Purification of Intrinsic Factor.*† (23872)

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Intrinsic factor (IF) is a heat-labile substance found in normal human gastric juice and absent in persons with pernicious anemia. It has been demonstrated by using radioactive Vit. B_{12}, that the function of IF is to cause absorption of Vit. B_{12} from intestinal tract of man(2,3). Since hog stomach and hog duodenum contain IF activity, numerous investigators have been concerned with the purification and isolation of IF(4).

A highly purified intrinsic factor preparation obtained from desiccated hog stomach has been described(5). In our report, fresh hog duodenum was employed and an IF preparation termed RAS (reprecipitated ammonium sulfate) fraction, fully active at 5 mg, was obtained in 100 g quantities suitable for further purification. This RAS fraction was further purified by ion exchange on a cellulose derivative and an IF fraction active at 1 mg was obtained.

Method. Preparation of AS fraction. Hog duodenum was obtained at slaughter houses from freshly killed hogs. The outside of tissue was washed for a few seconds in water at about 30°. Loose adhering fat was removed from outside of tissue and contents of intestine, if present, were expelled manually. If the intestine was not processed for IF immediately, the tissue was quickly frozen in flat layers and kept frozen in a chill room at −10° until used. The duodenum in 10 kg batches was ground in meat grinder, mixed with 30 l of 2% NaCl solution for 30 minutes at 10-15°, then settled for 30 minutes. The suspension was filtered through 2 layers of cheesecloth. The material remaining on cheesecloth was re-extracted with 10 l of 2% NaCl solution for 15 minutes at 10-15° and allowed to settle for 15 minutes. This second suspension was also filtered through 2 layers of cheesecloth. The 2 extracts were combined and pH of combined extracts was adjusted to 9.0 with 1 N NaOH. After 30 minutes, the solution was adjusted to pH 1.5 with 1 N HCl. A precipitate formed and after 30 minutes was removed with Sharples centrifuge Model M-86-P-IE. The resulting supernatant was adjusted to pH 4.5 with 1 N NaOH and 194 g of $(NH_4)_2SO_4$/l of supernatant was added slowly with stirring. The solution stood for 4 hours and was then centrifuged in the Sharples centrifuge. The precipitate was discarded and to resulting supernatant, 174 g of $(NH_4)_2SO_4$/l of supernatant was added slowly with stirring. The solution was kept overnight and again centrifuged. The resulting supernatant was discarded and precipitate dialyzed in cellulose tubing to remove the $(NH_4)_2SO_4$. This fraction has been named ammonium sulfate (AS) fraction. Up to this point the procedure is a modification of the method of Prusoff *et al.*(6). *Preparation of RAS fraction.* The dialysate was then centrifuged at 15,000 times gravity for 15 minutes in angle head centrifuge at 2-5°. The precipitate was discarded and supernatant reprecipitated with $(NH_4)_2SO_4$ using 368 g/l. After standing overnight at 4° the precipitate was removed by centrifugation at the same temperature. The resulting precipitate was dialyzed and centrifuged at 15,000 times

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TABLE I. Evaluation of RAS Fraction in a Pernicious Anemia Patient in Relapse (Patient C.D.).*

Initial red blood cell count	2,510,000
Red blood cell count after 21 days	3,380,000
Reticulocyte peak	11.4%
Expected reticulocyte peak	11.0%

* This patient was given orally 5 mg of RAS fraction together with 10 μg of vit. B_{12}.

gravity to remove any insoluble material. The clear supernatant was dried from frozen state and termed reprecipitated ammonium sulfate (RAS) fraction. *Activity of RAS fraction.* RAS fraction was tested in a pernicious anemia patient in relapse (Table I), according to method of U.S.P. Anti-Anemia Board(7). It was also tested in pernicious anemia patients in remission (Table II) by modified

TABLE II. Determination of Activity of RAS Fraction by the Modified Urinary Excretion Test.

