JCI The Journal of Clinical Investigation

IMMUNOLOGIC STUDIES WITH INTRINSIC FACTOR IN MAN

Manuel E. Kaplan, … , Jack Remington, Victor Herbert

J Clin Invest. 1963;42(3):368-382. https://doi.org/10.1172/JCI104723.

Research Article

Journal of Clinical Investigation
Vol. 42, No. 3, 1963

IMMUNOLOGIC STUDIES WITH INTRINSIC FACTOR IN MAN *

By MANUEL E. KAPLAN,† RALPH ZALUSKY, JACK REMINGTON,‡ AND VICTOR HERBERT

(From the Thorndike Memorial Laboratory and the Second and Fourth [Harvard] Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

(Submitted for publication September 1, 1962; accepted November 15, 1962)

Since the fundamental defect in pernicious anemia is inadequate or absent secretion of gastric intrinsic factor (1), specific therapy with intrinsic factor would seem, logically, to be the preferred treatment. After the demonstration (2, 3) that desiccated hog stomach could substitute for human gastric juice to facilitate the absorption of vitamin B_{12} in man, many patients with pernicious anemia were treated orally with extracts of hog pyloric mucosa and vitamin B_{12}. In most cases, gratifying hematological improvement was noted initially. With continued administration of these preparations, however, a significant number of patients suffered hematologic relapse (4–9). In these individuals, it was found that sources of porcine intrinsic factor no longer facilitated vitamin B_{12} absorption (6–8, 10). This phenomenon of refractoriness to orally administered hog intrinsic factor concentrates (HIFC) was among the reasons that led the U.S.P. Anti-Anemia Preparations Advisory Board to recommend, in 1959, that such preparations be excluded from the U. S. Pharmacopoeia (11).

Certain observations suggest that the refractory state is immunologic in origin. The present studies were undertaken in an attempt to demonstrate a causal relationship between immunization to HIFC and the refractory state, and have been previously reported in preliminary form (12).

* Supported in part by grant A-795 from the U. S. Public Health Service and in part by a grant from Eli Lilly and Co., Indianapolis, Ind.

† Investigation carried out during the tenure of a postdoctoral fellowship from the National Heart Institute, U. S. Public Health Service. Present address: Department of Hematology, The Mt. Sinai Hospital, New York, N. Y.

‡ Present address: Palo Alto Medical Research Foundation, Palo Alto, Calif.

MATERIALS AND METHODS

I. *Anti-HIFC sera*

Purified hog intrinsic factor concentrate [1] (P-HIFC) was used as the stimulating antigen.

A. *Rabbits.* To 0.5 mg of P-HIFC dissolved in 1.0 ml of saline an equal volume of complete Freund's adjuvant [2] was added, and the mixture was emulsified thoroughly. Two albino rabbits each weighing 2 to 3 kg were injected weekly for 4 weeks with P-HIFC in adjuvant as follows: initially, 0.25 ml was administered into each foot pad; subsequently, 1.0 ml was injected into multiple subcutaneous and intramuscular sites. After a rest period of one month, the animals received intraperitoneally 0.5 ml of P-HIFC in saline without adjuvant and one week thereafter were bled. Sera were separated by centrifugation, pooled, and stored at −20° C until used.

B. *Patients.* Two elderly males (J. W. and J. D.) with treated pernicious anemia, previously diagnosed on the basis of *1)* macrocytic anemia with megaloblastic bone marrow, *2)* gastric anacidity after maximal histamine stimulation, *3)* low vitamin B_{12} and normal folate serum levels, and *4)* subnormal absorption of Co^{60}-vitamin B_{12} corrected by orally administered HIFC, were injected repeatedly with P-HIFC in the following manner: to a 100 mg per 100 ml concentration of P-HIFC in sterile saline was added sufficient 2% merthiolate [3] solution to result in a 1 : 1,000 concentration. Aerobic and anaerobic cultures of this preparation were consistently sterile. The P-HIFC solution, 0.2 ml, was mixed with 0.3 ml of incomplete Freund's adjuvant [2] and injected subcutaneously into the right upper arm. No immediate or delayed effects were noted other than persistent "soreness" at the injection site. One week thereafter, 0.5 ml of P-HIFC without adjuvant was administered subcutaneously into the left upper arm. No immediate untoward effects were noted; however, patient J. W. reported mild itching at the injection site, beginning within 4 to 6 hours and per-

[1] We are indebted to Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N. Y., for generously providing the following hog intrinsic factor concentrates: *a)* 3596 C-46-1 (P-HIFC) active at a daily oral dose (D.O.D.) of 0.3 mg; *b)* WES 671 A, D.O.D.= 5 mg; *c)* WES 186-1, D.O.D.= 50 mg.

