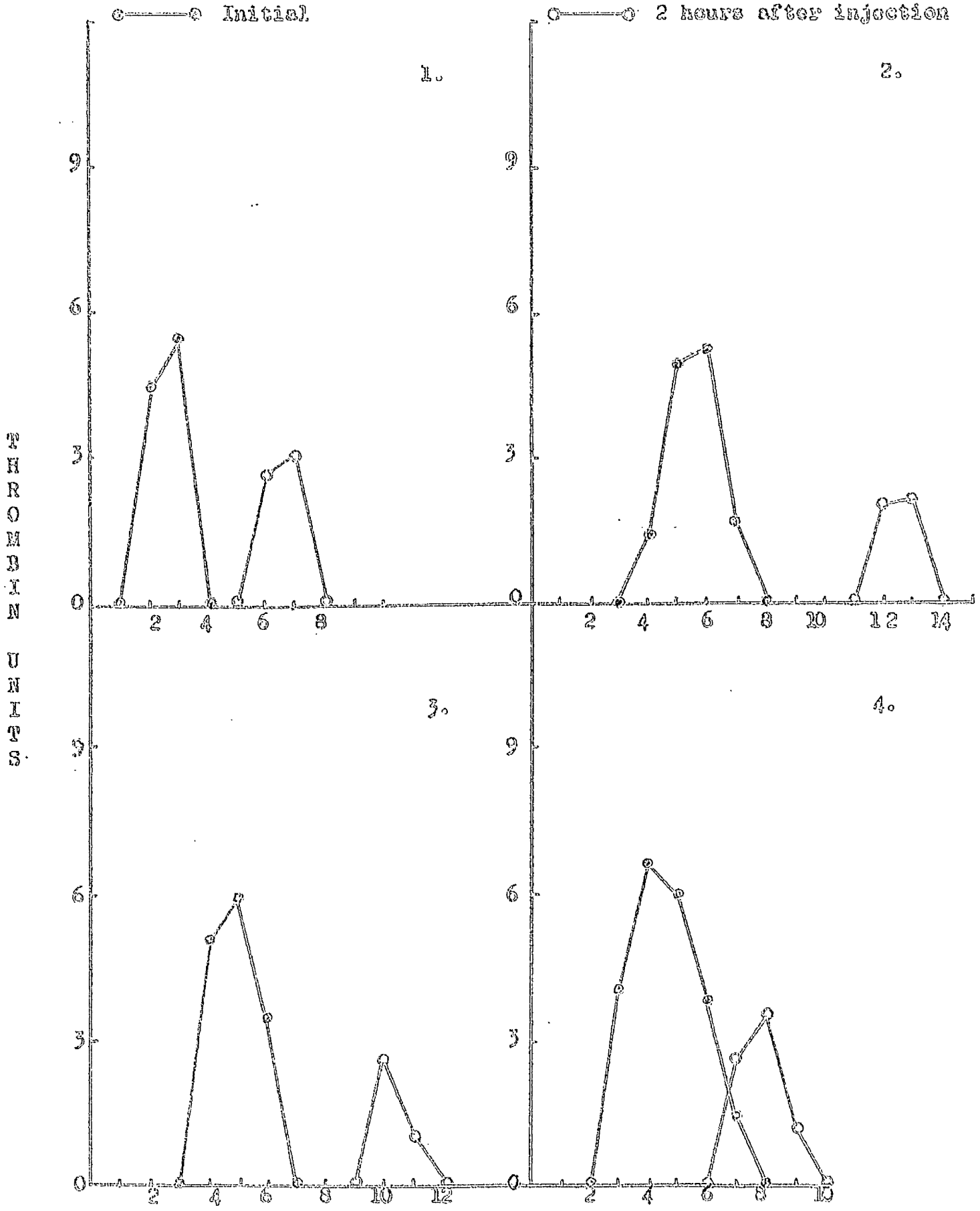
k - carrageenans gave colloidal precipitates.

A P P E N D I X 1


THROMBIN - FIBRINOGEN DILUTION CURVE
CLOTTING TIME (SECS)
165
150
120
90
60
30
0
2
4
6
8
10
12
14
16
18
20
THROMBIN UNITS

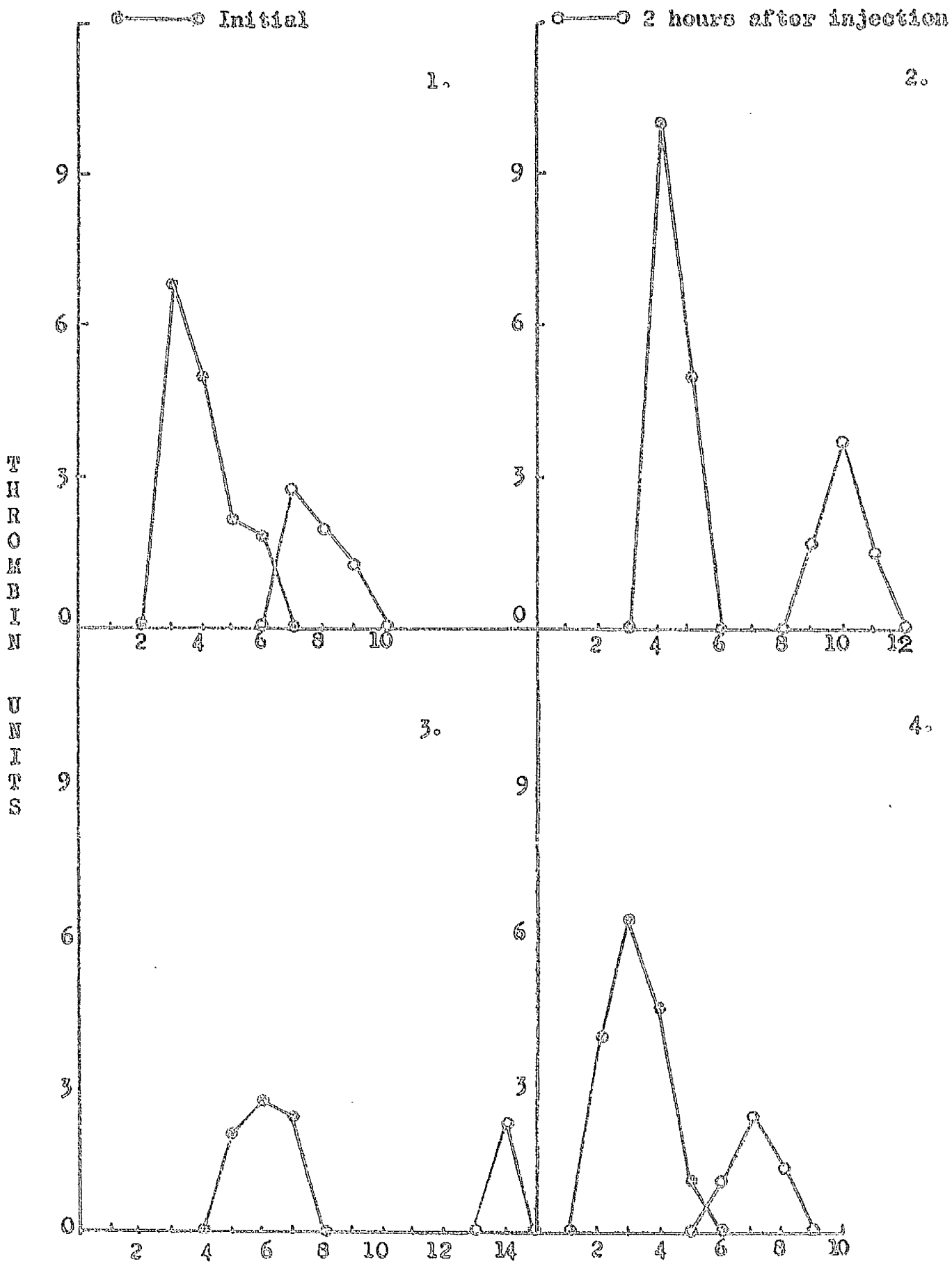
Chondrus crispus (k-CY) 5mg./kg.

o——o Initial o——o 2 hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (λ-CY) 5mg./kg.