	% excretion in urine
RAS fraction (10 mg)	5.7
Reference intrinsic factor 186-1 (50 mg)	5.9

urinary excretion test(8,9), and at dosage of 10 mg caused excretion of as much Vit. B_{12} as 50 mg of the reference IF #186-1. It was therefore 5 times as potent as the reference intrinsic factor sample, #186-1. The reference IF #186-1 used was previously studied by Ellenbogen *et al.*(8), Williams *et al.*(10), Baker and Mollin(11), and Callender and Evans(12), and it was shown that 50 mg of 186-1 was equivalent to 2-3 daily oral doses. Therefore, RAS fraction is active clinically at daily oral dose of 5 mg. *Electrophoretic and sedimentation studies of RAS fraction.* Electrophoretic analyses in phosphate-saline buffer of pH 6.1 and 0.1 ionic strength and acetate buffer of pH 4.1 and 0.1 ionic strength at

FIG. 1. Descending electrophoretic diagrams of RAS fraction. Left: Electrophoresis performed in phosphate-saline buffer of pH 6.1 and 0.1 ionic strength. Protein conc., 1.2%. Time of exposure, 80 min. Right: Electrophoresis performed in acetate buffer of pH 4.1 and 0.1 ionic strength. Protein conc., 1%. Time of exposure, 80 min.

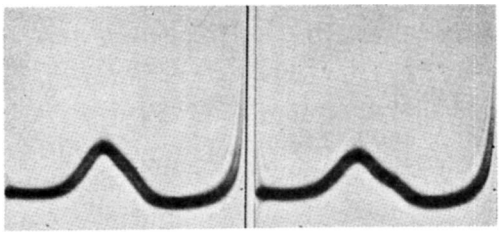
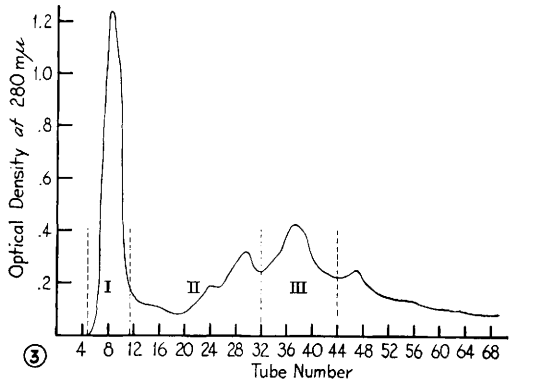
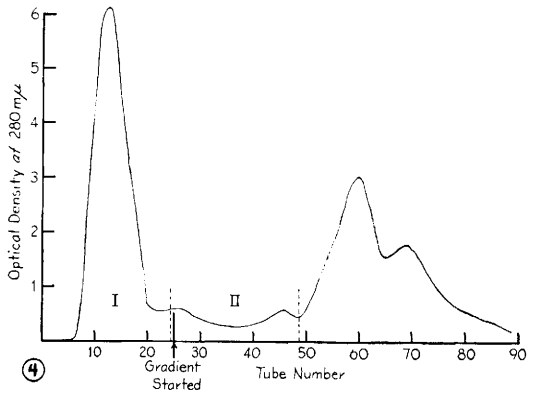


FIG. 2. Sedimentation diagram of RAS fraction. Ultracentrifugal analysis in Spinco ultracentrifuge at 59,780 rpm using synthetic boundary cell. Phosphate buffer pH 6.4, .002 ionic strength, with saline 0.14 ionic strength. Left: 24 min. Right: 32 min.

4° revealed that RAS fraction was heterogeneous (Fig. 1). Migration of components was towards opposite electrodes at these 2 pH ranges, indicating that IF present in RAS fraction had an isoelectric point between pH 4.1 and 6.1. Ultracentrifugal analysis in Spinco ultracentrifuge at 59,780 rpm with a synthetic boundary cell indicated that RAS fraction contained at least 2 major components, one with low sedimentation constant of about 0.7 S. (Fig. 2). To separate the high molecular weight (HMW) component and the low molecular weight (LMW) component of RAS fraction, centrifugation was carried out at 100,000 times gravity at 4° with Spinco Model E ultracentrifuge. In an initial attempt, centrifugation was carried out for 37 hours resulting in approximately 2-fold concentration of LMW component, although there was definite activity in both the LMW and HMW components. Centrifugation for 64 hours resulted in concentration of activity of HMW component. It is possible that intrinsic factor activity was associated with a component which could not be observed by ultracentrifuge analysis. In no instance was separation of components complete. *Cellulose ion-exchange purification of RAS fraction.* The anion exchange cellulose absorbent DEAE-Solka Floc (DEAE-SF)—100-200 mesh—was prepared according to methods of Peterson and Sober(13,14) and used to separate RAS fraction into 3 fractions. One hundred mg of RAS fraction was dissolved in 3 ml of 0.025 M phosphate buffer, pH 7.5, and applied to top of 1.4 x 24 cm column and allowed to run into the adsorbent by gravity. Three 1-ml portions of the buffer were used