[2] Difco Laboratories, Detroit, Mich.

[3] Thimerosol, Eli Lilly and Co., Indianapolis, Ind.

TABLE I

Source and serological characteristics of the sera studied

Code	Patient	Oral HIFC	BDB* Titer	BDB* Date	Date of serum collection	Electrophoretic mobility in serum of Co^{57}-HIFC	Homogenate uptake in cpm minus saline control
HIFC							6,785
A Refractory	WS†	Discontinued 1/56	1:16	1/59	3/59	β	11,572
B	Normal	0			?	β	13,077
C Refractory	LW [1]‡	Daily	1:100	2/59	6/59	β	12,535
Refractory	LW [2]	Discontinued 10/59	1:100	2/59	6/61	β	
D Refractory	FC [1]	Daily	1:2	8/59	8/59	None	14
H Refractory	FC [2]	Discontinued 10/59	1:2	8/59	1/61	β	11,006
Refractory	FC [3]	Discontinued 10/59	1:2	8/59	6/61	β	
E	Myelogenous leukemia	0			?	β	14,083
F	Normal	0			?	β	13,266
G Refractory	PC [1]	Discontinued 10/59	1:8	6/59	1/61	β	8,992
Refractory	PC [2]	Discontinued 10/59	1:8	6/59	6/61	β	
Refractory	SS	Discontinued ? date	0	?/59	6/61	β	

* BDB is bis-diazotized benzidine.
† The initials identify the patients previously proved refractory by Lowenstein and associates (13).
‡ Numbers in brackets indicate separate serum specimens from the same subject. All homogenate uptake studies were performed on the same day with samples of the same liver.

sisting for 2 to 3 days. Subsequent injections of 0.1 or 0.2 ml of P-HIFC in saline were administered intradermally in order to observe the development of skin sensitivity. Patient J. W. received a total of 1.1 mg and patient J. D. a total of 0.9 mg of P-HIFC. Generous blood samples were obtained from both patients before immunization was initiated, immediately before each injection of P-HIFC, and 1 week after the series of injections was completed. Sera were separated by centrifugation and stored in separate vials at −20° C until used.

II. *Sera from patients previously proved refractory*

The sera were supplied by Drs. Lowenstein and Cooper from 5 patients with pernicious anemia unresponsive to oral HIFC previously reported in detail by their group (13). In all, 12 sera were provided by the Montreal group; the first 8, characterized only by the serial code letters "A" to "H," included sera from subjects without pernicious anemia as well as from patients with pernicious anemia previously demonstrated to be refractory to oral HIFC. After completion of the studies herein reported, the donor of each serum was identified. The remaining sera were known to be recently obtained specimens from 4 previously refractory patients; 3 of the 4 had earlier contributed sera included in the first group of 8.

In Table I are designated *a*) the source and collection date of each serum studied and *b*) whether the patient was receiving oral HIFC at the time his serum was obtained. In addition, the results of the most recently performed hemagglutination titration studies (BDB = bis-diazotized benzidine) performed by Lowenstein, Cooper, Brunton, and Gartha (13) are included.

III. *Serological studies*

A. *Agar diffusion.* Precipitating antibodies to P-HIFC were sought in the sera of the patients and rabbits immunized with P-HIFC and in the sera of 5 patients previously proved refractory to oral HIFC. For this purpose, the double diffusion methods of Ouchterlony (14) and Preer (15) as well as the microimmunoelectrophoretic technique described by Scheidegger (16) were utilized.

B. *Precipitation of Co^{57}-B_{12}-HIFC complex by rabbit antisera.* A Co^{57}-B_{12}-HIFC complex was prepared by the addition of 1.75 μg of Co^{57}-labeled vitamin B_{12} [4] with a

[4] Kindly provided by Dr. Alpert, Merck Sharp & Dohme Research Laboratories, Philadelphia, Pa.