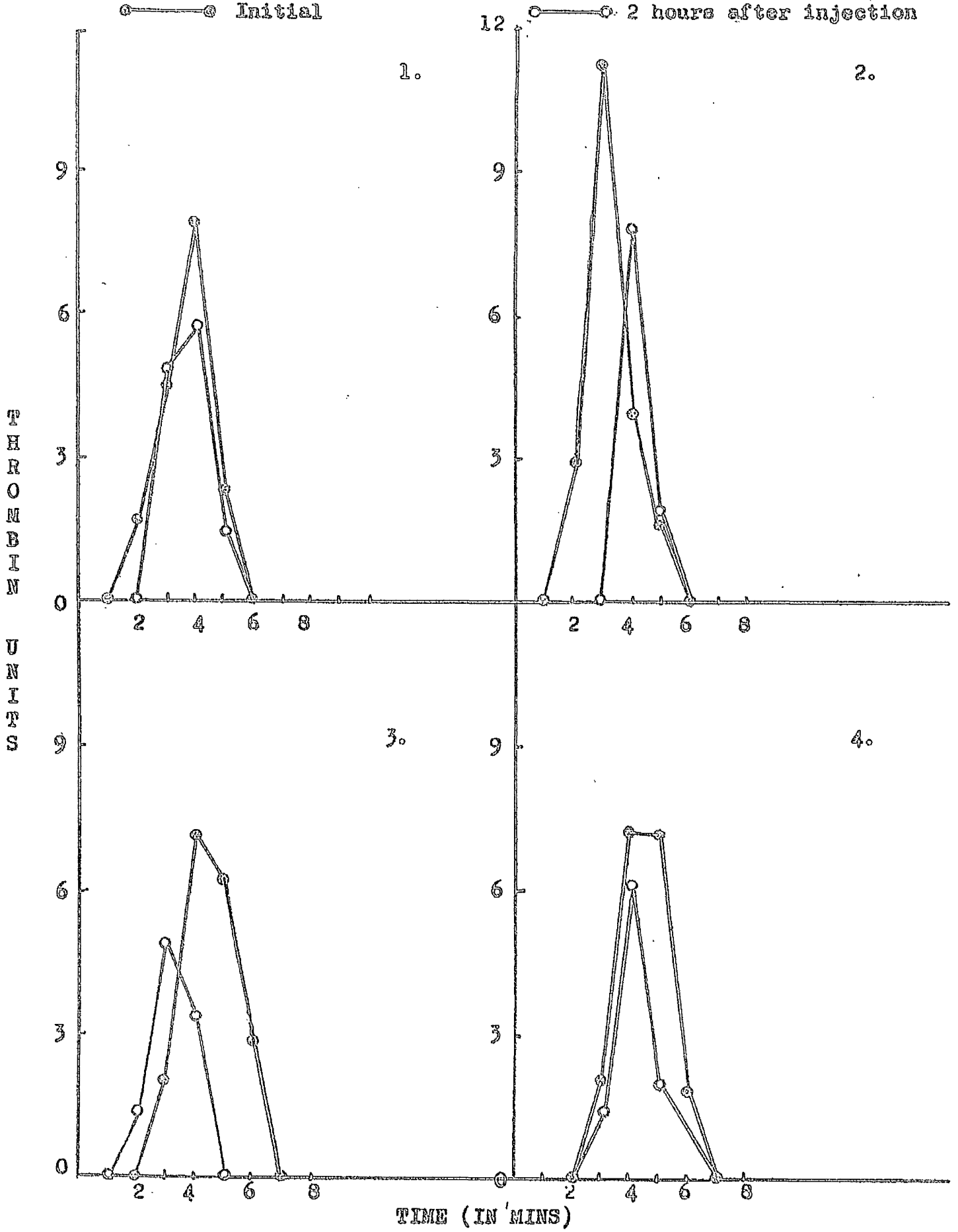


THROMBIN GENERATION TEST.

Chondrus crispus (k-CNI) 5mg./kg.

o——o Initial

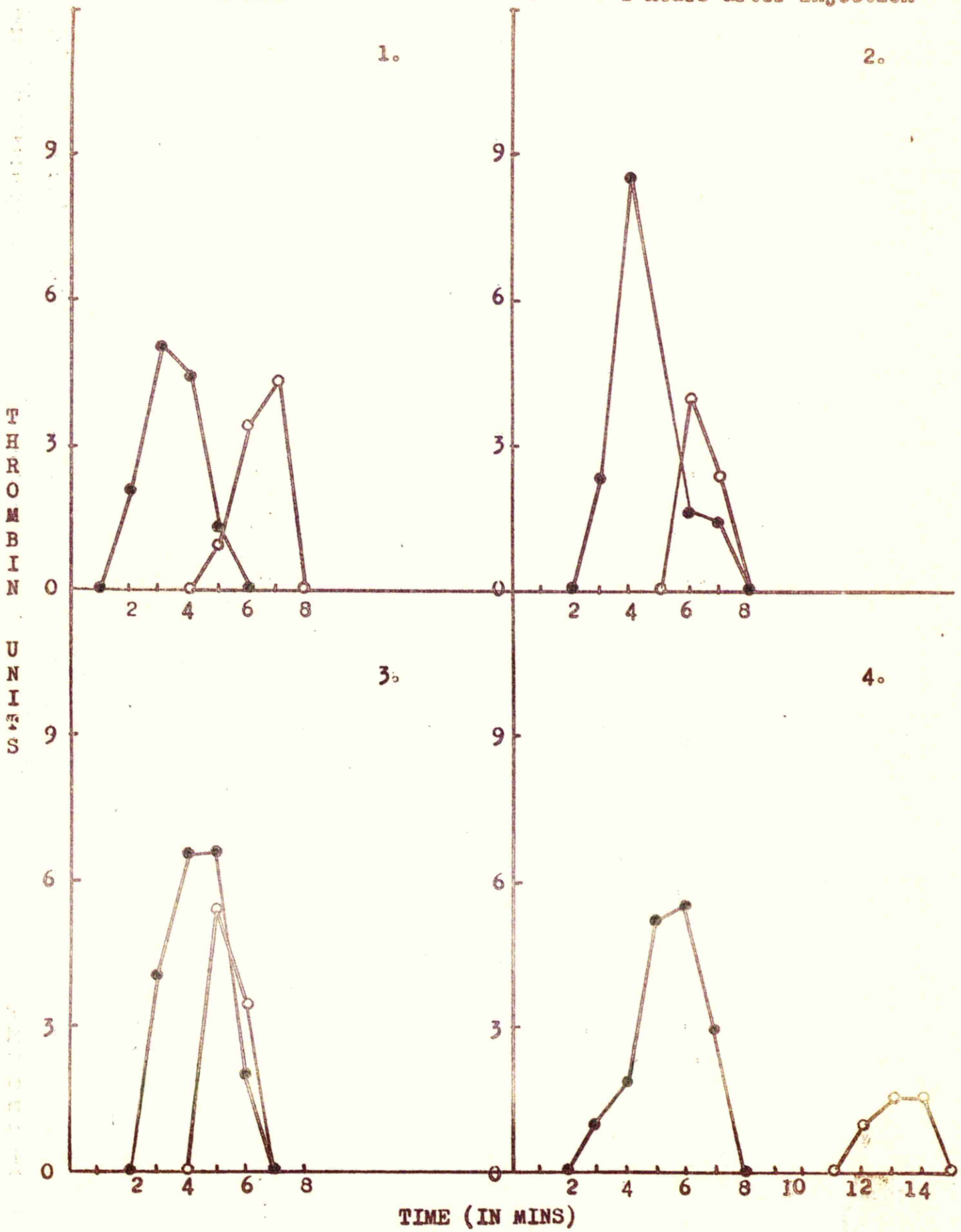
o——o 2 hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (λ-CMI) 5mg./kg.

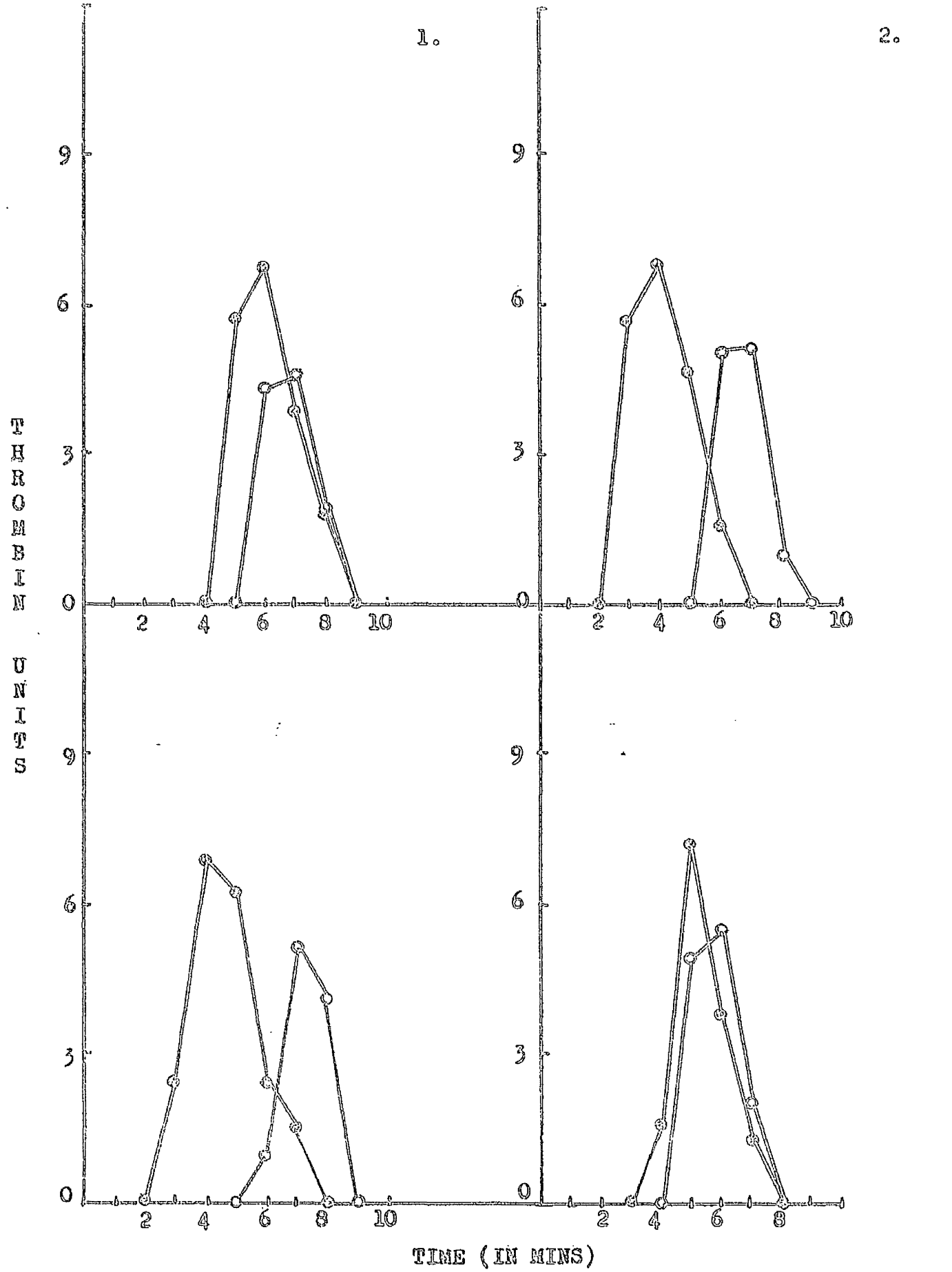
●——● Initial ○——○ 2 hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (k-CSE) 5mg./kg.