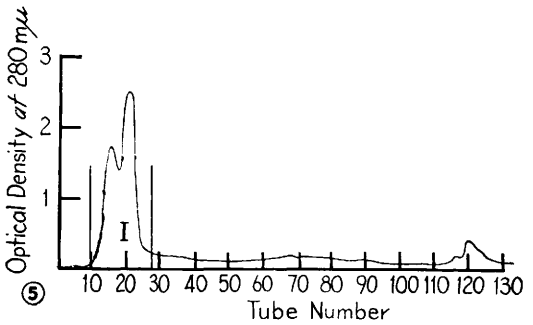


FIG. 3. Effluent diagram of 100 mg of RAS fraction on 1.4 × 24 cm column. See text for conditions of chromatography.

FIG. 4. Effluent diagram of 2 g of RAS fraction on a 2.8 × 32 cm column. First buffer (0.025 M phosphate—pH 7.5) was used until tube 25. pH gradient was started at tube 25 with pH 5.5 NaCl phosphate buffer.

FIG. 5. Rechromatography of column fraction II on 2.5 × 26 cm column. pH gradient was not started until tube 40. First buffer (0.025 M phosphate, pH 7.5) used as effluent until tube 40. First peak contained 52.7% of O.D. units of combined column fraction II before rechromatography.

to wash the sides of column after protein solution was added. A pH and ionic strength gradient was established with pH 5.5 NaCl-phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M NaCl) and allowed to run by gravity with a 10 to 40 in. buffer head. Elution was carried out in chill room at 4°. Flow rates were 4 ml/hr and 4 ml/tube were collected with the aid of a drop counter. The effluent fractions were examined in a Beckman DU spectrophotometer at 280 mμ.

Results. Fig. 3 shows an elution diagram obtained when RAS fraction was chromatographed on DEAE-SF. Chromatographic components were divided into 3 fractions as indicated by dotted vertical lines. Tubes from each of these 3 fractions were combined, dialyzed and lyophilized and then tested by the modified urinary excretion test(8.9). Results of assays are shown in Table III. Both fractions I and III showed little if any activity in urinary excretion test at 10 mg whereas fraction II representing about 20% of total protein of effluent showed full activity at 3 mg which should be equivalent to clinical activity at 1 mg (17 times as active as 186-1).

Larger amounts of RAS fraction (2-4 g) were chromatographed on 2.8 x 32 cm columns. The large inactive fraction (I) which came off the column first, emerged even before a pH gradient was established with pH 5.5 buffer (Fig. 4). As with smaller columns, intrinsic factor activity was present far down the descending slope of column fraction I and continued through the middle section of the curve (column fraction II). This middle section of the effluent diagram was somewhat flatter in these larger columns than column fraction II obtained in the smaller column (*cf.* Fig. 3).

Recovery of protein as measured by optical density was usually between 90 and 100%.

Column fraction II from 8 additional chromatographic separations, totaling 4.1 g, were combined and rechromatographed on DEAE-SF. Fig. 5 shows the effluent diagram obtained on rechromatography of fraction II. Activity of rechromatographed fraction II was present in the first peak this time, even though the pH gradient was started well past the flat portion of effluent diagram. No further increase in potency, however, was achieved.

TABLE III. Assay of Fractions from Cellulose Ion-Exchange by the Modified Urinary Excretion Test.*

Material	Dose (mg)	% excretion in urinary excretion test: Vit. B_{12} Co^{60} only	Vit. B_{12} Co^{60} + fraction	Vit. B_{12} Co^{60} + 50 mg of reference IF sample, 186-1
Column fraction I	10	.4	1.9	12.3
II	10	.4	19.2	12.3
II	3	.7	6.8	6.7
III	10	.4	2.8	12.3

* See effluent diagram in Fig. 3.

In the ultracentrifuge the active peak from rechromatography appeared heterogeneous (Fig. 6). Approximately 70% of this material showed a sedimentation constant of 2.9 S, 15% with 1.0 S and 15% with 11 S.

Discussion. One of the main difficulties in fractionation of IF has been in obtaining large quantities of a preparation of intermediate potency, *i.e.*, about 5 times as active as the reference sample #186-1. Preparation of RAS fraction represents a solution to this problem.

This RAS fraction prior to chromatography on DEAE-SF is about twice as potent as the IF obtained by Robbins and Shields(15) after purification on cellulose ion exchanger.