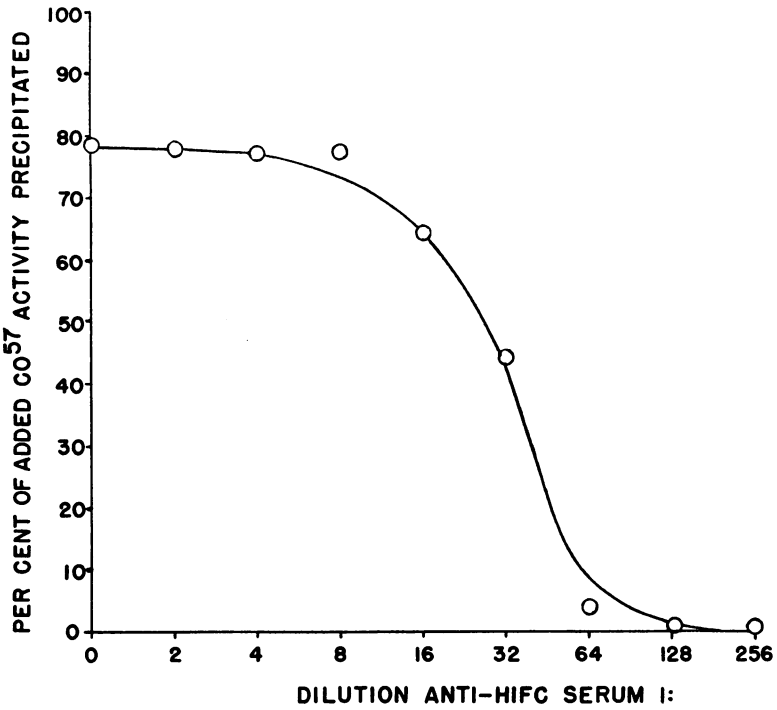

FIG. 1. PERCENTAGE OF Co^{57} ACTIVITY PRECIPITATED FROM A SYSTEM CONTAINING Co^{57}-B_{12}-HIFC (HOG INTRINSIC FACTOR CONCENTRATE) AND SERIALLY DILUTED RABBIT ANTI-HIFC SERUM.

specific activity of 990 μc per mg to 6.0 ml of a 550 mg per 100 ml aqueous suspension of HIFC (WES 671-A). Although it would have been preferable to use P-HIFC as the antigen in this precipitin system, an insufficient supply of the latter material would not permit this. After dialysis for 24 hours against cold (4° C) water, the contents of the dialysis bag were centrifuged in the cold and the scanty precipitate discarded. The clear supernatant fluid was diluted 1 : 5 in saline, and 0.5 ml samples thereof were pipetted into a series of test tubes. Three ml of the pooled serum obtained from rabbits after immunization with P-HIFC were diluted serially in twofold steps, from 0 to 1 : 1024. To each tube containing Co^{57}-B_{12}-HIFC was added 2 ml of a given dilution of rabbit antiserum; a control tube containing normal, undiluted rabbit serum was included. After incubation at 37° C for one hour, the tubes were allowed to stand at 4° C for 48 hours. The precipitates were spun down, washed twice in cold saline, and counted against 0.5 ml of the Co^{57}-B_{12}-HIFC standard in a well-type scintillation counter.

IV. *Electrophoretic studies*

A similar Co^{57}-B_{12}-HIFC complex was prepared by the addition of 0.5 ml (0.484 μg) Co^{57}-B_{12} to 2.5 ml of a 100 mg per 100 ml aqueous suspension of HIFC (WES 671-A). After dialysis and centrifugation as previously described, 20 μl of the Co^{57}-B_{12}-HIFC solution was added to 0.25 ml samples of various human sera and the resultant mixtures were incubated at 37° C for 1 hour. Fifty μl of each mixture was applied to an 8- by 11-inch sheet of Whatman no. 1 filter paper and electrophoresis at 180 v performed in Veronal buffer (pH 8.6, 0.05 M) for 16 hours at 25° C. Thereafter, the filter paper was oven dried and cut into 1¼-inch wide longitudinal strips corresponding to the migration paths of the various sera. With each electrophoretic run, one control strip was stained with bromphenol blue in order to localize the various serum components. The distribution of radioactivity of the remaining strips was measured by cutting each strip into ¼-inch segments and counting them in a well-type scintillation counter.

