purified properdin to determine recovery value of the added properdin. This recovery value can then be used to calculate the true properdin content of such serum.

Summary. 1. A sensitive, reproducible assay for properdin is described using spectrophotometric determinations of C'3 content. 2. RP and R3 reagent sera for the properdin assay may be readily prepared from guinea pig serum. 3. RP and R3 from both human and guinea pig sources may be used interchangeably in the reported properdin assay. 4. Inulin was found to be more satisfactory than zymosan as a properdin binding and C'3 inactivating agent.

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Isolation of Anticoagulant Fractions from Crude Fucoidin. (22960)

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Studies on methylpentose-containing polysaccharide material of plant origin have been in progress in this laboratory for more than two years(1,2). During this work, it occurred to us in view of the known anticoagulant activity of some other sulfated polysaccharides(3), that fucoidin, a polymer of L-fucose monosulfate, present in large amounts in the brown marine algae *Phaeophyceae,* might possess anticoagulant activity. This was found to be the case. The present paper describes the preparation of fucoidin fractions and gives data on some of their chemical properties and anticoagulant activities.

Material and methods. Source and preparation: Air dried *Fucus vesiculosus* was obtained from Woods Hole Marine Laboratories, Mass., between April and July, 1956. The preparation of crude material was based on older procedures(4). Purification procedures were guided by determinations of the anticoagulant activity employing the protamine titration test. Crude fucoidin was prepared as follows. About 1 kg dried *Fucus vesiculosus* was ground for 28 hrs. in a ball mill. To the powder obtained, 4 l of tap water were added and the mixture heated with occasional shaking in boiling water bath for 18 hrs. Toluene was added and the material left at room temperature overnight. The supernatant fluid amounted to about 500 ml to which 1 l of water was added. The fluid was then freed of insoluble matter by filtering through muslin. One hundred grams of lead acetate in 300 ml water were added and the precipitate removed by filtration. Barium hydroxide was added to the supernate until it became pink to phenolphthalein. The resulting precipitate was allowed to settle, the supernate siphoned off, discarded, and 300 ml of 4N H_2SO_4 were added for every 700 ml of precipitate containing fluid. The mixture was then shaken mechanically 4 hrs. This was followed by dialysis for 3 days against running tap water. The nondialyzable material was concentrated *in vacuo* to 500 ml, freed of insoluble material by spinning at 12000 g for 45 min. and the supernate poured into 10 vol. of 100% ethanol containing 0.3% sodium acetate. The precipitate was washed with

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TABLE I. Fractionation of Crude Fucoidin at Various Concentrations of Ethanol.

Fraction	% ethanol	% methyl-pentose	% recovery
I	42	25.8	30
II	47.5	32.2	4.7
III	52.5	44	14.6
IV	59	48.5	14.1
V	63	59	7.3
VI	69	65.5	7.1
VII	78	65.5	9.4

100% ethanol and dried *in vacuo* over P_2O_5. This material, about 1.5-2.0% of the air dried algae, was designated as crude fucoidin. This crude material was purified by one of the following two methods: a) crude fucoidin was made up to 2% in water and fractionated with ethanol containing 0.3% of sodium acetate. Total recovery based on crude fucoidin was about 87%, which was divided between the different fractions as shown in Table I. b) To 250 ml of a 1.5% solution of crude fucoidin in water, 13 ml of a 10% aqueous solution of cetyl dimethyl benzyl ammonium chloride was added drop wise with continuous stirring. The resulting precipitate was washed with 100% ethanol and dried *in vacuo* over P_2O_5; the yield was 2.7 g. A 2% solution was made by dissolving a fine powder of this preparation in 25 ml of 3.6 M aqueous solution of $CaCl_2$; insoluble matter was discarded. The material was fractionated by addition of 100% ethanol. Precipitates obtained at final ethanol concentrations of 45% and 67% were dialyzed and dried as described above. The yield was 32% and 40% respectively on weight basis of crude fucoidin.