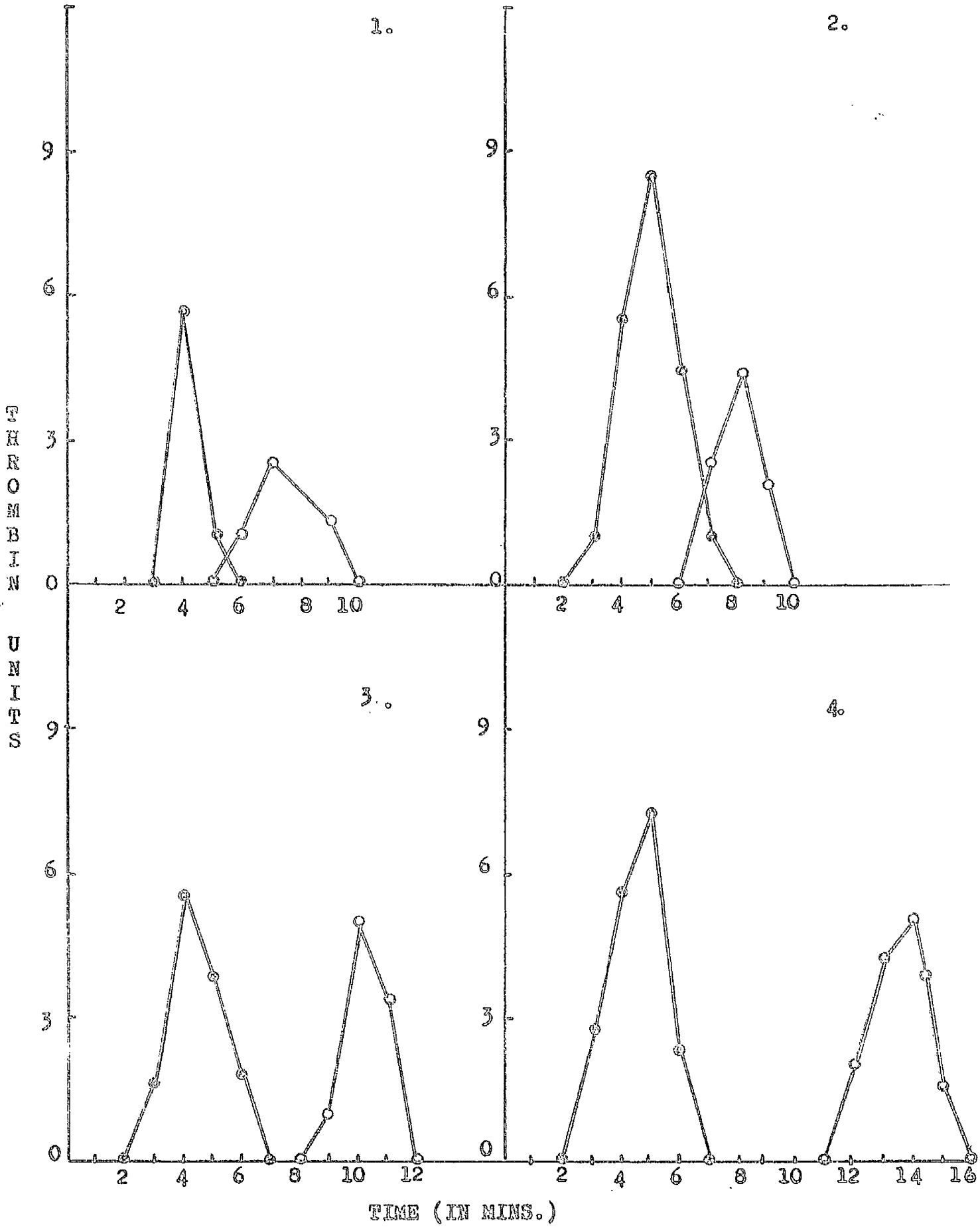
⊖——⊖ Initial ○——○ 2 hours after injection



THROMBIN GENERATION TEST

Chondrus crispus (λ-CSE) 5mg./kg.

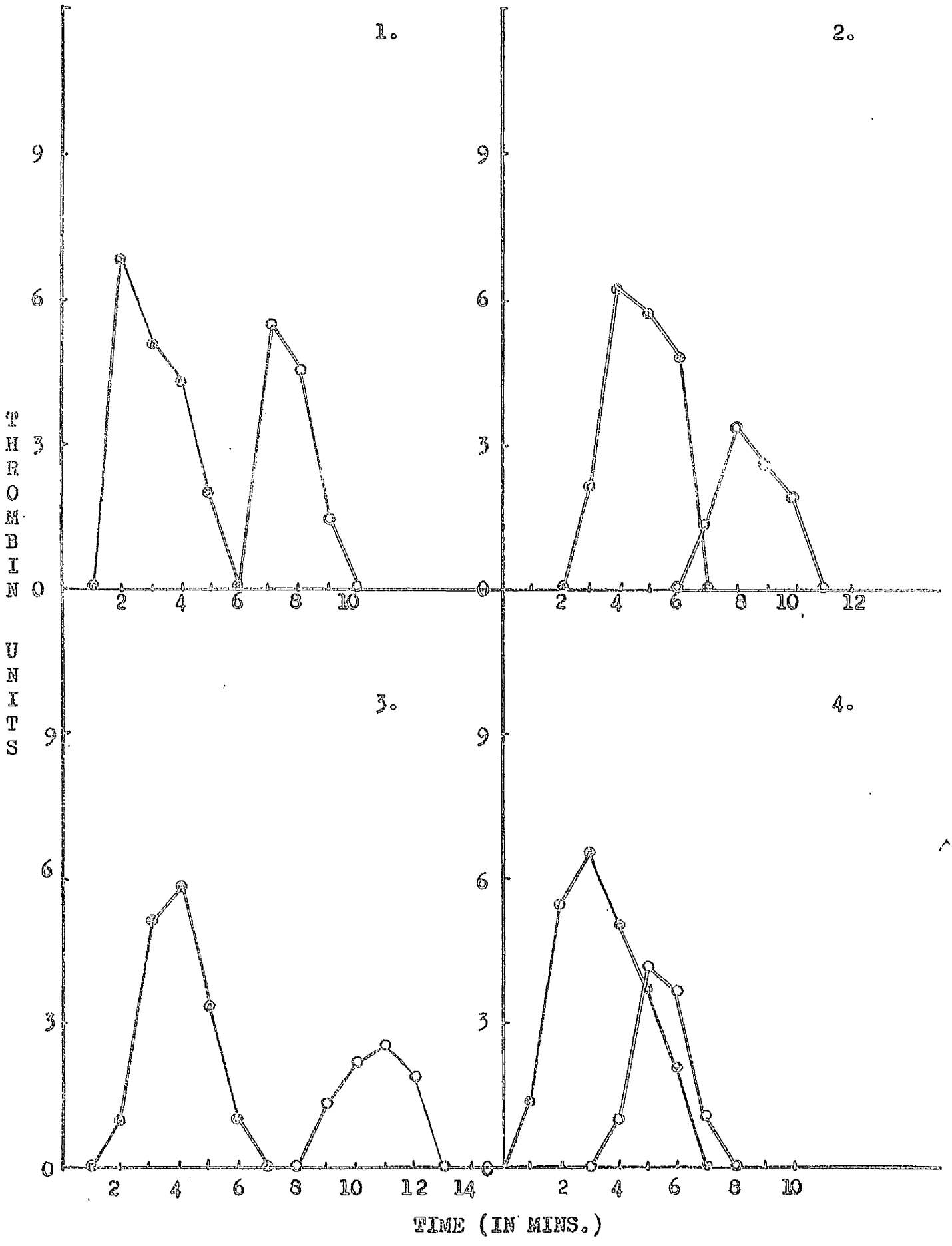
Initial — 2 hours after injection



THROMBIN GENERATION TEST.

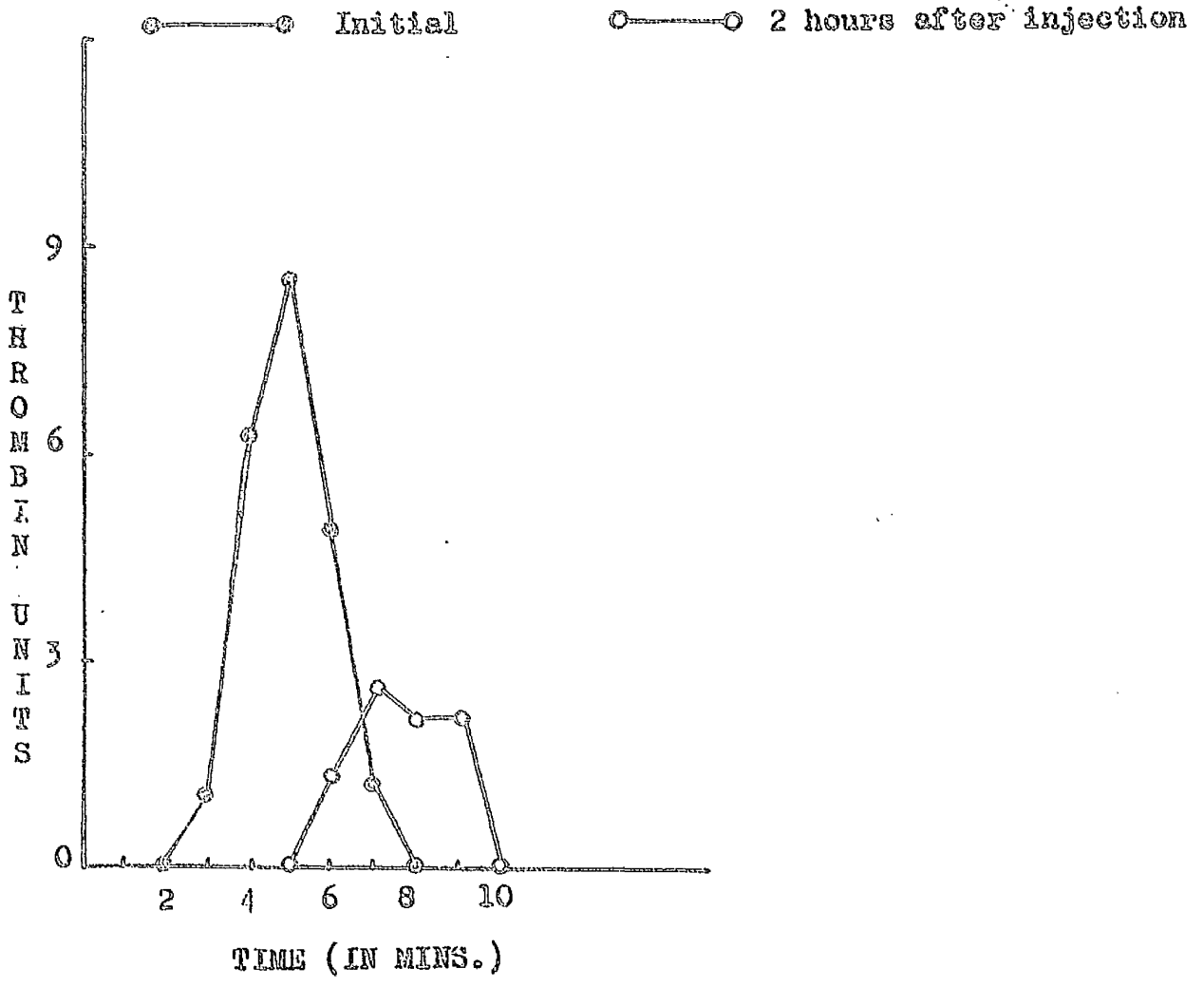
Chondrus crispus (k-CNS) 5 mg./kg.

