Chemical and Biological Properties of Fractions Derived from Hog Intrinsic Factor Concentrate by Disc Electrophoresis.* (31634)

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The existence of an "intrinsic factor" elaborated by the gastric mucosa of various species of animals and required for physiologic absorption of vitamin B_{12} in man has long been known. Yet the precise chemical nature of the material remains obscure(1-3).

The active groups of the molecule responsible for its B_{12} binding action, and the specific enhancement of B_{12} absorption have not been established, and its cellular origin has not been determined.

A number of potent hog intrinsic factor concentates (HIFC) have been prepared from hog gastric antra, and serve as "starting materials" in the purification and study of intrinsic factor's properties. One such preparation, designated 3908C-46-1, was described by Highley and Ellenbogen in 1964 as active in man at a dose of 500 μg, binding approximately 5.0 μg of vit B_{12} per mg. It was found to contain an appreciable amount of polysaccharide, including 3% sialic acid(4).

We have obtained further purification of this HIFC by recently developed methods of disc electrophoresis in polyacrylamide gel(5), and have studied the chemical and biologic properties of the isolated fractions.

Materials and methods. Disc electrophoresis. 100-500 μg samples of the HIFC (3908C-46-1)‡ were suspended in the sample gel segment of 7 × 63 mm polyacrylamide gel columns(5). Standard gel (7.5% acrylamide monomer) was used to form the "running" portion of the columns.§ Separation was carried out at room temperature (22°C) in a Tris-glycine buffer (0.22 M, pH 8.2). The columns were run at 5 milliamperes per tube until the bromphenol blue tracer dye reached a premarked point 5 mm from the anodic end of the column. Some columns were frozen immediately and stored at —20°C. Others were fixed and stained promptly.

Histochemical staining. Columns were stained as follows: (a) amido Schwarz (amido black 10B) 0.55% in 7.5% acetic acid for one hour, followed by differentiation in repeated changes of 15% acetic acid for several days, was used to identify proteins(6); (b) after fixing a column for one hour in 7.5% acetic acid at room temperature, oxidation was carried out for one hour with 0.2% periodic acid at 22°C. The periodic acid was completely removed by electrophoresis of the column in a solution of 7.5% acetic acid for one hour at room temperature (10 milliamperes per column). Polysaccharide was colored magenta by exposure of the column to a 0.5% solution of Schiff's reagent for one hour at 4°C(6); (c) acidic (carboxyl and sulfate) polyanions were demonstrated by staining with 0.2% alcian blue in 3% acetic acid (pH 2.5) for 4 hours, followed by differentiation in repeated changes of 3% acetic acid for several days(7); (d) 0.2% alcian blue in 0.5 N HCl (pH 0.5) was used to demonstrate sulfate polyanions by staining for 4 hours, followed by differentiation with repeated changes of 0.5 N HCl(8).

The amido Schwarz stained columns were analyzed with the Spinco Analytrol Model R at 610 mμ to determine the quantity and distribution of protein in the columns (Fig. 3).

Bioassay of intrinsic factor activity of fractions. The segments which contained each fraction were excised from the frozen columns using the stained column as a guide. As the columns swell approximately 10% during staining, it was necessary to reduce the size of the stained columns proportionally. By

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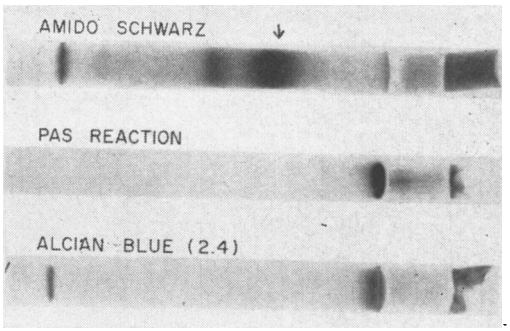

FIG. 1. Stained polyacrylamide gel columns of HIFC. Note that F-III (arrow) stains intensely for protein but not at all by the PAS or alcian blue methods.

placing the columns in a modified photo enlarger it was possible to project an image of the stained columns identical with the size of the unstained columns. Segments of the unstained columns corresponding to fractions seen in the stained columns were excised (Fig. 1).

The excised segments from each column were ground with mortar and pestle and suspended in 100 ml of normal saline. Extraction was continued overnight with gentle agitation at 4°C, and the resulting solution stored at −20°C.