Biological determinations: Clotting time, prothrombin consumption, platelet counts, recalcification time, one stage prothrombin, labile factor and protamine titrations were determined according to standard procedures (5). Antithrombin activity of fucoidin and heparin was determined by adding various amounts of these materials to 0.1 ml of a 1% fibrinogen solution (Armour Laboratories). The fibrinogen was then transformed to fibrin by adding 10 units (0.1 ml) of topical thrombin (Parke Davis Laboratories). All biological activities are expressed on the basis of weight of vacuum-dried (over P_2O_5) material and are compared to those of 1% preparation of Na-heparinate (Abbott Laboratories) which contained 1000 U.S.P. units/ml. Blood employed for *in vitro* studies was obtained from healthy human donors selected at random. Healthy rabbits of either sex weighing 2-3 kg were employed. They were fed an unrestricted diet of Purina chow and water supplemented with fresh carrots.

Physical and chemical determinations. P was determined according to the method of Zilversmit and Davis(6), Ca according to Clark and Collip(7), Na and K by means of Baird flame photometer. Hexosamine estimations were made according to the method of Elson and Morgan(8). Methylpentose was determined spectrophotometrically(9) employing a Beckman DU spectrophotometer. L-fucose (Mann Biochemicals) recrystallized from methanol was used as standard. Reducing sugar was determined by the method of Folin and Malmros(10). Relative viscosity was measured in Ostwald viscosimeter. Hydrolysis of fucoidin was carried out with 1 N H_2SO_4 in sealed tubes at 100°C, the hydrolysates were neutralized with a stoichiometric amount of $Ba(OH)_2$ and concentrated. Paper chromatographic technics employed were those described previously(2).

Results. Fucoidin which precipitated at final ethanol concentration of between 49-64% uniformly was found to be the most active biologically. *All data given in this report refer to fractions obtained in this range of ethanol concentration.*

Anticoagulant activity. Fractions that proved most potent (40-70% of heparin activity) in the protamine titration test were also found to be the most active in all other tests employed.

The *in vitro* inhibition of coagulation of blood and recalcification of plasma are shown in Fig. 1. Fucoidin possesses 85 to over 100% of the potency of heparin in the recalcification test and it has 10% heparin activity in whole blood coagulation inhibiting test. Fucoidin delays action of thrombin on fibrinogen considerably more than does heparin (Fig. 2). As shown in Fig. 3, it is less potent

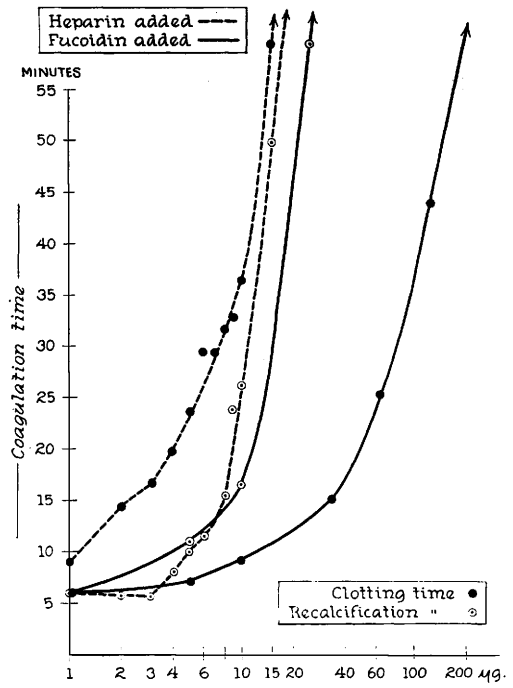

FIG. 1. Influence of fucoidin and heparin on blood coagulation and plasma recalcification.

an inhibitor in the one stage prothrombin test. Fucoidin did not precipitate fibrinogen or fibrin in any of these studies.

