Preparation of Partially Purified Porcine Intrinsic Factor. (23363)

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The preparation of highly purified porcine intrinsic factor has been described(1,2,3). The active principle appears to be mucoprotein in nature. The methods described for the preparation of porcine intrinsic factor use whole porcine stomach as the starting material and are detailed and complicated. This report describes a new and simple method for preparing highly purified porcine intrinsic factor from porcine stomach pyloric mucosa. The evaluation of this material both clinically and in the urinary excretion test will be discussed. Experiments on the further purification of porcine intrinsic factor on cellulose-ion exchangers will be described.

Methods. . A. Preparation of intrinsic factor. To each kg of fresh, frozen, ground porcine stomach pyloric mucosa was added 2 l of ice-cold water. The pH of the suspension was adjusted to 3.5 with 8N HCl and the mixture was stirred overnight at 2°C. The bulk of the residue was removed by filtration through cheesecloth. The remaining suspension was further clarified by centrifugation at 4000 rpm, at 2°C, for 1 hour. The extract of intrinsic factor was concentrated by either vacuum distillation or lyophilization. The solids content at this point, as determined by lyophilization, was 30 g/kg tissue. The concentrate (pH 4.0) was adjusted to 7% total solids and to pH 5.2 with 2N NaOH. A one molar sodium acetate-acetic acid buffer, pH 5.2, was added to this concentrate to give an ionic strength of 0.15 (sodium acetate) at a solids concentration of 6% at 2°C. Ninety-five % ethanol at −20°C was added to this extract to final concentration of 50% at −5°C. After standing overnight at −5°C, the precipitate was removed by centrifugation at 4000 rpm, at −5°C, for 30 minutes. It was suspended in ice-cold distilled water (4 x precipitate volume) and stirred overnight at 2°C. The solution of intrinsic factor (PI) was lyophilized. The yield, after lyophilization, was 5-6 g/kg tissue. Dialysis of intrinsic factor PI before lyophilization resulted in a loss of 60% of the solids.

B. Clinical evaluation. A number of intrinsic factor PI preparations were evaluated for clinical activity using a hematologic method in carefully selected pernicious anemia patients in relapse. The tests were carried out by physicians in accordance with recommendations of the Anti-Pernicious Anemia Board of the U.S. Pharmacopoeia(4). The patients were not used for more than one test. Each patient was hospitalized throughout the entire 3-week period of assay, with red blood cell counts and reticulocyte counts taken either daily or 3 times weekly. Thirty-five mg of intrinsic factor preparation PI was mixed with 15 μg crystalline vit. B_{12} and the mixture was given orally, suspended in water or orange juice, an hour or more before breakfast. The average red cell count for 2 or 3 days before therapy and the red blood cell count at the 21-day level were compared with the expected red blood cell response in the U.S. Pharmacopoeia recommended tables(5). The percent response was calculated by dividing the 21-day red blood cell response by the expected red blood cell response. The percentage of reticulocyte response was calculated in a similar way using U.S. Pharmacopoeia recommended tables(6). The clinical evaluation of intrinsic factor PI is summarized in Table I. The clinical data on 4 preparations indicated that a *full* red blood cell response (90-100% of U.S.P. response expected in 21 days) occurred in the majority of cases. The excellent clinical response in practically all cases indicated that the daily dose used, namely, 35 mg intrinsic factor PI and 15 μg vit. B_{12}, could be defined as one U.S.P. oral unit.* The dialyzed and lyophilized intrinsic factor PI (IV-D) appears to be fully active in a daily dose of about 15 mg intrinsic factor and 15 μg B_{12}.

* Intrinsic factor—vitamin B_{12} preparation used in Biopar Forté, Armour Laboratories.