Aliquots of each fraction were assayed for intrinsic factor activity by the guinea pig small intestine mucosal homogenate method, as described by Sullivan, Herbert and Castle (9). Assays were performed using several dose ranges of each fraction. Both negative and positive controls were included with each assay. For positive controls, we used a solution of HIFC 3908C-46-1 (1.0 μg/ml), and a National Formulary Intrinsic Factor Reference Standard (NFIF #1966, 125 μg/ml). For a negative control we used 1.0 ml of normal saline in lieu of the intrinsic factor sample. Duplicate assays were performed with all samples and controls. The results were averaged and expressed as picograms (pg) of ^{57}Co-B_{12} uptake per 1.0 ml homogenate (Table I).

A protein fraction (F-III), found by *in vitro* assay to contain all the intrinsic factor activity in the HIFC, was assayed also by the modified Schilling method. The patient used for testing had never been treated with hog intrinsic factor materials. The procedure of the National Formulary Antianemia Advisory Board was observed except that a dose of 0.5 μg of ^{57}Co-B_{12} was employed instead of 2.0 μg(10). A conventional Schilling response was obtained with 0.5 μg of ^{57}Co-B_{12} in this patient before testing the fractions. The "starting material," 3908C-46-1 (500 μg) and the NFIF reference standard (50 mg) were used for positive controls (Table II).

B_{12} binding capacity of fractions. The HIFC 3908C-46-1 was complexed *in vitro* with isotopically labelled B_{12}.‖ Sufficient ^{60}Co-B_{12} was added to an aqueous solution of HIFC to supersaturate it. The excess free

TABLE I. *In vitro* Assay of Intrinsic Factor Activity of Fractions of HIFC 3908C-46-1 Obtained by Disc Electrophoresis.*

Sample	Amount (ml)	B_{12} uptake (pg)
Saline control	1	16.5
NFIF(rs) #1966 (125 μg/ml)	1	39.7
HIFC 3908-46-1 (1 μg/ml)	0.5	45.5
	1	37.2
	2	35.5
	4	30.2
F-I	1	18.4
	2	13.2
	4	12.6
F-II	0.5	11.8
	1	18.3
	2	10.1
	4	13.8
F-III	0.5	14.0
	1	13.2
	2	30.6
	4	59.5
F-IV	0.5	13.2
	1	17.8
	2	14.6
	4	18.0
F-V	0.5	10.2
	1	16.8
	2	15.4
	4	18.0

* Guinea pig intestinal homogenate sytem contained 1 ml homogenate, 5 ml Krebs-Ringer's-Tris-maleate buffer, pH 7.4, with 10 mM $CaCl_2$/l, 2500 picograms (pg) Co^{57}-B_{12}, specific activity 5.12 μc/μg, giving a count of 10 C.P.M. per pg B_{12} in well-scintillation counter, plus control or unknown IF sample. Fractions I-V were obtained by eluting each segment from a column containing 100 μg HIFC 3908C-46-1 into 100 ml normal saline.

‖ ^{60}Co-B_{12}, E. R. Squibb, specific activity adjusted to approximately 1.0 μc/10μg B^{12}.

TABLE II. Results of *in vivo* Assay of F-III by the Modified Schilling Method.

Intrinsic factor material	Dose	Co^{57}-B_{12}	% Urinary excretion of ingested dose B_{12} 1st 24 hr	2nd 24 hr	Total
None		.5 μg/.5 μc	.10	×	×
NFIF(rs) #1966	50 mg (stnd. dose)	.5 μg/.5 μc	8.80	×	×
HIFC 3908C-46-1 (100× potency of NFIF, 500 μg act. dose, binding 2.0 μg B_{12})	515 μg	.5 μg/.5 μc	8.45	2.50	10.97
F-III (#1)	532 μg (B_{12} binding) or 426 μg (GPIMH assay)	.5 μg/.5 μc	2.42	1.10	3.52
F-III (#2)	642 μg (B_{12} binding) or 574 μg (GPIMH assay)	.5 μg/.5 μc	5.40	2.80	8.20

^{60}Co-B_{12} was removed by overnight dialysis in Visking membranes against distilled water at 4°C. The remaining HIFC-^{60}Co-B_{12} complex was lyophilized and used in preparing sample gel segments for disc electrophoresis. Columns containing the complex were stained for polysaccharide (PAS reaction), protein (amido Schwarz), or serially sectioned at 2 mm intervals. Each 2 mm segment was counted in a well-type scintillation counter, and the gamma activity correlated graphically with the stained fractions (Fig. 2).

