Fractions with High Activity for Intrinsic Factor and Combining Vit. B_{12} With Receptor Substances.* (24836)

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Although attempts have been made to isolate intrinsic factor (IF) from hog mucosal extracts in pure form, no homogeneous preparations have apparently been obtained(1). One of the principal difficulties has been the absence of a rapid *in vitro* assay for intrinsic factor activity. Miller and Hunter(2), reported that uptake of Vit. B_{12} by liver slices was stimulated by IF concentrates and suggested that it could be used as an assay for IF. This method was further developed by Herbert(3). Miller(4) and Miller and Hunter(5) have also reported that IF concentrates greatly increase the combination of B_{12} with receptor substances prepared from serum and liver tissue and proposed that this reaction be employed as an assay for IF activity. Cellulose-ion exchangers described by Peterson and Sober(6) have been used successfully to isolate proteins of blood serum (7) and egg white(8). The cellulose cation-exchanger, carboxymethylcellulose (CM-cellulose), has been employed in the present study for isolating fractions from hog stomach mucosal extracts which had high activity for increasing the combining of B_{12} by the receptor substances of Miller and Hunter(5) in an *in vitro* assay.† These fractions have also been found to have activity for IF by the Schilling technic in humans.

Methods. Starting materials with IF activity were extracts of hog stomach. Both initial aqueous extract and dry concentrate prepared therefrom were employed (Wilson Labs., Chicago, Ill.). Dried concentrate was so prepared that 10 mg when combined with 10 μg of Vit. B_{12} constitutes 1 U.S.P. (oral) unit. Material was prepared by dialysis against buffer of 0.1 *M* acetic acid titrated to desired pH with NH_4OH. Insoluble precipitate was removed, washed and washings combined with supernatant. CM-cellulose, containing 0.6 meq. of titratable acidity/g, was prepared by method of Peterson and Sober(6). Use of the exchanger was essentially similar to that previously described for the fractionation of egg white(8). All fractionations were carried out at room temperature. Assays for IF activity on humans were done by the Schilling technic (9). In this assay preparations required in the least amount have highest activities. Results of *in vitro* assays(5) are given in terms of radioactive counts/min/mg of preparation tested. The preparations which give highest counts have highest activities. Estimations of protein concentrations were routinely obtained by determination of absorbances at 280 mμ with a Beckman Model DU spectrophotometer. Sialic acid was determined by the method of Werner and Odin(10) except that sulfuric acid was employed rather than hydrochloric acid in the direct Ehrlich's test. The sialic acid used for standard was prepared from egg white. It had properties similar to crystalline material prepared from *Escherichia coli*.‡ Paper electrophoretic analyses were performed with horizontal strip apparatus using potassium phosphate, 0.1 μ, pH 6.9, as buffer. A constant current (350 volts; 8 milliamperes) was used for 18-24 hours. Moving boundary electrophoretic analyses were performed with American Instrument Co. portable electrophoresis apparatus.

Results. I. *Preliminary fractionation.* Approximate conditions required for initial adsorption and elution of fractions with *in vitro* activity were initially established.

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TABLE I. Fractionation of IF Concentrate on Carboxymethylcellulose.

Description	Fraction	Vol, ml	pH of eluate	Dry, mg	*In vitro* assay, count /min./mg $\times 10^3$	Total activity, count/min. $\times 10^6$
Initial fractionation	IF conc.			20,000	50	1,000
	A-1*	1200		3,960	111	438
	2	1450	3.9	1,885	90	170
	3	1115	5.0	780	344	268
Refractionation	3	1064		744	344	256
	4	1380	3.9	230†	9	<2
	5	165	4.0	20†	50	<1
	6	50	4.0–4.4	37	2,622	97
	7	175	4.4	40	925	37
	8	68	4.4–4.6	58	672	39
	9	38‡	4.6	46	457	21
	10	195	4.6–7.0	585†	48	28
Starting extract				3,795		438
1st fractionation (A-2 and A-3)				2,665		438
Material refractionated A-3				744		256
Refractionation (A-6 to A-9)				181		194

* A-1 was initial extract from IF concentrate. 1150 ml used for fractionation.
† Estimated from absorbance at 280 mμ.
‡ Concentrated from 423 ml by adsorption at pH 4.0 on 3 g CM-cellulose and elution at pH 9.0.