In regard to number of daily oral doses of IF necessary to give near maximum urinary excretion test, Robbins and Shields(15) have failed to note that some of the hematological data presented by Williams *et al.*(10) were after only 14 days of treatment. They have also failed to note that Baker and Mollin(11) found that 50 mg of IF caused absorption of 0.5 μg or more of Vit. B_{12} from 0.8 μg dose. When tested in the same patient, 50 mg of IF #186-1 caused excretion of 0.452 μg of Vit. B_{12} whereas 50 mg of standard used by Robbins and Shields caused excretion of 0.230 μg of Vit. B_{12} with a 2 μg oral dose.

Preliminary chromatographic experiments of RAS with DEAE-SF for further purification of IF have resulted in a 5-fold concentration. However, failure to obtain further purification upon rechromatography of column fraction II (Fig. 5) may indicate that further changes in pH, ionic strength or elution schedules may be necessary to obtain further resolution. All investigations thus far have been conducted using sodium phosphate buffers. It is quite possible that other buffer systems will provide better resolution. In view of the fact that the active peak after rechromatography contained 52.7% of total O.D. units, a 2-fold increase in potency is the best that can be obtained under conditions of this rechromatography.

It is quite likely that active fraction II of original chromatograms was displaced backwards by presence of fraction I, since in absence of fraction I (rechromatography of combined fraction II) the active fraction emerged as a sharper and more concentrated peak (Fig. 5).

Bacterial contamination was a problem frequently encountered in the use of larger amounts of IF for chromatography. Reluctance to freeze and thaw individual tubes for dialysis, assay, rechromatography and assay again necessitated exposure of dilute solution to 4° for as long as one week. Methods of preventing bacterial contamination are presently being investigated. Preliminary studies indicate that use of sterile distilled water and autoclaved glassware appear beneficial.

Another probable loss of activity has resulted from dialysis and lyophilization of very dilute solutions. That changes have taken

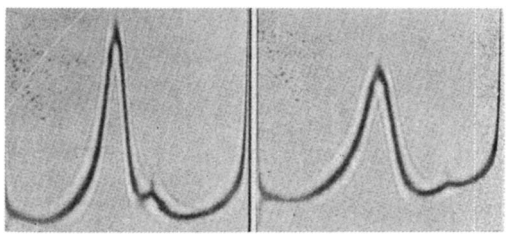


FIG. 6. Sedimentation diagram of column fraction I obtained from rechromatography of column fraction II on DEAE-SF. Ultracentrifugal analysis in a Spinco ultracentrifuge at 59,780 rpm using a synthetic boundary cell. Left: 8 min. Right: 16 min.

place after dialysis and drying is shown by the fact that dried IF was not completely soluble when an attempt was made to redissolve it. It is noteworthy that IF clinically active at 1 mg was heterogeneous. Unless there is more than one substance that can act as IF, this would indicate that pure IF is physiologically active at less than 1 mg.

Summary. A highly purified intrinsic factor preparation (RAS fraction) was prepared in quantity from fresh hog duodenum by saline extraction, pH adjustment and ammonium sulfate precipitation. This fraction was active in pernicious anemia patients in relapse at daily oral dose of 5 mg. Ultracentrifugal separation could not completely separate the components of RAS fraction. RAS fraction was further purified on a DEAE-cellulose ion exchanger to give a fraction which was active in urinary excretion test at a dose of 3 mg, equivalent to a daily oral dose of 1 mg in pernicious anemia patients in relapse. Rechromatography of this active fraction resulted in no further increase in potency. Ultracentrifugal studies of the most active fraction obtained from rechromatography showed essentially 3 peaks with sedimentation constants of 1.0 S, 2.8 S and 11 S.

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Effect of 17-Ethyl-19-Nortestosterone on Hyperglycemic Action of Glucagon in Humans. (23873)

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There are only a few known conditions in which human subjects will fail to respond to intravenous injection of pancreatic hyperglycemic factor (HGF or glucagon) with substantial rise in blood sugar. These are: a) if liver glycogen is low, as in starvation, severe liver disease or diabetic acidosis, and b) if liver glycogen is abundant but a specific enzyme deficiency prevents glycogenolysis, *e.g.* deficiency of glucose - 6 - phosphatase in glycogen-storage disease(1).

A chance observation in one patient led to the suspicion that 17 ethyl-19-nortestosterone (Nilevar†) might depress glucagon-in-

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