Estimation of technical losses of intrinsic factor activity during processing. Several samples of HIFC were incubated with an excess of ^{60}Co-B_{12} for 30 minutes at 22°C to allow saturation of binding sites. Excess B_{12} was removed by dialysis against distilled water overnight at 4°C. The samples were lyophilized and used to prepare sample gel segments for disc electrophoresis. At each step of the process, gamma counting was performed in a well-type scintillation counter to detect possible losses of HIFC-B_{12} complex due to (a) adherence to the dialysis bag, (b) adherence to the lyophilization tube, (c) loss during electrophoresis into upper (cathodic) buffer solution, and (d) loss due to incomplete elution from ground gel segments.

Results. Five fractions could be distinguished by histochemical staining in the polyacrylamide gel columns (Fig. 1 & 2), and were designated as follows: (F-I) the sample gel segment containing residual protein and neutral polysaccharide; (F-II) a polysaccharide fraction located in the cathodic end of the lower gel, which was stained by both the PAS reaction and by alcian blue at pH 2.5; (F-III) a broad protein band, devoid of stainable polysaccharide; (F-IV) an intermediate protein band; (F-V) a narrow protein band migrating with the tracer dye to a point 5 mm from the anodic end of the column.

The amido Schwarz stained columns revealed 3 distinct protein fractions in addition to some material remaining in the sample gel segment, which failed to migrate. Using the

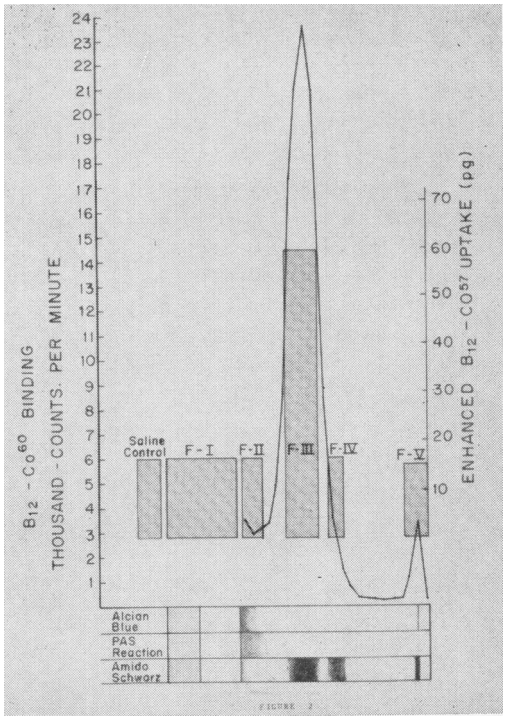

FIG. 2. B_{12} binding (line graph) and *in vitro* intrinsic factor activity (bar graph) correlated with the chemical staining of HIFC fractions in polyacrylamide gel columns.

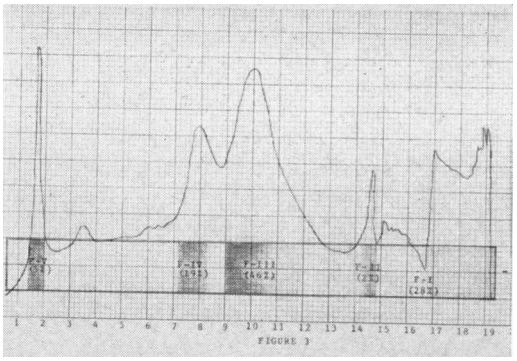

FIG. 3. Diagram of a polyacrylamide gel column of HIFC stained for protein (amido Schwarz) superimposed on the Analytrol scan of the column. Note the high peak corresponding to F-III as opposed to the low peak associated with F-II.

Spinco Analytrol Model R, traces of protein material were also detected at the beginning of the lower gel segment and between the intermediate and most anodic protein factions (Fig. 3).