Initial — 2 hours after injection



THROMBIN GENERATION TEST.

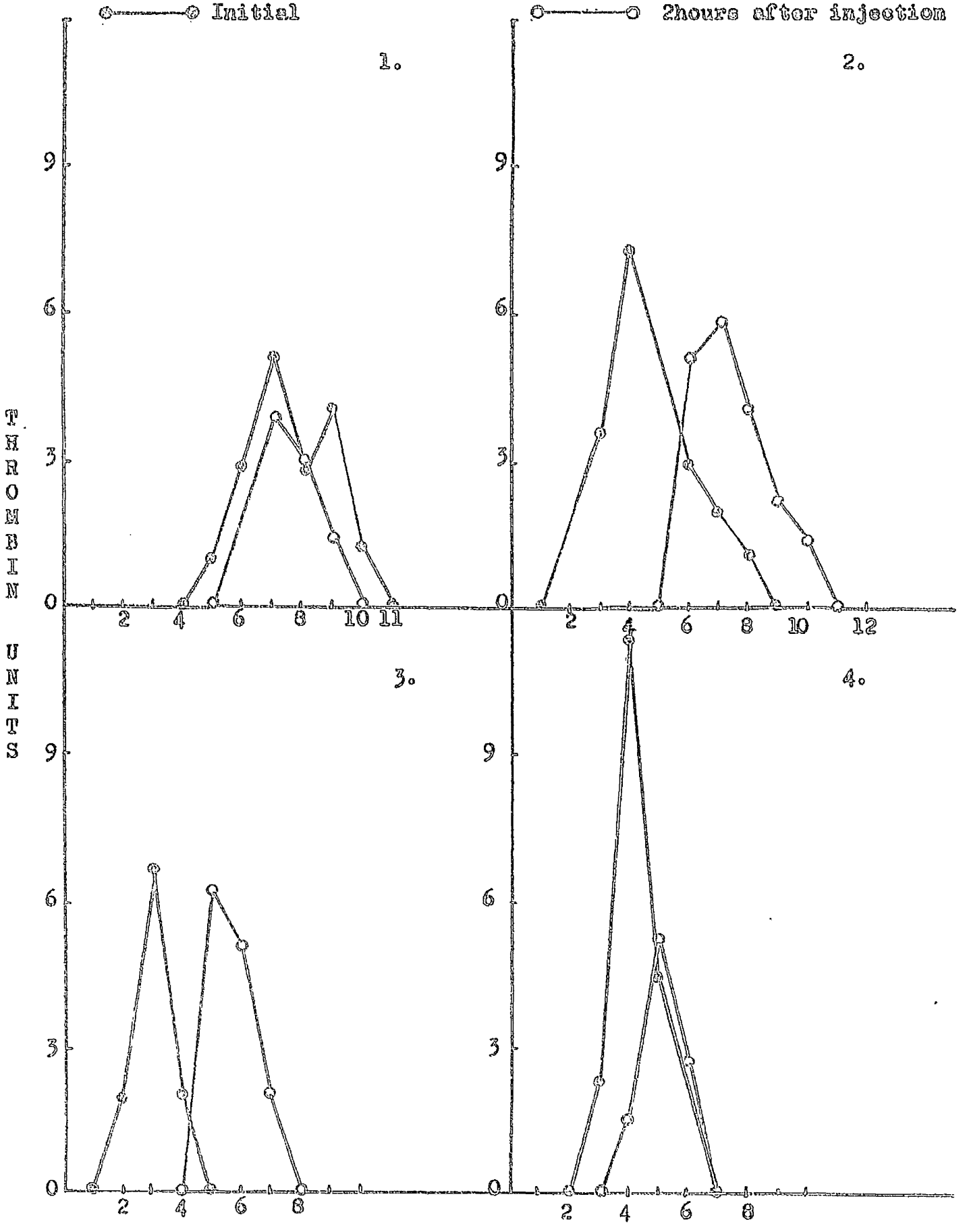
Chondrus crispus (λ-CNS) 5mg./kg.



THROMBIN GENERATION TEST.

Chondrus crispus (k-CY) 3mg./kg.

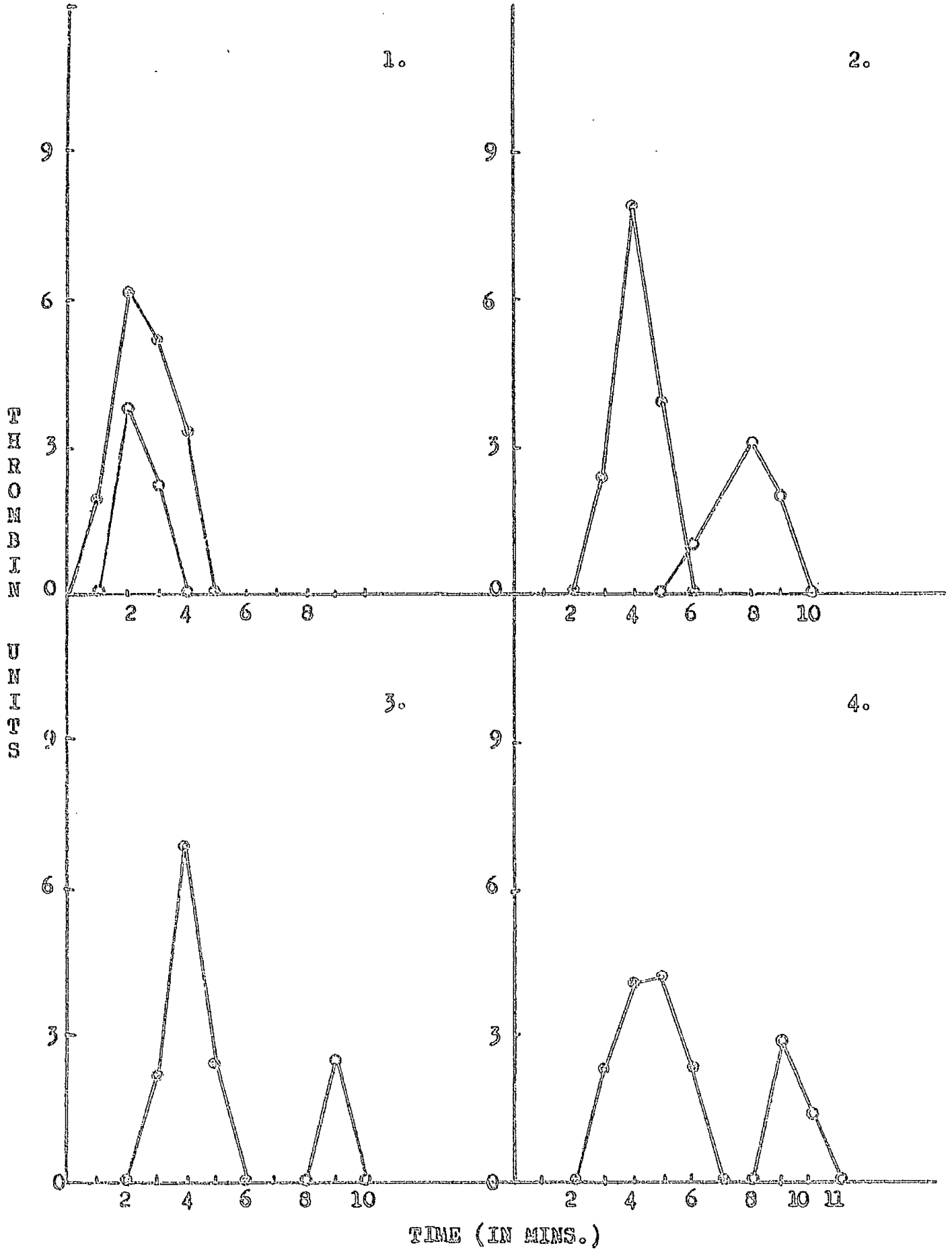
Initial — 2hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (λ-CY) 3mg./kg.

