TABLE I. Serum Cholesterol and Low-Density Lipoprotein Values before and after 4 Weeks Therapy with Versenate in 11 Patients.

				Low-density lipoproteins*					
		Cholesterol		Sf 0-12		Sf 12-400		A.I.†	
Age	Sex	Control	V'senate	Control	V'senate	Control	V'senate	Control	V'senate
58	♀	1012	1065	1407	1259	310	331	195	184
57	♀	270	299	438	541	180	125	75	74
66	♂	210	187	296	339	405	221	101	73
65	♀	240	302	456	517	225	193	85	86
68	♀	302	532	673	719	518	564	158	171
70	♀	299	302	526	517	406	437	124	128
72	♂	272	265	544	521	355	284	117	102
54	♂	253	233	485	493	210	196	85	84
29	♂	225	249	396	434	409	451	111	122
40	♂	493	651	920	1111	252	281	136	160
47	♂	325	302	639	634	283	322	113	120

* Ultracentrifugally analyzed at Inst. of Medical Physics, Belmont, Calif.

† Atherogenic Index $= \dfrac{\text{Sf 0-12 lipoproteins} + \text{Sf 12-400} \times 1.75}{10}$.

ble. There were no consistent changes in any of the lipid categories beyond those which may have occurred spontaneously.

Discussion. The findings of the present study showed no cholesterol or lipoprotein lowering effect of the medication. This implies that the results previously reported(5) were probably due to the lowered fat content of the diet rather than the chelating agent.

Summary. Calcium disodium EDTA, in total dose of 3 g daily for 4 weeks, had no effect upon serum cholesterol and low-density lipoproteins of 11 humans.

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In vitro Assay of Hog Intrinsic Factor Concentrates Employing Paper Electrophoresis and Co^{60}-B_{12}.* (24958)

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Almost thirty years ago the substance in normal gastric juice, known as intrinsic factor, was discussed by Castle(1). Many attempts have since been made to isolate the active principle in pure form. Progress has been made, although electrophoretic and chromatographic evidence suggests that even the very best preparation is only 10-20% pure. Isolation and purification work on intrinsic factor has been hampered by lack of adequate assay procedures. At first the only method available was based on extent of hematological response registered by a pernicious anemia patient in relapse(2). This is still the only procedure accepted by the USP Anti-Anemia Board for evaluating potency of commercial

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intrinsic factor concentrates (IFC). Recently assay methods have been proposed suitable for use with pernicious anemia patients in remission. These methods are based on increase of absorption of radioactive Vit. B_{12} brought about by ingestion of intrinsic factor. The procedures make use of hepatic uptake(3), fecal excretion(4), and urinary excretion data (5). The urinary excretion method of Schilling(5) has been used extensively and represents a real step forward. This method, however, suffers all disadvantages of a clinical assay and has all errors inherent in any biological procedure. Several attempts to develop an *in vitro* assay for hog IFC have been reported based on total Vit. B_{12} binding capacity(6). All these attempts have failed, because of presence of multiple binders in even the purest IFC measured. Hoff-Jorgensen(7) reports success in standardizing commercial intrinsic factor preparations employing a strain of *E. coli* isolated from feces of a pernicious anemia patient. Miller(8) used the stimulating effect of IFC on absorption of radioactive Vit. B_{12} by rat liver slices as a measure of activity. Herbert(9) extended this work to provide for sequential incubation of rat liver slices in intrinsic factor—containing buffer, followed by Co^{60}-B_{12}. Minard and Wagner(10) found that uptake of Co^{60}-B_{12} by rat liver homogenate can be used as *in vitro* assay of hog intrinsic factor of pyloric origin. There is still need for an assay of intrinsic factor activity which does not require a biological environment. This assay should apply to all samples possessing clinical activity, regardless of origin and method of preparation. In the present study attention was centered on Vit. B_{12} binding components present in hog IFC, since all evidence available suggests binding as a necessary, although not sufficient, condition of activity. Paper electrophoresis is used to separate various binders in the presence of sufficient radioactive Vit. B_{12} to saturate all binding sites. Tagging of Vit. B_{12} permits ready location and quantitation of the binders. A correlation of one particular binder with activity thus provides the basis for an *in vitro* assay of intrinsic factor activity based on physical data alone.