V. *Homogenate studies*

In the presence of a hog intrinsic factor source, homogenates of rat liver will take up large quantities of vitamin B_{12} from the surrounding medium (17). In order to explore the effects of various sera in this system, the standard homogenate technique was modified as follows: HIFC (WES 186-1) was prepared as a 5 mg per 100 ml suspension in saline. To a series of test tubes containing 0.5 ml of the test serum serially diluted in saline from whole serum to 1 : 32,000, 0.5 ml of the HIFC suspension was added (containing 25 μg of HIFC). After incubation for 1 hour at 37° C and overnight at 4° C, the tubes were centrifuged at 3,000 rpm in the cold for 30 minutes. The supernatant fluid was decanted and a sample thereof assayed for intrinsic factor activity in the

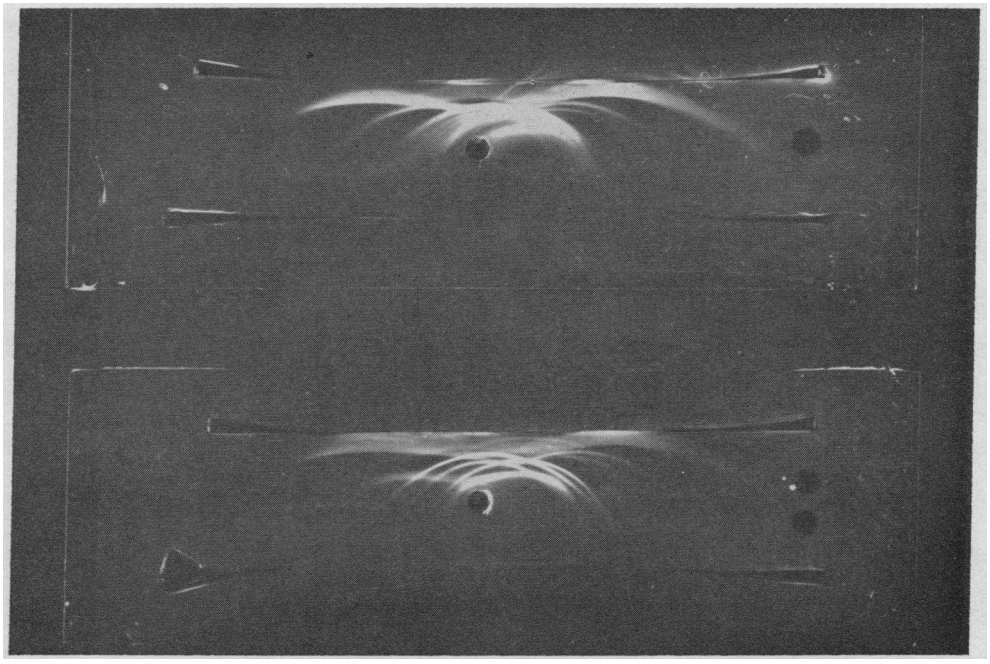

FIG. 2. IMMUNOELECTROPHORETIC SLIDES A AND B. The circular wells contained postimmunization serum from patient J. W. (upper) and J. D. (lower). The longitudinal reservoirs above each well were filled with goat antihuman serum and those below each well were filled with P-HIFC. The anode was to the right. In each case a faint precipitin line is seen between the P-HIFC reservoir and a serum component corresponding, in electrophoretic mobility, to the gamma globulins.

liver homogenate system by the single incubation technique (17). The following controls were always included: normal serum with HIFC, test serum without HIFC, and saline with HIFC. During the incubation of immune rabbit serum with HIFC, precipitates formed in tubes containing the higher concentrations of rabbit serum. These precipitates were washed twice in saline and dissolved in 0.1 ml 0.05 N NaOH. Chemical neutrality was re-established with addition of 0.05 N HCl and sufficient Krebs-Ringer Tris buffer, pH 7.4, added to result in a standard volume of 1 ml. These dissolved, buffered precipitates, which showed no tendency to reprecipitate spontaneously, were assayed simultaneously with the supernatant fluids in the liver homogenate system by the single incubation technique. The results were expressed as counts per minute above or below the value given by the control containing test serum without added HIFC.