Initial — 2 hours after injection

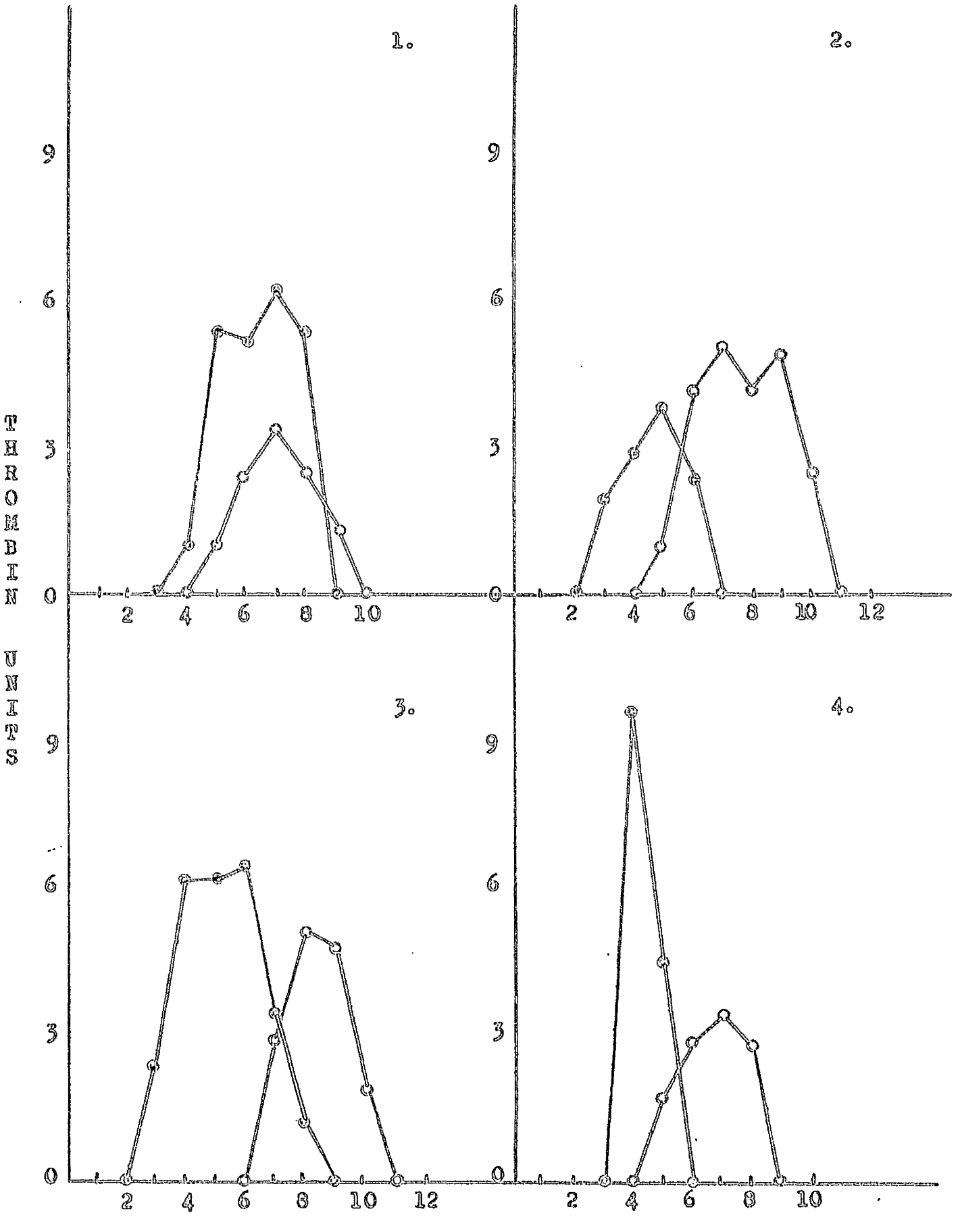


THROMBIN GENERATION TEST.

Chondrus crispus (k-CMI) 3mg./kg.

Initial

2 hours after injection

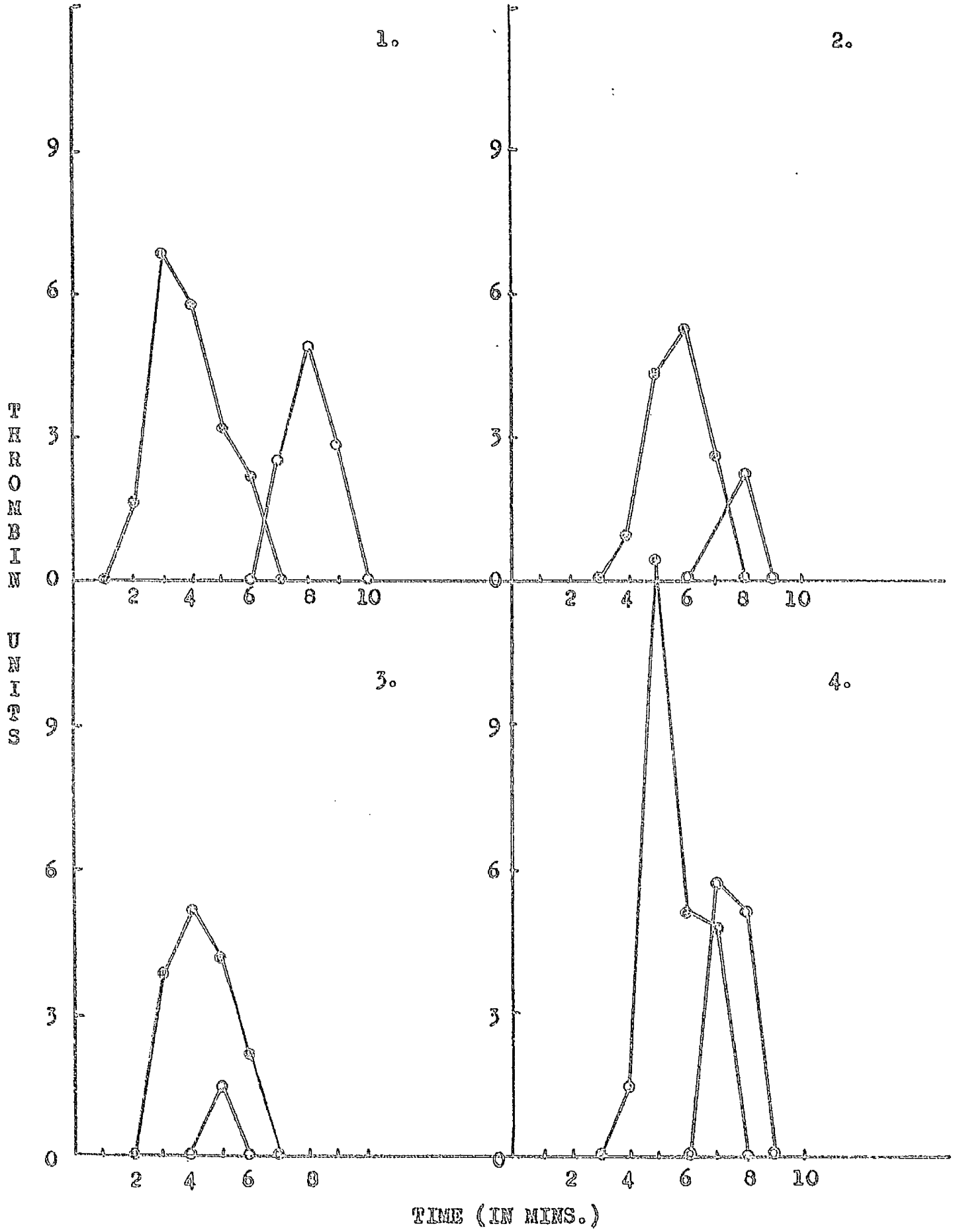


THROMBIN GENERATION TEST.

Chondrus crispus (λ-CMI) 3mg./kg.

⊙——⊙ Initial

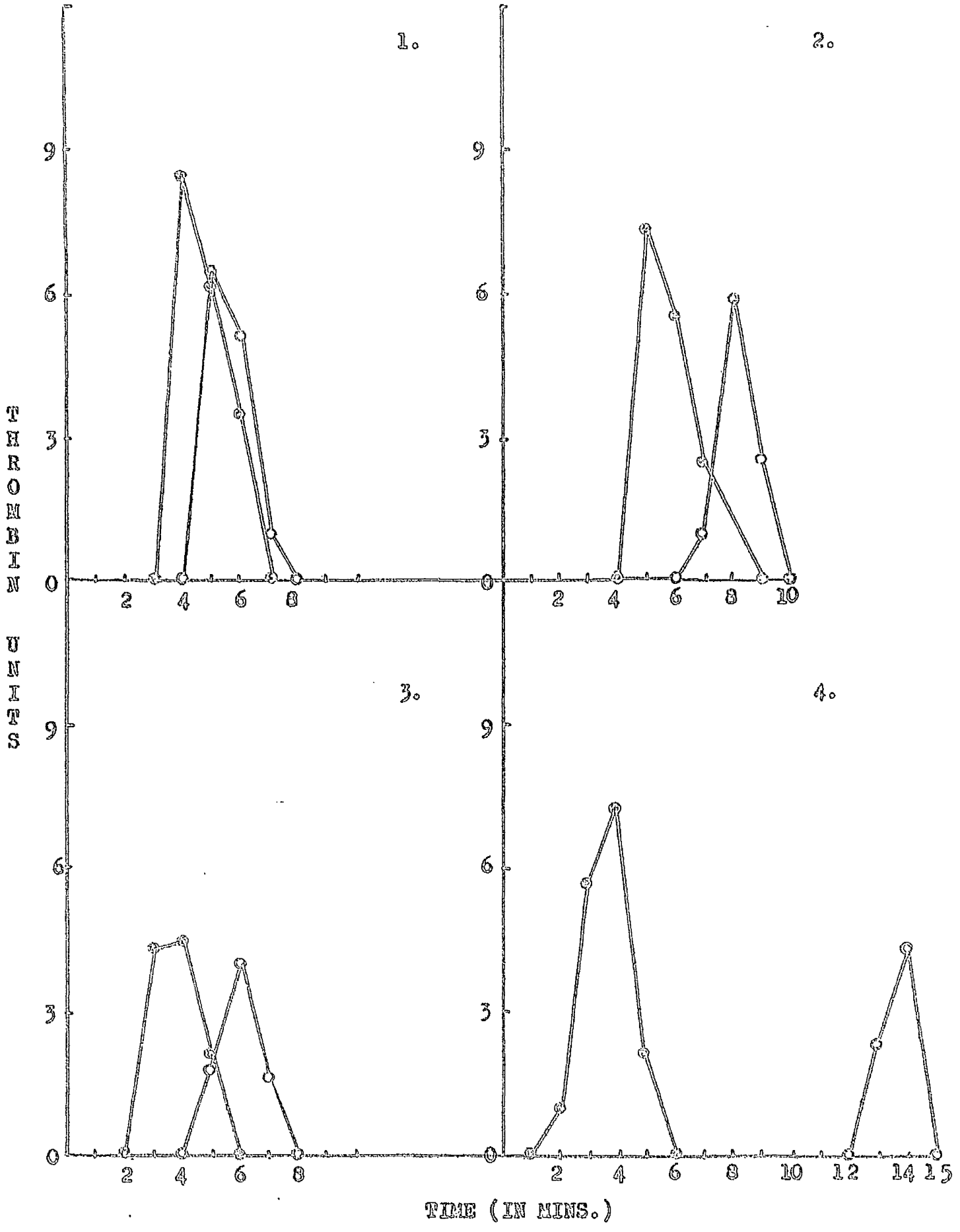
O——O 2 hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (k-CSE) 3mg./kg.