The influence of fucoidin on blood clotting mechanism *in vivo* was studied in rabbits. The results obtained in 12 rabbits injected intravenously are listed in Table II. In all rabbits clotting time was prolonged. This effect lasted from 2¼ to 10 hours. The average was 6 hours. Even more impressive was the prolongation of recalcification time. From an average of about 4 minutes, it was usually delayed to more than 30 minutes. In many animals, the prolonged recalcification time persisted after clotting time of whole blood had returned toward normal. In five animals, clot retraction was markedly impaired. In one of these, it was still poor after clotting time had returned to normal. A similar observation was also made after heparin administration. One of the 12 rabbits developed transient thrombocytopenia each time that it was injected with the standard dose of fucoidin. After intravenous injection, thrombocytes dropped from pretreatment level of 372,000 to 16,000 at 3 hrs., rose to 66,000 at 6 hrs. and returned to 250,000 at 7 hrs.

Table II further demonstrates a significantly prolonged prothrombin time after injection of fucoidin. At no time was there a demonstrable change in the content of labile factor. Prothrombin consumption was prolonged from an average of 27.5 seconds to 38.2 seconds for 3-4 hours after injection of fucoidin and in general remained prolonged as long as clotting time was delayed.

The anticoagulant effect of fucoidin was undiminished on repeated injections. No rabbit showed toxic symptoms at any time nor was there sloughing at the site of injection, even if the material was given in high doses intramuscularly or extravasated into the soft tissues. There was no impairment of appetite or vitality of the rabbits.

Physical and chemical. All analyses were performed on the most active fraction which had been thoroughly dialyzed against 300 vol. deionized water. Calculations were made after subtraction of 20.45% moisture, which

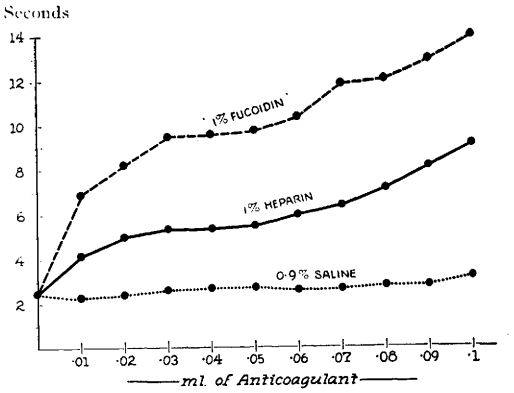

FIG. 2. Inhibition of thrombin-fibrinogen reaction.

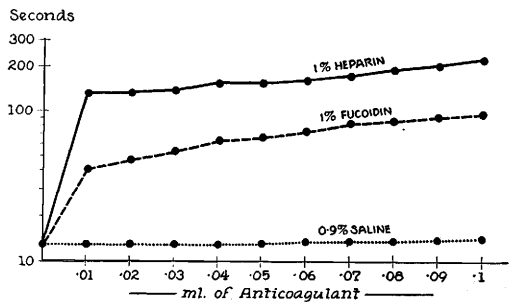

FIG. 3. Action of fucoidin and heparin on one stage prothrombin time.

TABLE II. Influence on Clotting Factors in Rabbits Given 45 to 50 mg Fucoidin Intravenously.

	Normal range	Hr after inj. of fucoidin ¾–1½	3–4
Clotting time (min.)	10–25 (19)	39–147 (69)	21–112 (44)
Recalcification time (sec.)	120–390 (205)	(> 2000)	(> 2000)
Labile factor (sec.)	8.3–10.8	7.4–10.9	8.3–10.7
Prothrombin time (sec.) (one stage)	9.2–12 (10.5)	10.5–16.5 (14.1)	10.2–12.5 (12.0)
Platelets	218,000–786,000 (430,000)	136,000–480,000* (310,000)	208,000–464,000* (320,000)
Prothrombin consumption	16.7–42 (27.5)	16.7–63 (33.8)	22–56 (38.2)

Figures in parentheses give arithmetic means.
* One out of 12 rabbits reacted with a thrombocytopenia upon inj. of fucoidin. The effects in this animal are described in text.

could be removed by drying fucoidin over P_2O_5 at 40°C at a pressure of 0.1 mm mercury.