II. *Principal fractionations.* In experiment presented in Table I, 20 g of powdered concentrate were fractionated. The soluble and dialyzed extract contained 20.8% of original dry weight and 43.8% of total original apparent activity. This extract (A-1) of dry concentrate was mixed with 15 g of CM-cellulose at pH 3.9 and filtered and washed on a Buchner funnel. The combined filtrate and washings were the pH 3.9 fraction, (A-2). The exchanger cake was resuspended in buffer at pH 5.0, adjusted with NH_4OH, then filtered. The cake was washed with pH 5.0 buffer, combined eluate and washings (A-3) redialyzed at pH 3.9 and refractionated through a column of 3 g of CM-cellulose. Fractions A-4 to A-10, obtained by stepwise elution on a fraction collector, were dialyzed and activities determined by the *in vitro* method. As is apparent, the most active fraction emerged from the column between pH 4.0 and 4.4 with decreasing amounts between pH 4.4 and 4.6. Three-fourths of the total activity put on column emerged in pH interval of 4.0 and 4.6 but less than a quarter of dry weight was in this fraction.

In Table II, results are given of fractionation of 40 g of dry concentrate. In this instance, the extract (B-1) was passed through 40 g of CM-cellulose as a compacted cake on a Buchner funnel under slight vacuum. Fractions B-2 to B-11 were obtained by step-wise changes in eluting buffer. Approximately 100 ml fractions were removed manually and absorbance read at 280 mμ. As in the previous fractionation, the activity peaked at pH range of 4.2 to 4.4 with decreasing amounts to pH 5.0. In this fractionation, 95% of activity put on the exchanger was recovered in the pH 4.1 to 5.0 interval but only 10% of dry weight.

Five other fractionations were also carried out on various amounts of the dry concentrate (2 to 20 g). In complete agreement with above described runs, peaks of activity by *in vitro* assay were obtained between pH 4.1 and 4.4 with decreasing amounts to pH 5.0. Specific activity of peak fractions varied from 7.5×10^5 to 2.01×10^6 counts/min/mg. Likewise, approximately 75% of activity and 25% of solids placed on the column were recovered in fractions eluted from pH 4.1 to 5.0.

III. *Direct isolation from aqueous extract.* Two fractionations were made starting with aqueous intrinsic factor extracts. The first fractionation of 300 ml of dialyzed extract was made on a 3 g column of CM-cellulose starting at pH 4.0 and eluting in a stepwise fashion. Again the active fraction by *in vitro* as-

TABLE II. Fractionation of IF Concentrate on Carboxymethylcellulose Employing Buchner Funnel.

Fraction	Vol, ml	Elution pH	Dry, mg	*In vitro* assay, count /min./mg × 10^3	Total activity, count/min. × 10^6
IF conc.			40,000	50	2,000
B-1*	1700		11,000†	67	740
2	1815	3.9	2,568	11	27
3	350	3.9	252	40	10
4	775	3.9–4.1	434	168	73
5	350	4.1–4.2	147	272	40
6‡	995	4.2–4.4	239	1,201	287
7	700	4.4	119	487	58
8	48§	4.4	57	368	21
9	1515	4.4–4.7	258	531	137
10	1100	4.7–5.0	317	511	162
11	1500	5.0–6.0	1,830	56	102
Material on column			11,000		740
" eluted, pH 3.9–6.0			6,221		917
" " , " 4.1–5.0			1,137		705

* B-1 was initial extract from IF concentrate.
† Estimated from absorbance at 280 mμ.
‡ Fractionation interrupted overnight after this fraction before the next fraction was obtained.
§ Concentrated from 964 ml by adsorption on 4 g. CM-cellulose at pH 4 and elution at pH 9.

say occurred between pH 4.0 and 4.5. Specific activity at peak was 4.96 X 10^5 counts/min/mg. A second isolation of intrinsic factor was made from undialyzed extract. One l of the extract was centrifuged to remove insoluble precipitate and the supernatant was adjusted to pH 4.7 and passed through a compacted cake of CM-cellulose (50 g) on a Buchner funnel. The eluate was adjusted to pH 4.2 with 0.1 *M* acetic acid and water added to maintain the ionic strength of buffer. This was passed through a second 50 g cake of exchanger on Buchner funnel. After washing exchanger cake with pH 4.2 buffer, the active fraction was eluted at pH 9.0. (Elution was done with buffer of high pH, to obtain a rapid elution of the protein). This eluate was refractionated as above with the exception that 12 g cakes of exchanger were used. The yield obtained was 148 mg. An *in vitro* assay was not obtained on this sample (G-1); however, several human assays were performed (Table III).