TABLE I. Clinical Evaluation of Intrinsic Factor PI.*

Preparation No.	Intrinsic factor dose, mg	B_{12} dose, μg	Reticulocytes: Initial, %	Reticulocytes: Peak, % at Day	Reticulocytes: % of expected ret. response	Red blood cells, millions/mm³: Initial	At 14 days	At 21 days	Clinical response,† % of expected R.B.C.
I	35	15	.3	16.4 5	46	1.17	3.14	4.08	100
	"	"	.5	18.1 8	60	1.40	2.49	3.08	93
	"	"	5.6	20.7 8		1.73		2.41	42
	"	"	1.9	8.2 8	71	2.45	2.96	3.88	100
	"	"	.6	11.1 8	31	1.37	2.21	3.25	100
	"	"	1.0	24.2 7	100	1.70		3.60	100
II	"	"	4.0	15.6 7	53	1.49	2.00	2.62	64
	"	"	2.9	16.6 8	55	1.43	3.00	3.46	100
	"	"	.9	29.8 6	87	1.25		2.87	82
	"	"	.9	30.6 9	84	1.20	2.80	3.70	100
III	"	"	.7	7.5 8	30	1.49	2.46	3.25	100
	"	"	.6	22.9 8	100	2.09	3.46	3.80	100
	"	"	.8	19.4 6	62	1.35	2.10	2.80	80
	"	"	.7	10.0 12	50	1.90	3.20	2.94	65
	"	"	2.2	26.6 9	80	1.30	2.80		100 (14 days)
	"	"	2.0	14.5 10	76	1.96	2.74	2.62	51
	"	"	2.8	14.8 8	97	2.20		3.29	71
IV	"	"	.8	16.2 8	89	2.00		3.56	100
IV-D	15	"	.4	23.7 8	78	1.34	1.71	2.46	78
	"	"	3.4	23.0 8	78	1.32	2.60	3.05	97

* One U.S.P. unit of intrinsic factor and vit. B_{12} is equivalent to amt of intrinsic factor which, when given with 15 μg B_{12} in a single daily oral dose to a pernicious anemia patient in relapse, will give a *full* red blood cell response (90-100% of expected response) in 14 or 21 days according to U.S.P. calculations(4,5).

† Based on red blood cell response in 21 days.

C. *Urinary excretion test.* The technic used in the 24-hour urinary excretion test has been described(7). The test used to evaluate intrinsic factor PI involved the use of an oral dose of 1 μg vit. B_{12} Co^{60} and an intramuscular dose of 1000 μg non-radioactive vit. B_{12}. The results of the urinary excretion tests on intrinsic factor PI-IV are summarized in Table II. The 27 control tests gave a urinary excretion of 1.7 ± 1%. The 14 subjects receiving 15 mg intrinsic factor PI (0.43 U.S.P. unit) showed a urinary excretion of 11.0 ± 4.6% and the 10 subjects receiving 50 mg intrinsic factor PI (1.43 U.S.P. units) showed a urinary excretion of 18.1 ± 5%. The patients receiving 50 mg intrinsic factor PI excreted approximately 0.18 μg vit. B_{12}, which appears to be close to the maximum excretion of vit. B_{12} when using a 1 μg vit. B_{12} oral dose. The amount absorbed is probably 3-4 times the amount excreted(7,8). Therefore, excretion of 0.18 μg vit. B_{12} corresponds to an absorption of about 0.54-0.72 μg vit. B_{12}. Since the maximum absorption which could be expected from 1 μg vit. B_{12} oral dose in normal subjects is about 70-90%(9), the 50 mg intrinsic factor PI dose gives close to maximum absorption of vit. B_{12}. Three subjects previously tested with 50 mg intrinsic factor PI were then tested with 350 mg intrinsic factor PI (10 U.S.P. units). They showed a urinary excretion of 14.7 ± 1.5%. Extraordinarily high or unphysiological doses of intrinsic factor appear to depress absorption of vit. B_{12} Co^{60}. Our data show that less than 0.5 U.S.P. unit of intrinsic factor PI will give significant and measurable urinary excretion of vit. B_{12} and that 1.5 U.S.P. units of intrinsic factor PI will give close to maximum excretion of vit. B_{12}. These results are in contradiction to those reported by Williams *et al.*(10) who found that 2-3 U.S.P. units of intrinsic factor are needed to give significant urinary excretion values. The difference may be due to the dosage of vit. B_{12} Co^{60} used (1 μg *vs.* 2 μg) or to the intrinsic factor preparations used in the 2 studies. It is more likely that the discrepancy is due to the incorrect assignment of potency by Williams *et al.*(10) to their standard preparation. It is obvious from their pub-

TABLE II. Urinary Excretion Tests Using Intrinsic Factor PI-IV and 1 μg Vit B_{12} Co^{60} in 27 Patients.