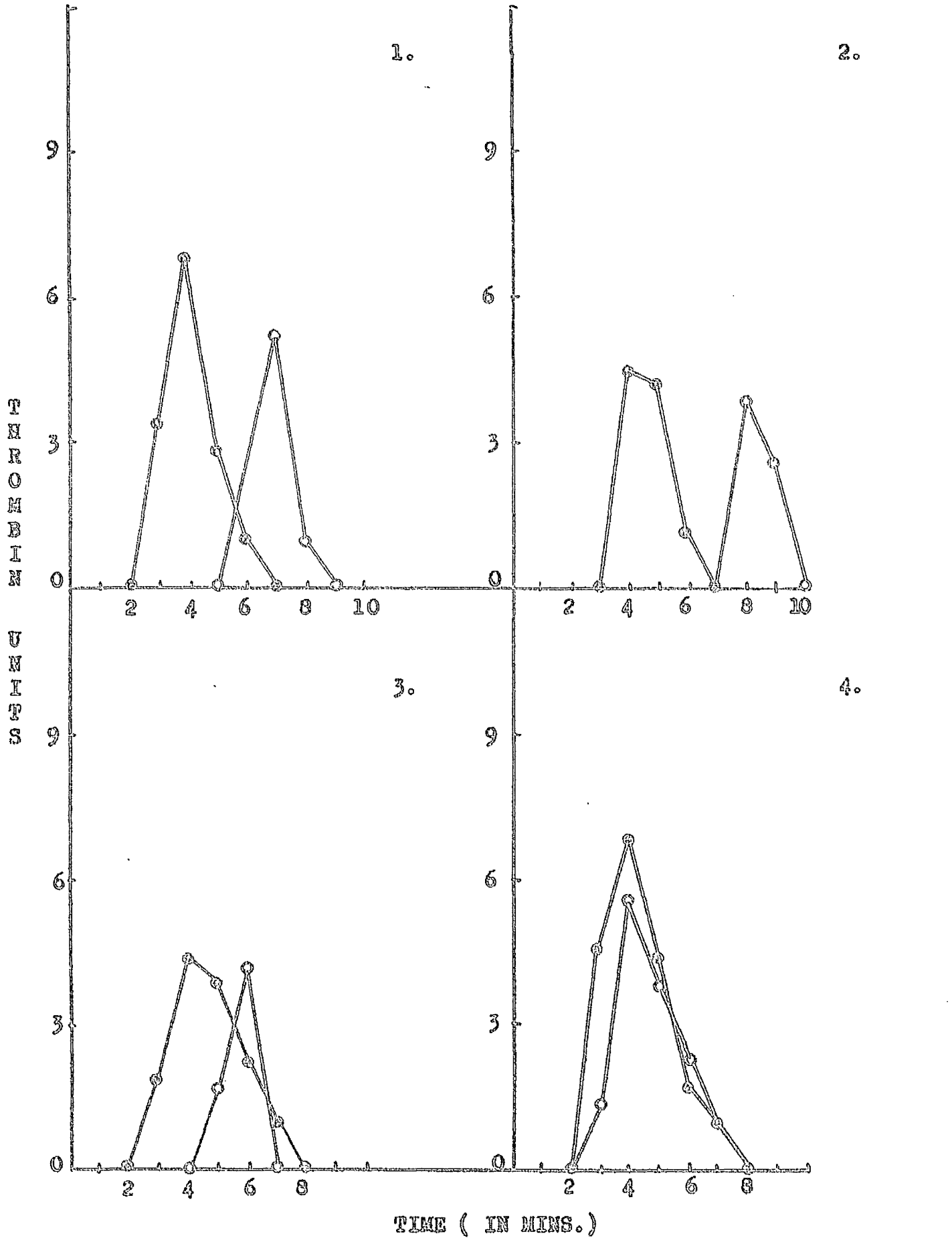
Initial — 2 hours after injection



THROMBIN GENERATION TEST.

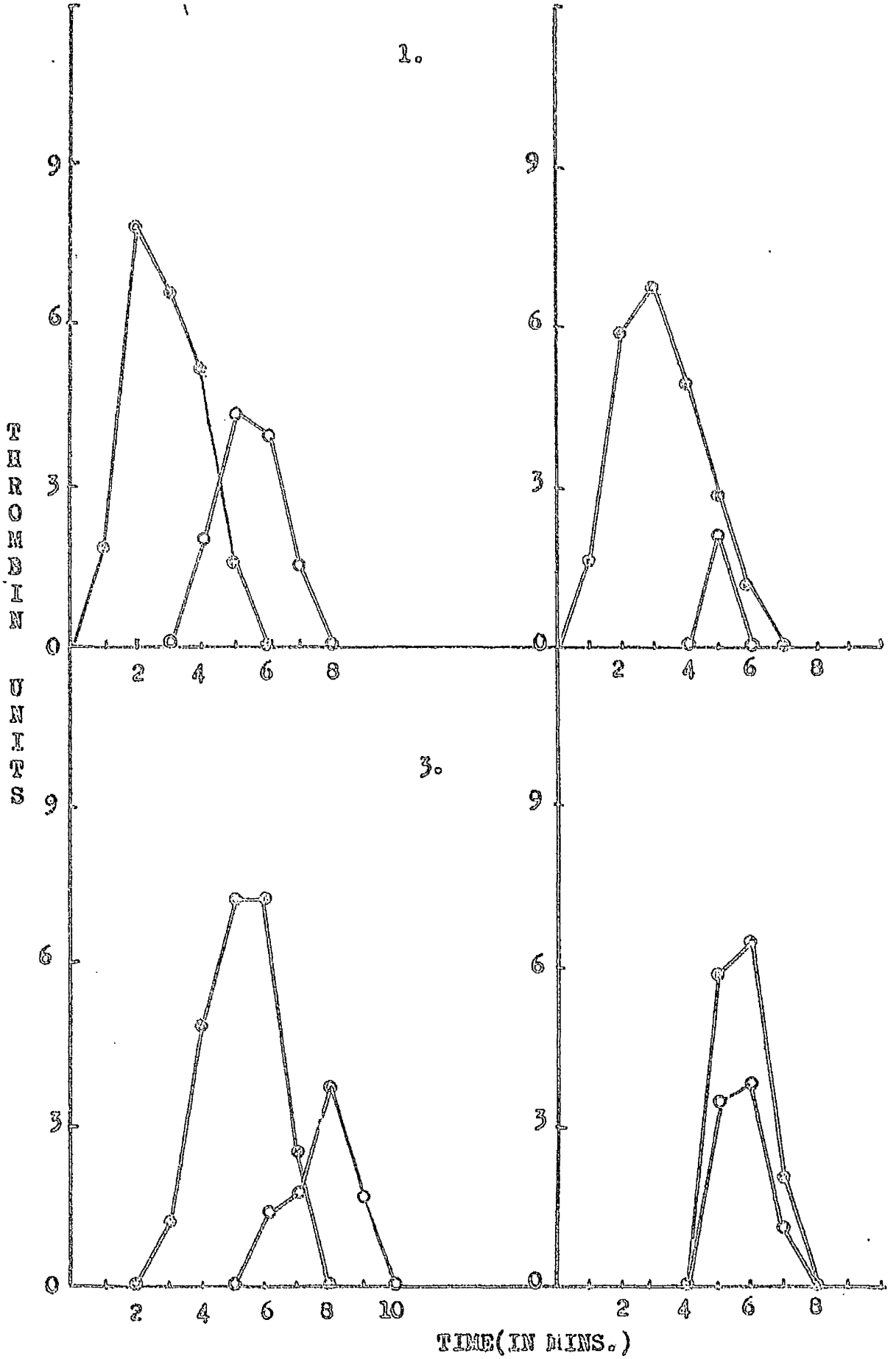
Chondrus crispus (λ-CSE) 3 mg./kg.

o——o Initial o——o 2 hours after injection



Chondrus crispus (k-CNS) 3mg./kg.

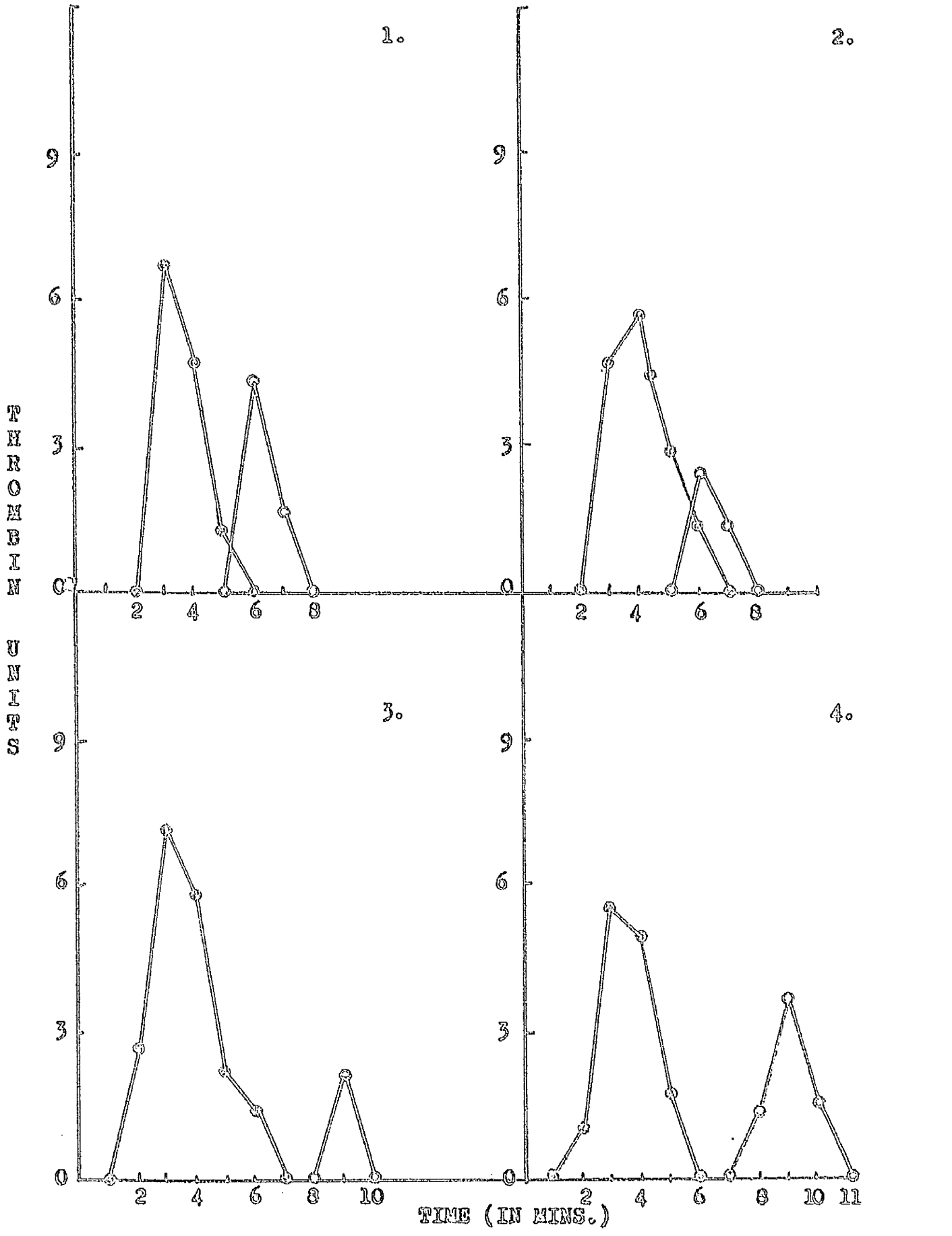
⊙——⊙ Initial ○——○ 2 hours after injection



THROMBIN GENERATION TEST.