Methods. Electrophoretic assay. The test solution was made in 6.2 phosphate buffer at ionic strength 0.05. Concentration of intrinsic factor was 40 mg/ml; concentration of radioactive vitamin was 10 μg/ml. The samples ranged in activity from 2 mg to 100 mg. The tagged vitamin was paraben free and had a specific activity of 0.97 μc/μg. Electrophoretic separation of B_{12} binders was carried out in horizontal paper electrophoresis unit (Reco), employing strips of Whatman No. 1, 50 cm x 2.5 cm. A volume of 25 λ of test solution was applied as a thin line 35 cm from the anode. Separations were carried out for 24 hours at a potential of 350 volts. After electrophoresis, the strips were air dried and autoradiographed, according to conditions outlined below. The processed films were analyzed in recording densitometer (Spinco Analytrol equipped with a B-2 cam.) Where data on protein distribution was desired, the electrophoretic strips were stained with napthol blue black and read in the densitometer. The autoradiographic procedure called for contact between strip and Kodak No-Screen X-ray film (1⅞″ x 16″) for 17 hours. The films were developed using standard technic. An internal standard of radioactive B_{12} was run with each film. All areas were corrected for variations in film exposure and processing conditions as revealed by response of the standard.

Results. Protein distribution data. In our early studies data were collected comparing distribution of proteins with distribution of Vit. B_{12} binders. A sample active clinically at 5 mg/day gave results shown in Fig. 1. It will be seen that major protein components are not the same as the major B_{12} binding components. This explained why our previous efforts to correlate clinical activity with Tiselius electrophoretic data had failed.

Correlation of Vit. B_{12} binding with clinical activity. Nine samples of hog IFC ranging in clinical activity from 1.5 mg to 75 mg were used. These samples were clinically assayed at 3 levels by a modified Schilling technic(11) to make certain that the activity level of each sample was established beyond doubt. The clinical data are given in Table I. Data on distribution of B_{12} binders in each of these samples were obtained following standard

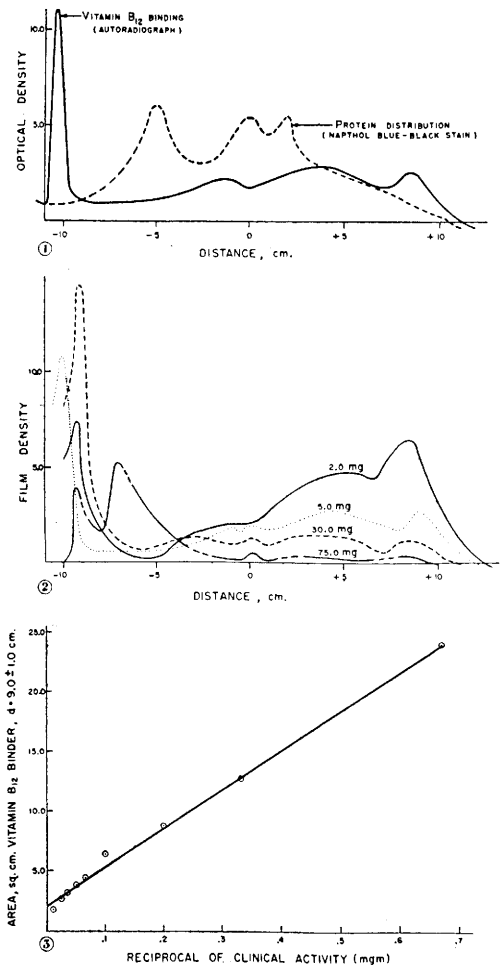

FIG. 1. Distribution of proteins and B_{12} binders in sample of hog intrinsic factor active clinically at a level of 5 mg.

FIG. 2. Electrophoretic distribution of B_{12} binders in samples of hog intrinsic factor of varying activity.