Duplicate columns stained for polysaccharide showed its accumulation entirely in the upper end of the lower gel segment. No stainable polysaccharide was associated with the protein bands which were located anodically. Normal human serum glycoproteins with the possible exception of serum albumin, show definite PAS reactivity(11). The polysaccharide fraction (F-II) stained intensely with the PAS reaction. The alcian blue technique produced staining of this zone at pH 2.5, but not at pH 0.5, indicating the presence of free carboxyl groups, presumably sialic acids, and suggesting absence of sulfate polyanions(8). When stained immediately after separation, the bromphenol blue tracer dye stains with alcian blue (Fig. 1). This is not seen when the column is fixed overnight in 7.5% acetic acid prior to staining.

Data from the photometric scan of the amido Schwarz stained columns reveals the relative amounts of protein found in each of the 5 fractions as follows: (F-I) 28%, (F-II) 2%, (F-III) 46%, (F-IV) 19%, and (F-V) 5%. F-II, containing all the stainable polysaccharide material, retains only a small amount of associated protein (Fig. 3).

When disc electrophoresis was repeated using HIFC complexed with ^{60}Co-B_{12}, no detectable changes in the staining pattern for the fractions of the HIFC were noted (Fig. 2 line graph). A tall peak of B_{12} binding was observed in the zone identified as the F-III (Fig. 3). This represents 80% of the total B_{12} binding capacity of the material introduced. F-II, the polysaccharide fraction, accounted for only 5% of the B_{12} binding capacity of the starting material.

Approximately 85% of the ^{60}Co-B_{12} tagged HIFC was lost in processing due to the following technical factors: (a) 25% loss due to adherence to wall of tube used to prepare sample before polymerization of sample gel segment in column, (b) 20% loss from polymerized sample gel segment into upper (cathodic) buffer solution by diffusion or reverse migration, and (c) 40% loss due to incomplete elution from ground gel segments. The total loss from these sources correlated well with the approximately 15% recovery in the F-III eluate of the total B_{12} binding capacity of the HIFC processed. Also *in vitro* assay (GPIMH) of the same eluate of the F-III indicated a recovery of activity of approximately 10% of the amount of HIFC processed.

By *in vitro* assay of all 5 fractions, it was found that F-III enhanced B_{12} uptake approximately 4 times the saline control, which represents the maximal response of the system(12). All other fractions (I, II, IV, V) were inert (Table I; Fig. 2 bar graph), despite testing over a wide range of concentrations. These observatons confirm the belief that the bulk of intrinsic factor-like activity was present in the protein fraction, F-III, but do not exclude the possibility that traces of active material were present in other fractions.

In vivo assay was carried out on the F-III fraction to ascertain its activity in man (Table II). After establishing the standard response for this patient with and without administration of a certified active dose of hog intrinsic factor an assayed quantity of F-III was mixed with the 0.5 μg of ^{57}Co-B_{12} in the modified Schilling test. The dose of F-III was determined by comparing its B_{12} binding effect against the binding capacity of a known amount of the "starting material,"

3908C-46-1. A similar comparison was established for the *in vitro* activity (GPIMH method) for F-III and the HIFC 3908C-46-1. The first *in vivo* assay of F-III elicited a 48-hour excretion of only 3.5% of the ingested dose of ^{57}Co-B_{12}. However, the *in vitro* assay indicated that this quantity of the fraction may have been sub-optimal in comparison with the standard dose of HIFC employed in the Schilling test. The *in vivo* test was repeated using 574 μg of F-III in terms of guinea pig ileum assay. Significant enhancement of vit B_{12} absorption was demonstrated, comparable to that observed with 515 μg of starting material. It is to be emphasized that these figures are based on a biological assay; it is likely that the quantity of carbohydrate-free fraction III was considerabl less than 500 μg in gravimetric terms.

Discussion. Previous work has suggested that the polysaccharide moiety is an essential constituent of the "native" intrinsic factor molecule. Although active preparations have been obtained with negligible blood group activity, preparations heretofore reported have generally contained hexosamine and sialic acids(13).

Disc electrophoresis of the HIFC produced a complete separation of the major polysaccharide component from the protein fractions, suggesting a weak association between the moieties, which are cleaved by the molecular sieve effects of acrylamide gel disc electrophoresis. These studies indicate that the major polysaccharide component is unnecessary for B_{12} binding, and *in vitro* intrinsic factor activity, exhibited by the protein fraction, F-III.