Chondrus crispus (λ-CNS) 3mg./kg.

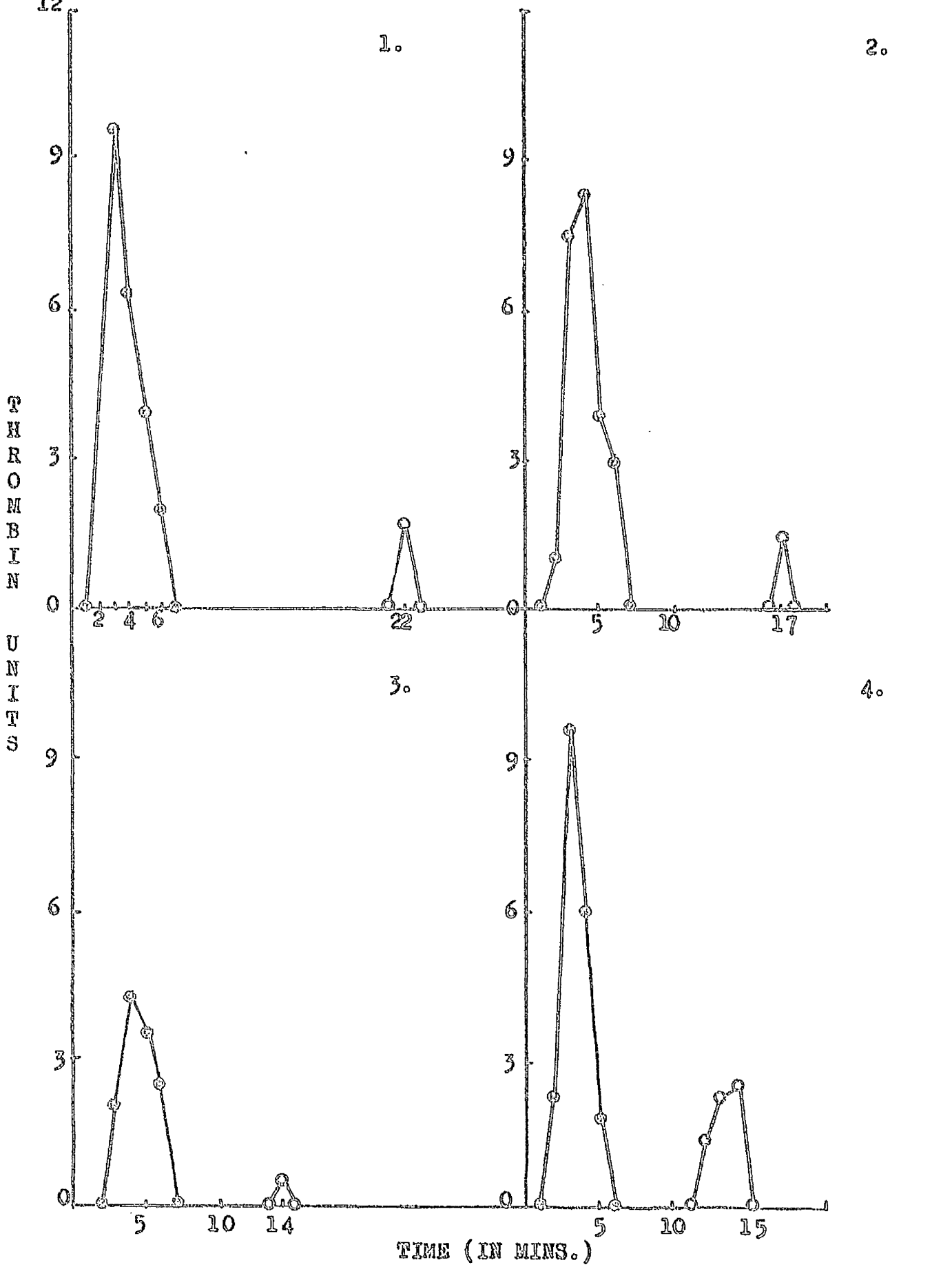
o——o Initial o——o 2 hours after injection



THROMBIN GENERATION TEST.

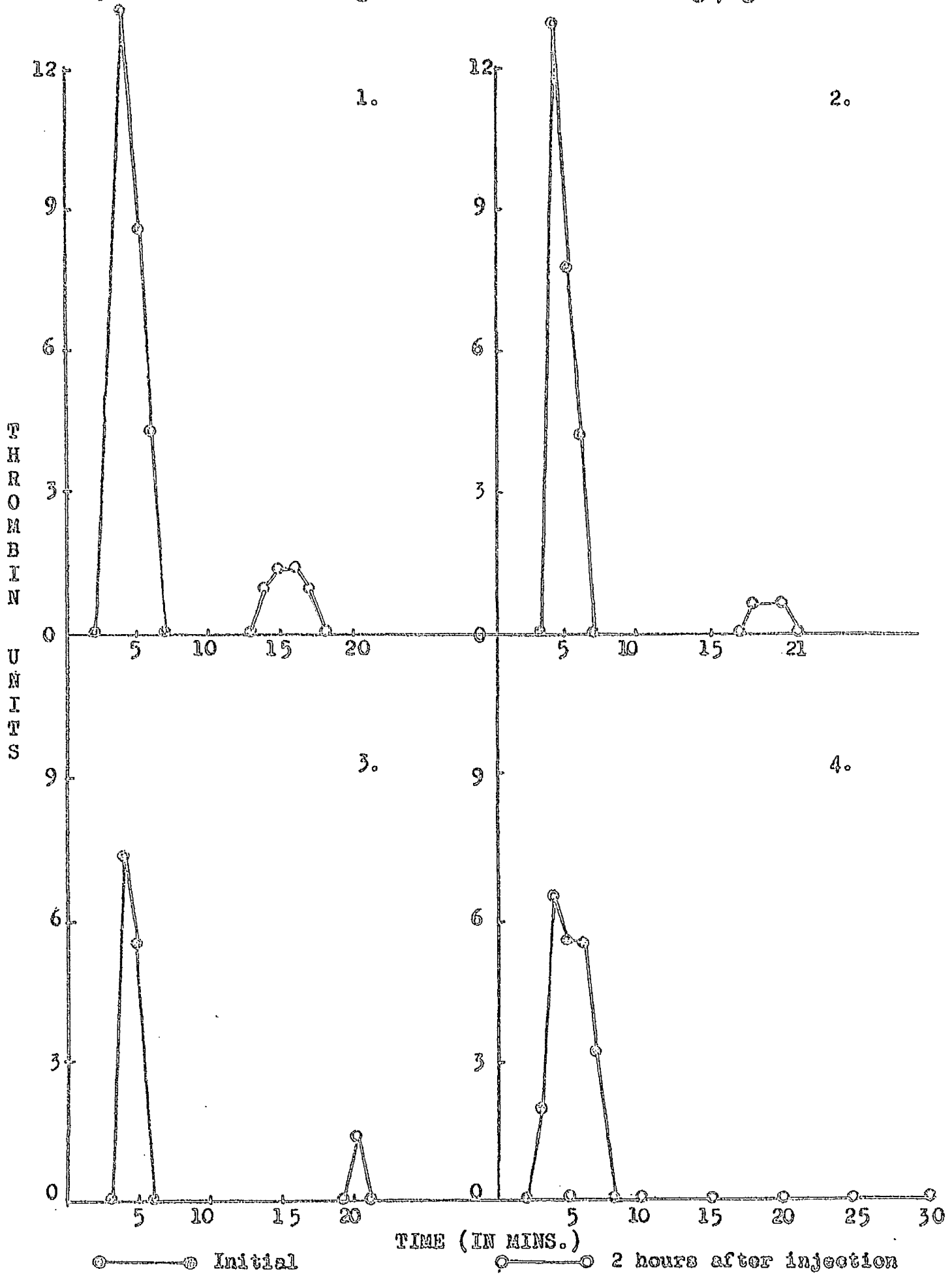
Polyides rotundus 3 mg./kg.