Relative viscosity of a 1% solution of fucoidin in 0.85% saline was $\eta = 1.2$ (35°C). Ultraviolet absorption of 0.8% solution in water showed very weak absorption between 256 and 280 mμ. Otherwise no absorption maximum was found between 220-600 mμ. Specific rotation was $[a]^{25°}_D = -119.6°$ ($\bar{c}$ 1 in water).

The analytical data were: fucose 44.2%, S 9.6%, Na 6.9%, K 0.76%, Ca 1.65%, N 0.71% (micro Kjeldahl), P<0.05%. Sulfated ash 25%. No hexosamine was demonstrable.

Paper chromatograms of hydrolysates of the most highly purified and potent preparations of fucoidin showed no carbohydrates other than fucose and a component which moved between 2-0-methylfucose and fucose and gave color reactions of methylpentoses (2).

The reducing values of hydrolysates rose rapidly from zero within the first hour of hydrolysis and reached a steady value after about 3 hours.

Discussion. The experiments reported here show that purified fucoidin fractions from *Fucus vesiculosus* are potent inhibitors of blood coagulation *in vitro* and *in vivo*. It is remarkable that the most active material contained after hydrolysis only 2 demonstrable sugars, namely fucose and a faster component located between fucose and 2-0-methylfucose on paper chromatograms(2). In agreement with Lunde *et al.*(11) but in contradistinction to Percival and Ross(12), the main inorganic ion of fucoidin was sodium.

The methylpentose content of the fractions increased with increasing ethanol concentration (Table I), while the most potent anticoagulant material was obtained at medium alcohol concentrations. Absorption observed in the ultraviolet range (256-280 mμ) indicates a small amount of non-carbohydrate impurity. The sulfur content of active preparations was 80% of that reported for heparin (13).

Fucoidin is characterized by its high moisture content. While the analytical data presented here have been corrected for an easily removable moisture content of 20.45%, Percival and Ross(12) have reported that crude fucoidin, dried as described in this paper, still contains 15.4% moisture.

As shown in this report, fucoidin like other acid polysaccharides(14) combines with certain basic substances. Its anticoagulant activity was neutralized by protamine. Fucoidin is also precipitated and inactivated by the long chain quaternary ammonium salt, cetyl dimethyl benzyl ammonium chloride. Dissociation of fucoidin-base complexes with strong salt led to restitution of activity.

Sulfuric acid esters of carbohydrates other than heparin have previously been employed as anticoagulants. Some of these have been found to be satisfactory substitutes for heparin in anticoagulant therapy such as synthetic dextran sulfate which exhibited no toxic effects(15,16) while others, such as syn-

thetically sulfated polymannuronic acids and polygalacturonic acids have been reported to have certain undesirable side effects(17). Both of these are derivatives of uronic acids.

While it was possible occasionally to obtain a significant effect in rabbits of 2-3 kg weight by giving 15 mg fucoidin intravenously, it was necessary to employ higher concentrations of fucoidin than heparin in these animals to obtain reproducibly significant alterations of the *in vivo* clotting mechanism. When such an effect was obtained it lasted longer than after giving heparin. Fucoidin, however, appeared to be more potent than heparin as an antithrombin.

Some sulfated polysaccharides agglutinate platelets. Grosswall, Ingelmann and Mosimann have shown with sulfated dextrans that this undesirable side effect depends on a certain molecular size(18). Present studies in 12 rabbits showed with one exception, an insignificant drop in platelet count after injection of fucoidin (Table II). An insignificant drop of platelets was also observed in our preliminary studies with heparin in rabbits. The very transitory fucoidin-induced thrombocytopenia in one rabbit may have been caused by platelet agglutination, although we have not demonstrated this as the cause. A similar fall in platelet count has occasionally been observed in patients treated with heparin.† The rabbit which developed thrombocytopenia following fucoidin injection did not show a bleeding tendency.