FIG. 3. Standard curve used in electrophoretic assay of intrinsic factor concentrates to determine level of clinical activity.

autoradiographic procedure. Typical curves are shown in Fig. 2. It was noted that all samples contained more than one B_{12} binder, but more important, that they all contained a B_{12} binder that moved 9 $\pm$ 1 cm. If this figure is corrected for electroosmotic flow, the distance is 18 cm. This gives an electrophoretic mobility on paper of $-3 \times 10^{-5} cm^2/sec.$ volt. For convenience and since standard conditions are used, the uncorrected figure of 9 cm is preferred. A check showed that the amount of this binder appeared to correlate with clinical activity of the sample. The binding data are given in Table I. When the area of the peak at 9 $\pm$ 1 cm, a measure of B_{12} bound by that particular component, is plotted against reciprocal of the clinical activity in mg, a straight line is obtained, Fig. 3. Since samples involved in this study, all of hog mucosa origin, had been treated in a wide variety of ways and had been exposed to various types of environment, this finding of a linear relationship is meaningful.

Use of standard curve on unknowns. Using Fig. 3 as our standard curve, the work was extended to a large number of unknown concentrate samples which had never been assayed clinically. From electrophoretic assay data activity levels were predicted. Each sample was then assayed clinically by the Schilling technic at the predicted level. A few typical results are shown in Table II. It will be noted that all samples were fully active at the predicted levels. Close to 100 unknown hog IFC samples have been run so far ranging in activity from 2 mg to 100 mg. In every instance agreement between electrophoretic assay and Schilling data is excellent

Discussion. In arriving at final conditions of the method, the electrophoretic conditions of paper, pH, ionic strength and time have all been explored to make certain that maximum resolution of the B_{12} binders is obtained. A dialysis step was not included in our standard assay procedure, since all of the concentrates used to establish the assay had been dialyzed at least once during their preparation. Average specific conductivity of our test solutions (4%) was 5.0×10^{-3} mhos. Samples which have not been freed of highly conducting materials may need to be dialyzed before they are assayed.

In the autoradiographic step every effort has been made to reduce film exposure time. This includes a 20-fold concentration of the commercial solution of tagged vitamin. When B_{12} of higher specific activity becomes available, the exposure time can be reduced further. The present overnight regimen, however, is quite convenient.

In place of employing autoradiography, standard counting procedures can be used directly on electrophoretic paper strips. Count-

ing can be carried out by scanning 1 cm segments. The disadvantage of this method is the discontinuous nature of the count data, making it difficult to quantitate amounts of

TABLE I. Clinical and Electrophoretic Data on Standard IFC Samples.

Standard sample	Patient	Level, mg	Clinical data (Schilling) B_{12} Co^{60} recovered in urine, % Base	% Standard	% Sample	Electrophoretic data Activity level, mg	Distance, cm	Area, cm^2
1	R.T.	3.0	0.7	6.5	8.7	1.5	8.8	24.0
	I.W.	1.5	0.2	7.0	7.5			
	C.C.	1.5	0.7	6.0	7.7			
	E.M.	0.75	1.0	6.8	3.4			
2	A.H.	6.	2.1	6.0	8.6	3.	8.4	12.8
	C.C.	3.	0.7	6.0	6.2			
	E.M.	3.	1.0	6.8	6.1			
	I.W.	1.5	0.2	7.0	3.8			
3	B.H.	10.	0.0	12.2	22.7	5.	9.3	8.8
	A.S.	5.	3.2	8.9	9.7			
	B.H.	5.	0.0	12.2	12.0			
	F.J.	2.5	1.5	15.5	7.8			
4	C.C.	20.	0.7	6.0	7.5	10.	8.3	6.4
	L.D.	10.	3.5	18.3	15.0			
	C.C.	10.	0.7	6.0	7.0			
	A.K.	5.	1.0	10.4	5.8			
5	E.P.	30.	1.3	7.0	10.0	15.	9.0	4.4
	C.R.	15.	1.5	15.2	14.1			
	J.P.	15.	0.8	9.6	10.0			
	E.G.	7.5	7.3	27.2	11.5			
6	B.G.	40.	1.0	11.8	17.5	20.	8.2	3.8
	J.P.	20.	0.8	9.7	8.8			
	F.C.	20.	1.8	6.8	6.9			
	R.K.	10.	1.5	6.6	3.9			
7	J.P.	60.	0.8	9.7	8.0	30.	8.4	3.2
	J.F.	30.	0.0	14.0	10.9			
	L.D.	30.	3.7	18.3	14.2			
	C.J.	15.	2.7	16.7	4.6			
8	P.J.	80.	1.7	15.5	20.6	40.	8.5	2.6
	P.J.	40.	1.7	15.5	15.5			
	R.B.	40.	1.0	11.8	11.8			
	R.B.	20.	1.0	11.8	6.0			
9	R.K.	150.	1.5	6.6	16.5	75.	8.4	1.8
	J.P.	75.	0.8	9.7	14.0			
	F.C.	75.	1.8	6.8	6.9			
	F.W.	37.5	2.0	18.0	8.0			