⊙——⊙ Initial ⊙——⊙ 2 hours after injection



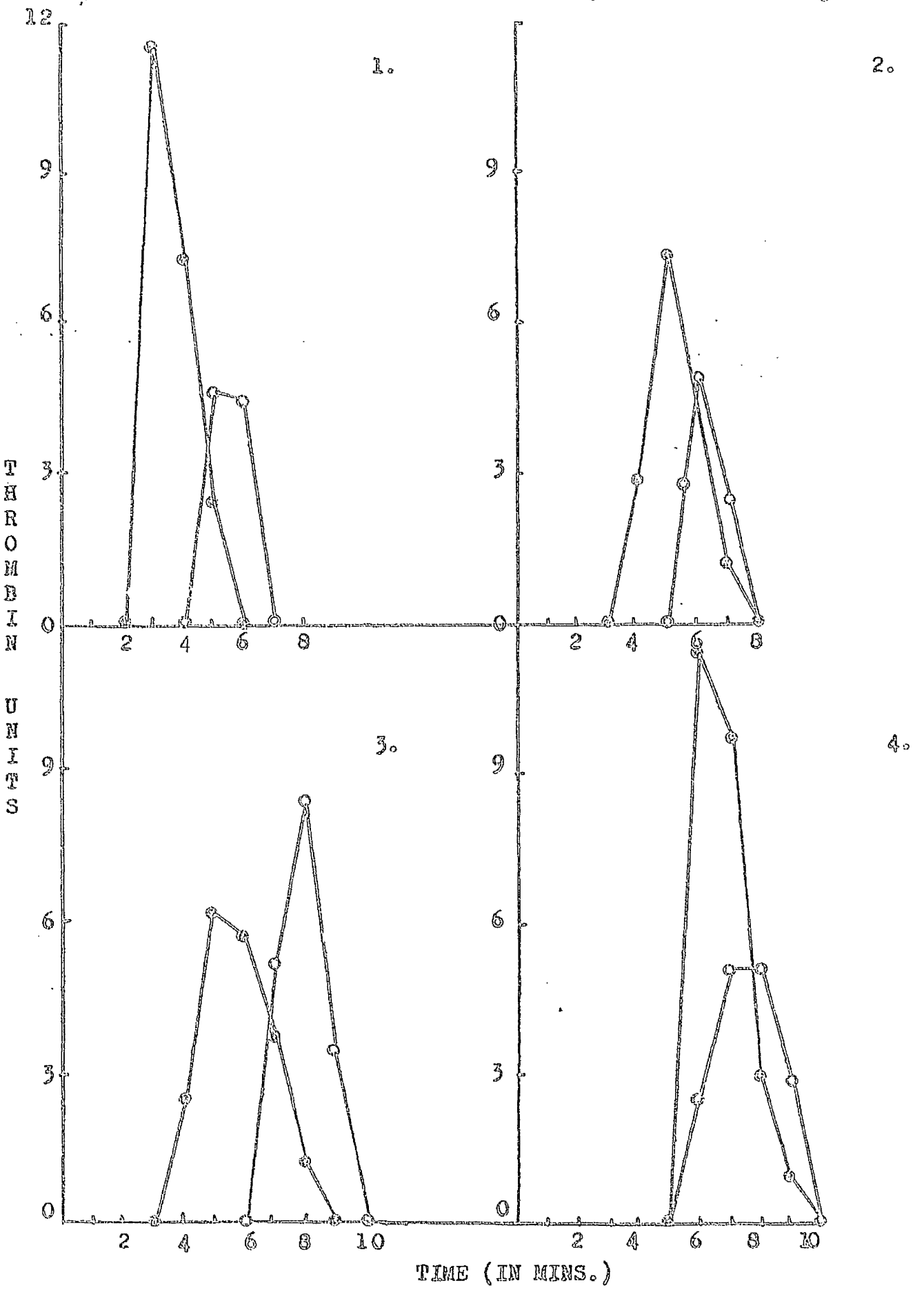
THROMBIN GENERATION TEST.

Degraded Eucheuma carrageenan 200mg./kg.



THROMBIN GENERATION TEST.

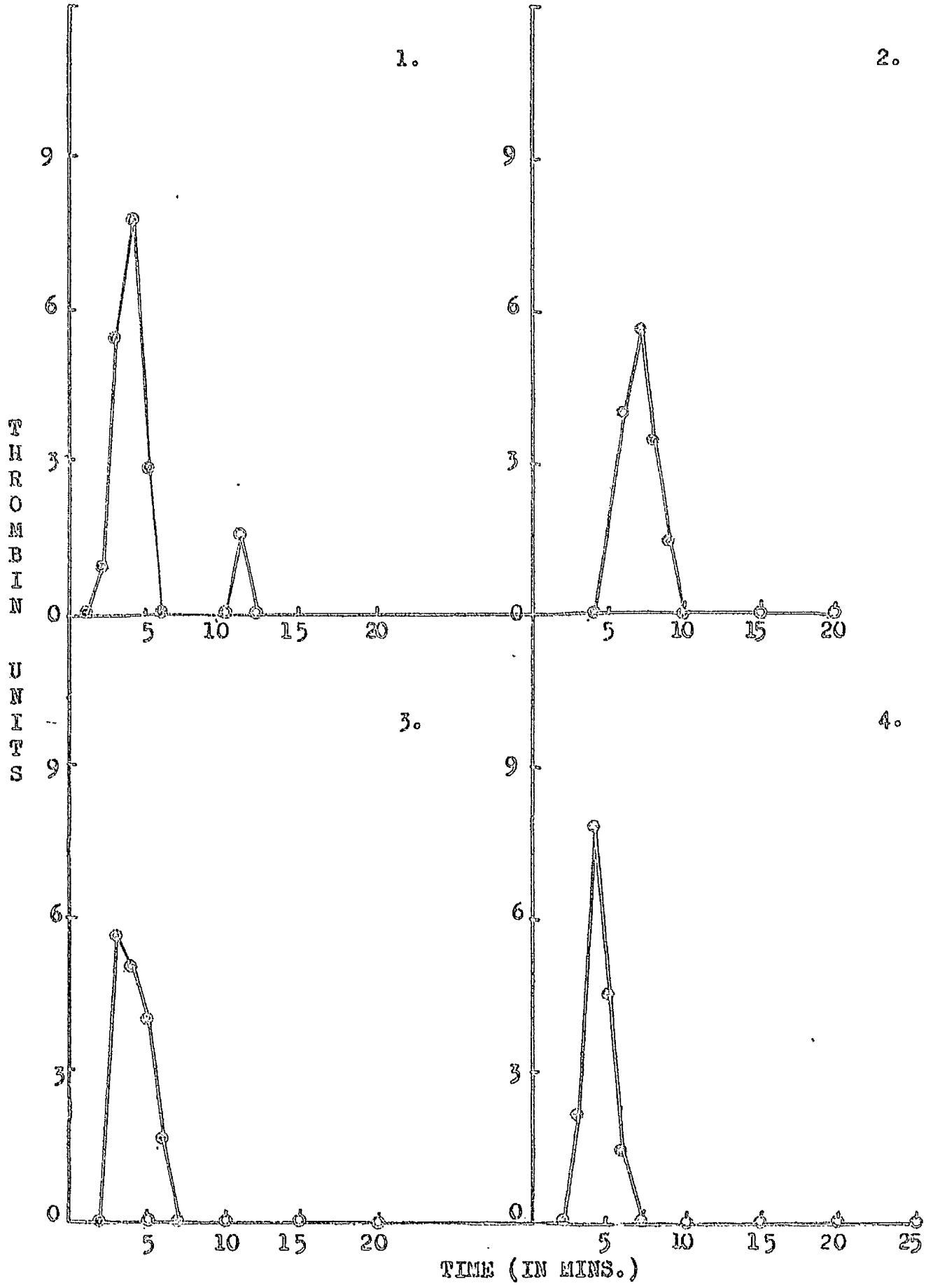
Heparin 75 i.u./kg.



THROMBIN GENERATION TEST.

Heparin 200i.u./kg.

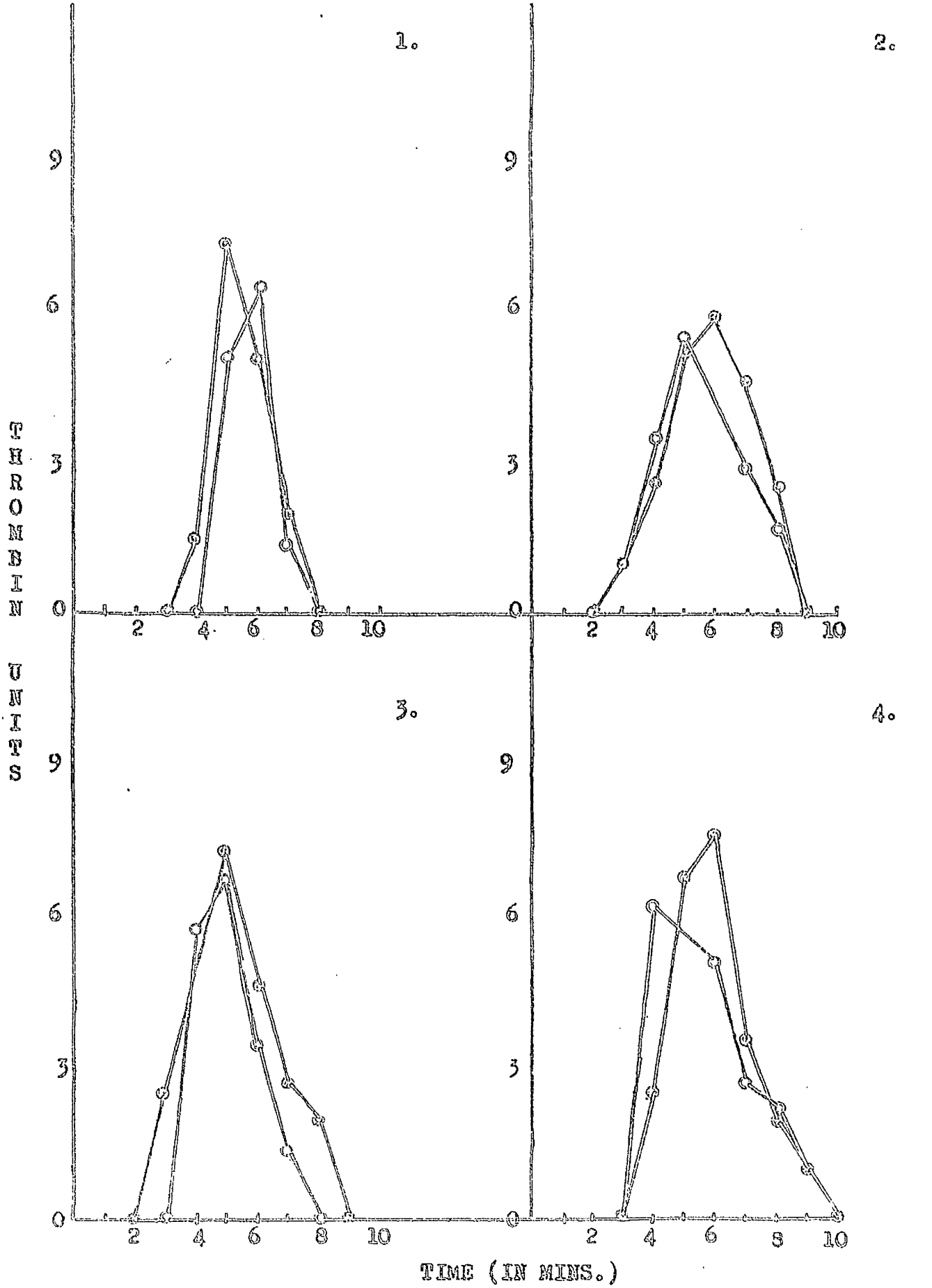
⊙———⊙ Initial ⊙———○ 1/2 hour after injection



THROMBIN GENERATION TEST.

Nembutal (Control) — Initial

25 mg./kg. — after injection



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