% B_{12} excreted			
Intrinsic factor (mg)			
0	15	50	350
2.1	6.9		
1.1	6.3		
3.8	14.1		
1.4	6.7		
.2			
1.0	13.0		
1.8			
2.4	12.6		
2.5	22.7		
.7	12.5		
1.7	9.4		
.8			
1.9	9.3		
2.2	7.5		
2.0		22.0	
3.7		21.0	14.2
.7		8.5	13.5
1.5		22.1	16.4
2.0		20.6	
.2		13.2	
1.2		13.9	
2.6		20.9	
2.4		23.6	
2.8		15.5	
.3	7.7		
1.0	16.4		
.7	9.6		
* 1.7 $\pm$ 1.0	11.0 $\pm$ 4.6	18.1 $\pm$ 5.0	14.7 $\pm$ 1.5

* Mean $\pm$ S.D.

lished data(10) that the intrinsic factor preparation used as the standard did not show full potency for unitage assigned. The 2-3 U.S.P. units used in the urinary excretion tests are probably less than one unit if the U.S. Pharmacopoeia recommended tables(5) are used.

D. *Further purification using cellulose-ion exchangers.* Intrinsic factor PI-IV was fractionated on a DEAE-cellulose ion exchanger (11) at pH 7.0 using gradient elution. The results of the experiment are summarized in the elution diagram in Fig. 1. The fractions were assayed in the urinary excretion test. The activity was adsorbed and eluted in fractions Nos. A_2, A_3 and A_4 (Table III). The unadsorbed fraction, A_1, was inactive. Since 60% of the solids are lost on dialysis, the recovered fractions account for approximately all of the non-dialyzable solids. The estimated activity of the adsorbed fractions was 12, 9 and 10 mg/U.S.P. unit, respectively. The fractions were 3-4 times as potent as the starting preparation and about 1.5 times as potent as the preparation obtained on dialysis of intrinsic factor PI (IV-D) which had an activity of about 15 mg/U.S.P. unit (Table I). There was a loss in recoverable activity. A comparison of intrinsic factor PI-IV and PI-IV-D in the urinary excretion test was then made and the results are summarized in Table III. The estimated U.S.P. potency of intrinsic factor PI-IV-D from the urinary excretion test is about 15 mg, which is about the same as that found in the standard U.S.P. assay (Table I). Fraction No. A_4 (Fig. 1), containing the bulk of the activity, was rechromatographed on a CM-cellulose exchanger(11) at pH 5.4 using gradient elution. The gradient was produced with 0.01 M acetate buffer, pH 5.4, and 0.5 M NaCl. Two