IV. *Comparison of activities by human and in vitro assays.* A summary of results obtained in comparison of activities of different fractions by *in vitro* and human assays is given in Table III. The peak of activity by *in vitro* assay appeared at pH 4.3 to 4.4. However, most active preparations by Schilling assay were obtained at a slightly higher pH range, 4.4 to 4.6.

V. *Chemical and physical properties.* Be-

TABLE III. Comparison of Activities by *In Vitro* and Schilling Assay.

Fraction	pH of eluate	*In vitro* assay, count/min./mg × 10^3	Schilling* assay	
			Active	Dose, mg
A-6	4.0–4.4	2,622	No	2.0
7	4.4	925	Yes	.56
8	4.4–4.6	672	"	.75
9	4.6	457	"	1.2
B-9	4.4–4.7	531	"	.75
B-9 + 10†	4.4–5.0	521	"	.88
C-1	4.4–4.8	650	"	.70
D-1	4.1–4.4	1,250	No	.60
E-1	4.1–4.3	2,000	"	.60
E-2	4.3–4.5	1,000	Yes	.56
F-1	4.3–4.5	894	"	.55
G-1	4.2–4.7		"	1.0

* An active fraction was that which gave at least half maximum urinary excretion of standard dose of radioactive B_{12}. The minimum amount to give this is believed to be approximately equivalent to daily oral dose for pernicious anemia patient in relapse (= 1 U.S.P. unit). Dosage for the active samples as the lowest tested and may not be the minimum amount needed for activity. Likewise, dosage for the inactive samples was the maximum tested. Larger doses of these samples might show activity. Figures are actual weights of doses.
† An equal aliquot mixture of these 2 fractions.

cause of current interest in sialic acid and its presence in mucoprotein, analyses for sialic acid were run on preparations listed in Table III. Sialic acid contents varied from 2.2 to 3.4% on dry weight basis. Paper electrophoretic analyses of fractions A-6, A-7, and A-8 indicated the latter was essentially one component when run in phosphate 0.1 μ pH 6.9, while A-6 and A-7 gave one major component and only a trace of a second. A moving boundary electrophoretic analysis was run on 0.5% solution of preparation E-2 in 0.1 *M* sodium acetate, pH 4.4 at 1°C for 21,300 sec. with potential gradient of 4.2 volt/cm. Under these conditions, only one component was evident and had a very low negative mobility. Both the electrophoretic analyses and the pH of elution from CM-cellulose indicate an approximate pK (or isoelectric point) of pH 4.5 for the material with intrinsic factor activity by the Schilling assay.

Discussion. Replicate fractionations of dry intrinsic factor concentrates and of aqueous extracts under similar conditions have all yielded very active intrinsic factor preparations. This reproducibility was unexpected in view of published report where DEAE-cellulose was employed(1). The reproducibility obtained in present studies was similar to that obtained when CM-cellulose was employed to fractionate egg white(8).

Further study is necessary for interpretation of apparent difference in optimal pH for elution of activity, as determined by *in vitro* assay and human assay. This difference is so small (less than 0.2 pH unit) that more assays are necessary to prove or disprove heterogeneity. It is possible that the difference is due to presence of an activator or inhibitor in one fraction which affects one assay but not the other or that there are 2 substances. These might differ, however, in only a very minor way such as presence of an additional acidic or basic group. A separation of a substance with 2 such closely related structures has been reported in ovalbumin from egg white. Ovalbumin A_1 and ovalbumin A_2, which differ by one phosphate group, were separated on CM-cellulose at an interval of less than 0.2 of a pH unit(8).

Summary. Extracts of hog stomach mucosa were fractionated on cellulose-cation exchanger, carboxymethylcellulose. Highest activity for increasing the combining of Vit. B_{12} with receptor substances of tissue was eluted between pH 4.2 and pH 4.4. Highest activity for intrinsic factor by the Schilling technic was eluted between pH 4.4 and pH 4.7. An approximate pK (or isoelectric point) of pH 4.5 was indicated for the material with intrinsic factor activity by the Schilling assay. These fractions contained 2 to 3% sialic acid.

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