Further chemical and biological studies are required to establish the mode of action and non-toxic nature of fucoidin fractions. It is not known at present whether the "fast moving component" is a part of the active molecule, although this is suggested by paper chromatographic evidence. The low nitrogen content of active preparations is probably due to impurities. Failure to detect hexosamine by the Elson-Morgan reaction suggests the absence of nitrogen-containing sugars and it seems reasonable to assume that the anticoagulant activity is connected with the O-sulfate groups, although all O-sulfated carbohydrates do not possess anticoagulant activity (*e.g.* agar). There is no indication that our purest fucoidin contains uronic acid.

Summary. A method of preparation, some physical and chemical properties and anticoagulant activities of fucoidin fractions from *Fucus vesiculosus* have been described. Certain fractions prepared from crude fucoidin proved to have potent anticoagulant effects. The nature of the anticoagulant action and the chemical composition place fucoidin in the group of heparinoids. The most active preparations contained no demonstrable carbohydrate other than fucose and a component which migrated on paper chromatograms faster than fucose and rhamnose but slower than 2-O-methylfucose. This component gave all color reactions of the methyl-pentoses.

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† Unpublished observations.

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Factors Influencing Excretory Patterns of Cesium-134, Potassium-42 and Rubidium-86 in Rats.* (22961)

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MacLeod and Snell(1) reported that cesium, potassium, and rubidium behaved similarly in nutrition of lactic acid bacteria. These elements are known to enter the solute complex, participating in ion antagonism, osmosis, permeability regulation, maintenance of colloidal state and similar physiological phenomena(2). Mraz *et al.*(3) observed an increase in excretion of cesium-134 by the rat when dietary potassium was increased. Hamilton(4) reported that cesium excretion was distributed equally between feces and urine. Hood and Comar(5) found that the rat excreted 12 times more cesium-134 in urine than in feces. Mraz and Patrick(6) reported that supplementation of a simplified (casein-corn starch) rat diet with natural feedstuffs decreased the ratio of urinary to fecal excretion of cesium-134 and potassium-42.

This study was initiated to ascertain the factors responsible for these changes in excretory patterns of cesium-134 and potassium-42. Rubidium-86 was also used because of its biological and chemical similarity to cesium and potassium.

Methods and materials. The basal diet employed was the simplified rat diet used by Mraz and Patrick(6). Vermiculite, alfalfa, beet pulp, bentonite, or Amberlite IRC-50 (cation exchange resin) were incorporated at 10% of the diet substituting for an equivalent amount of corn starch in the basal diet. Two potassium-deficient diets were formulated, one from the basal and the other from the 10% vermiculite diet. Four weanling albino rats (Rockland strain) were used per diet in each experiment. All rats were fed their respective diets for 7 days before subcutaneous administration of 0.5 ml of the radioisotope dosing solutions. This period was considered of sufficient length to permit the rat to adjust to the new diet. Five microcuries (μc) of cesium-134 or 20 μc of rubidium-86 were administered to each rat in the cesium and rubidium studies and a 72-hour balance trial made. The short half-life of potassium-42, 12.5 hours as compared to 2.3 years for cesium-134 and 18.6 days for rubidium-86, necessitated the use of 100 μc of the nuclide and only a 48-hour balance trial. All nuclides were administered in the chloride form. Cesium-134 was used at tracer levels, while potassium-42 and rubidium-86 dosages contained 0.38 mg potassium and 0.063 mg rubidium, respectively. *Feces and urine* samples were collected every 24 hours after administration of the nuclides. Excreta were digested with nitric acid and aliquots counted in scintillation well-type counter. In the interest of ease of recording and reading, fecal, urinary, and total excretory data have

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