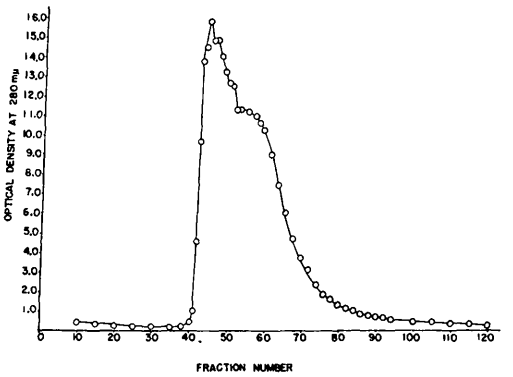

FIG. 1. Effluent diagram: 10 g intrinsic factor PI were dissolved in 300 ml 0.005 M phosphate buffer, pH 7.0. pH of the solution was adjusted to 7.0. Solution was dialyzed against 8 l of phosphate buffer for 48 hr at 1°C, was clarified by centrifugation since a precipitate appeared during dialysis. Supernatant solution was passed through a DEAE-cellulose column (41 $\times$ 2.5 cm) (Solka-Floc BW; .50 mEq/g) which had been equilibrated with 0.005 M phosphate buffer, pH 7.0. Column was washed with buffer and the wash collected in fractions 1-10. Gradient was begun at fraction 10 and produced by continuous introduction of .1 M Na_2HPO_4 + .5 M NaCl into a constant volume reservoir containing 100 ml of .005 M phosphate buffer, pH 7.0. Fraction volume was 6 ml and flow rate was 1.5 ml/min. Experiment was carried out at 25°C. Ordinate is optical density at 280 mμ and represents protein. Fraction pools 1-10 (Fraction No. 1), 42-46 (Fraction No. 2), 47-53 (Fraction No. 3), and 54-84 (Fraction No. 4) were dialyzed against distilled water at 1°C for 48 hr and lyophilized. Weights of fractions were as follows: No. A_1—1.50 g; No. A_2—.36 g; No. A_3—.56 g; No. A_4—1.20 g.

TABLE III. Urinary Excretion Tests on Various Fractions and Subfractions of Intrinsic Factor PI-IV.

Patient No.	No intrinsic factor (control), % of B_{12} excreted	Intrinsic factor PI-IV (standard) mg	Intrinsic factor PI-IV (standard) % of B_{12} excreted	Further fractionation of intrinsic factor PI-IV — Fraction	Yield*	mg	% of B_{12} excreted	Estimated U.S.P. units†	Purification factor‡
1	1.4	15	6.7	A_1	15 % of PI-IV	5	.4	0	0
2	2.0	50	22.0	A_2	3.6 "	"	9.5	.4	3
"	"	"	"	A_3	5.6 "	"	12.7	.6	4
"	"	"	"	A_4	12 "	"	11.3	.5	3.5
3§	1.3	15	3.3	PI-IV-D	40 "	6	4.1	.5	3
"	"	"	"	"	" "	15	7.1	.9	2
4	2.0	50	20.6	"	" "	6	12.0	.6	3.5
5	1.1	15	6.3	B_1	25% of A_4	5	6.4	.4	3
6	1.6	"	11.1	B_2	52 "	"	9.4	.4	2.5
				C_1	21% of PI-IV				
7	1.7	"	9.4	C_2	19 "	"	12.6	.6	4
"	"	"	"	D_1	27% of C_2	"	6.2	.3	2
8	1.9	"	9.3	D_2	11 "	"	11.5	.5	3.5
9	2.5	"	22.7	D_3	10 "	"	5.2	.1	.5
10	.7	"	12.5	D_4	24 "	"	.7	0	0

* % of starting material wt.

† Est. U.S.P. $= K \times \frac{\text{sample } \%}{\text{standard } \%}$ where K = 1.0 for 35 or 50 mg; K = .43 for 15 mg of standard.

‡ Purification factor = Est. U.S.P. $\times \frac{35}{\text{mg of sample}}$.

§ Patient tested with 2 rather than 1 μg $B_{12}Co^{60}$.

‖ No standard test done; value taken from series in Table II.

chromatographic peaks were obtained, one unadsorbed (B_1) and the other adsorbed and eluted (B_2). The activity appeared in both fractions (Table III), with no purification over the starting material.

In a third experiment, intrinsic factor PI-IV was fractionated on the DEAE-cellulose exchanger at pH 5.4 using gradient elution. The gradient was produced with 0.01 M acetate buffer, pH 5.4, and 0.5 M NaCl. Two chromatographic peaks were obtained, one unadsorbed (C_1) and the other adsorbed and eluted (C_2). The activity was adsorbed at pH 5.4 (Table III) and eluted in a single fraction, C_2. The estimated potency of fraction No. C_2 is about 9 mg/U.S.P. unit. The unadsorbed fraction was not assayed since in a preliminary experiment the unadsorbed fraction was inactive. Fraction No. C_2 was rechromatographed on DEAE-cellulose at pH 4.7 using gradient elution. The results of this experiment are summarized in the elution diagram in Fig. 2. The bulk of activity was unadsorbed (Table III). The most active fraction had about the same potency as the