TABLE II. Application of Electrophoretic Assay to Unknown Hog Intrinsic Factor Concentrates

Electrophoretic assay Sample	Distance, cm	Area, cm^2	Predicted level, mg	Clinical assay (Schilling) B_{12} Co^{60} recovered in urine, %* Patient	Base	Standard	Sample
26	9.5	15.0	3.	S.C.	3.3	8.4	7.7
C-10	8.5	6.0	10.	P.U.	5.5	17.1	16.5
27	8.3	4.1	15.	H.S.	2.5	14.2	16.0
17	8.3	2.8	40.	L.H.	.5	9.2	10.8
7221	8.6	4.2	15.	R.B.	1.0	7.5	9.3
46	9.3	11.0	4.	B.H.	.7	9.2	8.0
ED #3	8.7	9.3	5.	A.W.	2.1	6.0	8.6
45B	9.5	17.0	2.5	R.T.	.6	5.0	5.7
42	8.4	3.5	25.	W.W.	3.3	9.8	13.5
7223	8.8	3.1	30.	G.E.	1.4	8.4	8.6

* All clinical assays were obtained at the predicted levels.

the various binders. Use of a radiochromatogram scanner would overcome this objection.

The assay calls for use of excess of Vit. B_{12} sufficient to satisfy all binding sites and to permit a check for presence of unbound B_{12} on the autoradiograph. When substantially lesser amounts of B_{12} are used, no free B_{12} is seen, indicating that the binding equilibria favor strongly complex formation. No evidence suggesting preferential binding of Vit. B_{12} by any of the binders present has been observed.

Since the standard curve relating peak area and clinical activity is a straight line, its equation can be readily determined. This line is, however, very much influenced by the particular electrophoretic and autoradiographic conditions employed and by the choice of clinical assay procedures. A standard curve needs to be worked out for each laboratory using the assay. All unknown samples evaluated by the electrophoretic assay and checked clinically have been of hog origin. Some originated in the pylorus; others came from the duodenum. Extension of the assay to human gastric juice is now under study.

The correlative data presented do not include any inactive samples. A number of such samples, however, have been run, and in every instance the lack of any binder in the 9 cm. area has coincided with lack of any clinical response at high levels. In this work many active samples have been inactivated in the laboratory under various conditions of pH and temperature. The stability pattern for intrinsic factor which this work reveals checks very well published reports. At the present time the stability of intrinsic factor, in the presence and absence of Vit. B_{12}, is the subject of an extended study and will be reported separately. The combined data on protein and B_{12} binder distribution on our most active preparation permits an estimate of the ultimate level of activity of pure intrinsic factor. In a sample active at a level of 0.5 mg the protein distribution shows three major components, the percent believed to be associated with activity amounting to only 20%. On this basis it can be predicted that the ultimate level of activity for pure intrinsic factor will be of the order of 0.1 mg. It is of interest to estimate how much Vit. B_{12} is bound by pure