Staining properties of the separated polysaccharide fraction (F-II) reveal the presence of carboxyl groups, presumably sialic acids, shown to be present on chemical analysis of this material(14). The absence of alcinophilia at pH 0.5 argues against a significant sulfate content. These results can be tentatively correlated with histochemical data on the hog pyloric antrum, the regional source of porcine intrinsic factor preparations. The greater part of the luminal two-thirds of the pyloric glands produce sulfomucin. Basal portions of these glands often contained polyanion with only carboxyl groups (15). The cellular elements of the basal one-third of the hog pyloric glands also bind tritiated-B_{12} avidly by autoradiography. These observations suggest that vit B_{12} binding by hog gastric mucosal cells corresponds to cells producing sulfate-free mucins.

Faillard *et al* tested intrinsic factor preparations after neuraminidase digestion, and reported strong activity when assayed *in vitro* but markedly reduced activity *in vivo* (16). They hypothesized that neuraminic acid present in the polysaccharide may protect against destruction of intrinsic factor in the intestinal tract by digestive enzymes and bacteria. Our results indicate that the major polysaccharide component of porcine intrinsic factor, recently reported by Highley and Ellenbogen to represent more than 30% of the "pure" porcine intrinsic factor molecule by weight(17), is not essential for B_{12} binding, nor is it essential for *in vitro* and *in vivo* specific activity exhibited by the protein component.

Summary. HIFC was separated by polysaccharide disc electrophoresis into a distinct polysaccharide fraction (F-II), and 3 distinct protein fractions. Disc electrophoresis of the HIFC complexed with isotopically labelled vit B_{12} revealed that only one of these fractions (F-III), a protein devoid of stainable polysaccharide, bound B_{12} avidly. When the losses due to processing are considered. F-III represents approximately 80% of the binding activity in the starting material. Following elution, F-III shows intrinsic factor activity by *in vitro*(guinea pig intestinal mucosal homogenate method) bioassay, and *in vivo* by Schilling's method in a patient with pernicious anemia. All other fractions were inert *in vitro*. Thus a protein moiety, free of polysaccharide, was responsible for both B_{12} binding and the specific enhancement of absorption. The sialic acid-containing major polysaccharide fraction (F-II) showed no significant B_{12} or *in vitro* activity.

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Inhibition of Coronary Atherosclerosis in the X-Irradiated, Cholesterol-Fed Rat by Chondroitin Sulfate A.* (31635)

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Considerable data are available indicating that administration of chondroitin sulfates, which are widely distributed in connective tissues, cartilage and other tissues(1) have an inhibitory effect on the occurrence of atherosclerosis. As early as 1955, Kurita(2) reported that intravenous injections of chondroitin sulfate C at a level of 5 mg/kg of body weight daily reduced serum cholesterol levels and inhibited atherosclerosis in cholesterol-fed rabbits. Ohdoi(3) observed that sodium chondroitin sulfate inhibited the elevation of serum cholesterol, total lipids and the beta/alpha lipoprotein ratio as well as the formation of atheromatous aortic lesions in cholesterol-fed cockerels when administered orally at a level of 20 mg/kg of body weight per day. Murata(4) found that daily intravenous injections of 5 mg/kg of body weight of a chondroitin polysulfate which was prepared by sulfation of chondroitin sulfate from shark cartilage (chondroitin sulfate C) significantly reduced the serum total lipid and serum cholseterol levels of cholesterol-fed rabbits and had an ameliorating effect on the severity of cholesterol-induced atherosclerosis. More recently Morrison *et al*(5) observed that chondroitin sulfate A (prepared from bovine nasal septa and tracheae) when administered subcutaneously at a level of 20 mg/kg of body weight per day reduced serum total lipids and the incidence and severity of atheromatous aortic lesions in squirrel monkeys (*Saimiri sciurea*) fed a cholesterol- and butter-containing diet. It has been demonstrated the x-irradiation of the thorax (5 weekly exposures of 500 r each) increases the incidence and severity of coronary atherosclerotic lesions in rats fed a cholesterol-containing diet when compared to non-irradiated rats on the same diet or x-irradiated rats on a cholesterol-free diet(6, 7). Similar findings have also been obtained in the rabbit(8). Data are presented here on the effects of a single dose of total body x-irradiation upon the incidence and severity of coronary atherosclerotic lesions in rats fed a cholesterol-containing diet and the response to chondroitin sulfate A administration thereon.

Methods. One hundred and forty-four male rats of the Holtzman strain which had

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