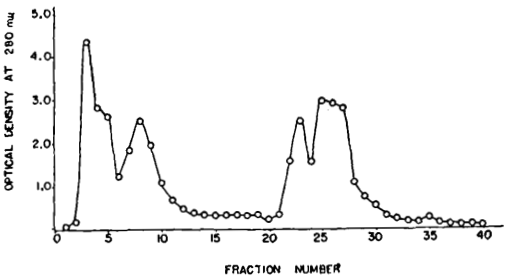

FIG. 2. Effluent diagram: 500 mg intrinsic factor Fraction No. 2 was dissolved in 10 ml .01 M acetate buffer, pH 4.7. Solution was dialyzed against 200 ml of the acetate buffer for 48 hr at 1°C. It was passed through a DEAE-cellulose column (37 × 1.1 cm) which had been equilibrated with 0.01 M acetate buffer, pH 4.7. Column was washed with buffer and gradient was begun at fraction 15 and produced by adding 0.01 M acetate buffer, pH 4.7, plus 0.5 M NaCl into a constant volume reservoir containing 100 ml 0.01 M acetate buffer, pH 4.7. Fraction volume was 6 ml and flow rate was 0.75 ml/min. Experiment was carried out at 25°C. Ordinate is optical density at 280 mμ and represents protein. Fraction pools 3-6 (Fraction No. 1), 7-11 (Fraction No. 2), 22-24 (Fraction No. 3), and 25-29 (Fraction No. 4) were dialyzed against distilled water at 1°C for 48 hr and lyophilized. Weights of fractions were as follows: No. D_1—138 mg; No. D_2—54 mg; No. D_3—50 mg; No. D_4—120 mg.

TABLE IV. Carbohydrate Analysis of Intrinsic Factor Fractions.

Fraction No.	Intrinsic factor activity, mg/U.S.P. unit	Carbohydrate as Mannose, %	Galactose, %
PI-IV	35	5.4	5.8
PI-IV-D	15	8.2	10.6
A_1	Inactive	12.8	16.5
A_2	12	8.6	11.1
A_3	9	6.5	8.4
A_4	10	4.2	5.4
B_1	12	6.1	7.9
B_2	14	2.2	2.7
C_1		12.8	16.4
C_2	9	6.3	8.1
D_1	18	10.9	14.0
D_2	10	3.1	3.9
D_3	70	7.7	10.0
D_4	Inactive	6.7	8.6

starting material. The number of peaks obtained in this experiment indicated that fraction No. C_2 was heterogeneous. There was also a considerable loss in recoverable activity. The active molecule may have split into more than one component on further purification or some prosthetic group may have been split off. The most active fraction obtained by the DEAE-cellulose chromatographic method had an estimated potency of 9 mg per U.S.P. unit.

The various fractions obtained were analyzed for carbohydrate by the method of Gurin and Hood(12). The results are summarized in Table IV. The 520/420 ratios indicate either mannose or galactose. There appears to be some correlation between intrinsic factor activity and carbohydrate content since the most active fractions contained the smallest amount of carbohydrate.

Summary and conclusions. A simple method is described for preparation of highly purified intrinsic factor PI from porcine stomach pyloric mucosa. In pernicious anemia patients in relapse, 35 mg of intrinsic factor PI when given with 15 μg vit. B_{12} is defined as one U.S.P. oral unit. Dialysis of intrinsic factor PI gives a material with a potency of about 15 mg/U.S.P. unit. In the urinary excretion test, one-half U.S.P. unit of intrinsic factor PI gives significant and measurable urinary excretion of vit. B_{12} Co^{60} when given orally with 1 μg vit. B_{12} Co^{60}. Approximately 1.5 U.S.P. units of intrinsic factor PI will give maximum urinary excretion of vit. B_{12} Co^{60}. Intrinsic factor PI can be further purified on a DEAE-cellulose ion exchanger to give a material with an estimated potency of 9 mg/U.S.P. unit.

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