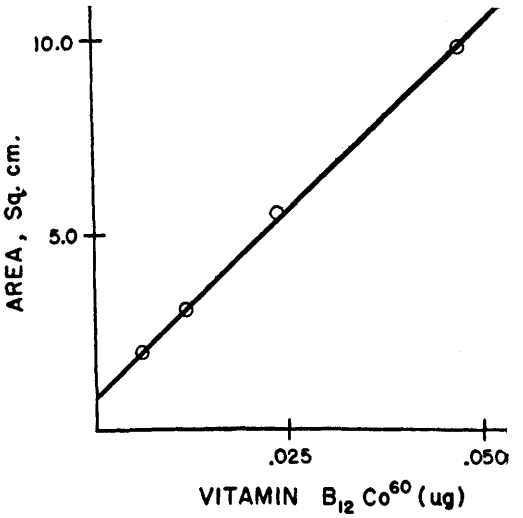

FIG. 4. Autoradiograph response to varying amounts of tagged B_{12}.

intrinsic factor, assuming that the particular binder measured at 9.0 cm is the active principle. For this purpose various amounts of tagged Vit. B_{12} were applied to some of our paper strips and autoradiographed. This gave the standard curve shown in Fig. 4. From Table I and Fig. 4 (extrapolated) the area under the 9 cm peak of standard sample 1 corresponds to 1.5 μg of B_{12}. Assuming pure intrinsic factor is active at a level of 0.1 mg, this sample, active at 1.5, mg contains 6.7% intrinsic factor. Since 1 mg of sample was applied to the paper strip, 0.067 mg of intrinsic factor was responsible for binding the 1.5 μg of B_{12}. On this basis one milligram of intrinsic factor will bind 23 μg of Vit. B_{12}. If the molar binding ratio of intrinsic factor to B_{12} is 1:1, molecular weight of intrinsic factor should be around 60,000. These are rough estimates, but these figures for B_{12} binding capacity and molecular weight of intrinsic factor agree well with other published estimates (6,12). The estimates refer to the intact hog intrinsic factor found in mucosal lining of the hog. It is recognized that the active principle may be a much smaller molecule, which in nature is either a component part of a large protein, or bound thereto.

Summary. Simultaneous use of paper electrophoresis and radioactive Vit. B_{12} permits separation and quantitation of the various B_{12}

binding components present in commercial intrinsic factor concentrates of hog origin. The amount of one particular binder has been found to correlate with the clinical activity of the concentrates as measured by the urinary excretion method of Schilling. This provides the basis for a simple *in vitro* assay of potency of hog intrinsic factor concentrates ranging in level of activity from 2.0 mg to 75.0 mg. It is predicted that pure hog intrinsic factor will be active clinically at a level of 0.1 mg; calculations suggest that it will bind 23 μg B_{12}/mg and have a molecular weight of 60,000.

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Histochemical Localization and Metabolic Significance of Glucose-6-Phosphate Dehydrogenase System in Adrenal Cortex.* (24959)

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It has been suggested that the adrenal cortex is one of the few mammalian tissues which utilizes the oxidative mechanism of pentose phosphate pathway for metabolism of carbohydrate in preference to the more usual glycolytic pathways of Embden and Myerhof and the citric acid cycle(1,2). Haynes(3,4) provided evidence that adrenocorticotrophin (ACTH) may act to promote steroid synthesis through a stimulating effect on adrenal phosphorylase with increased production of both glucose-1-phosphate and glucose-6-phosphate. The latter compound enters the pentose phosphate pathway *via* a triphosphopyridine nucleotide (TPN) mediated dehydrogenation to 6-phosphogluconic acid, with reduction of TPN to TPNH. The 6-phosphogluconic acid is in turn oxidatively decarboxylated, again using TPN as an electron acceptor with production of ribulose-5-phosphate and reduced TPN (TPNH). The system at this stage is acting as a TPNH generator. This is particularly significant since TPNH is required in the adrenal cortex as reductant for hydroxylations of the steroid molecule. These data in metabolic studies were obtained by quantitative methods which do not preserve structural relationships in the tissue. It was felt that use of histochemical technics for demonstration of some enzyme systems involved might contribute useful data on localization of these systems within the cortex and thus supplement the quantitative observations.

Materials and methods. Adrenal tissue was obtained from 10 250 g, female, white rats fed a standard laboratory diet and acclimated

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