After it had become apparent that antisera to HIFC prevented the latter from enhancing radioactive vitamin B_{12} uptake in the homogenate system, the mechanism of inhibition was investigated by means of the sequential incubation technique (17). Samples of liver homogenate were incubated for 30 minutes at 25° C in *a*) HIFC, *b*) rabbit anti-HIFC serum, or *c*) saline. After the homogenates were washed in buffer, anti-HIFC serum was added to the homogenate previously exposed to HIFC, HIFC added to the homogenates previously incubated with anti-HIFC or with saline, and reincubation was performed as described above. The homogenates were again washed and Co^{60}-B_{12} then added as a third incubation step. Final washing and measurement of homogenate radioactivity were performed in the usual way. Two controls were included in the sequential incubation studies: HIFC was incubated for 1 hour at 37° C with rabbit anti-HIFC or with normal rabbit serum before being mixed with homogenate; and after initial incubation and washing, Co^{60}-B_{12} was added subsequently as a second incubation step.

VI. *Vitamin B_{12} absorption studies*

On repeated occasions vitamin B_{12} absorption was measured by a modification (18) of the method of Schilling (19) in the two patients with pernicious anemia. An oral test dose of 2 μg Co^{60}-B_{12} containing 0.25 μc of radioactivity was administered. Immediately and 24 hours thereafter, 1,000 μg of unlabeled vitamin B_{12} was injected subcutaneously. Urine was collected for 72 hours after administration of the oral Co^{60}-B_{12}. Since little Co^{60} activity was excreted during the final 24 hours, the urinary excretion of Co^{60}-B_{12} was calculated and expressed as the percentage of the orally administered dose recovered within the first 48 hours. In the normal subject, this was greater than 5%. When the effect of a given serum on vitamin B_{12} absorption was to be assessed, the following protocol was adopted. To 50 mg HIFC (WES 186-1 dissolved in 4 ml of saline, 1 ml of saline containing 2 μg Co^{60}-B_{12} was added and the resulting sus-

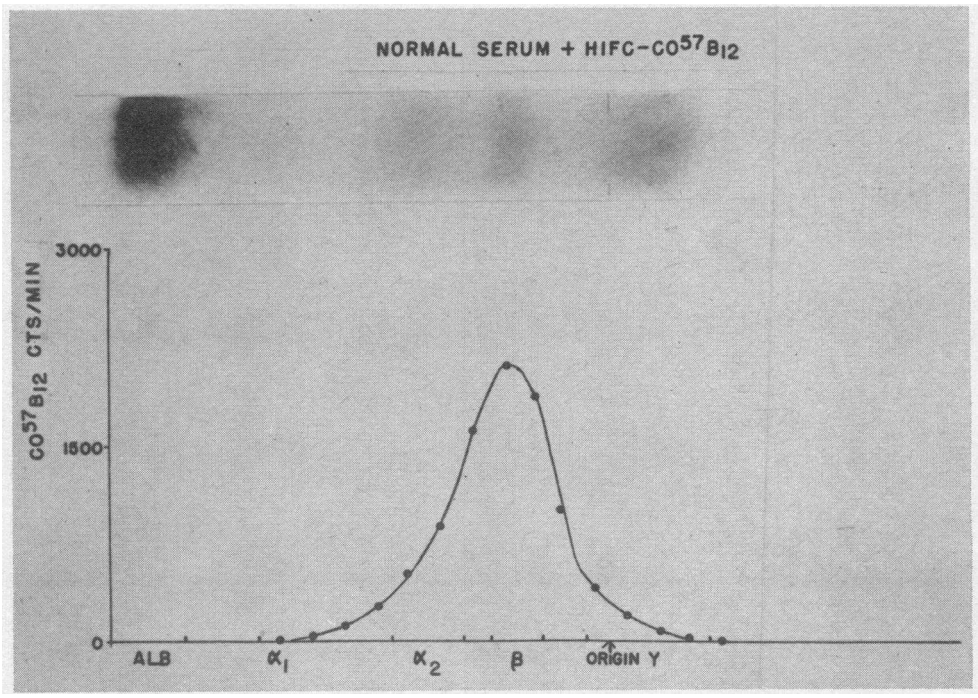

FIG. 3. PAPER ELECTROPHORESIS OF NORMAL SERUM WITH Co^{57}-B_{12}-HIFC. The electrophoretic distribution of radioactivity is shown.

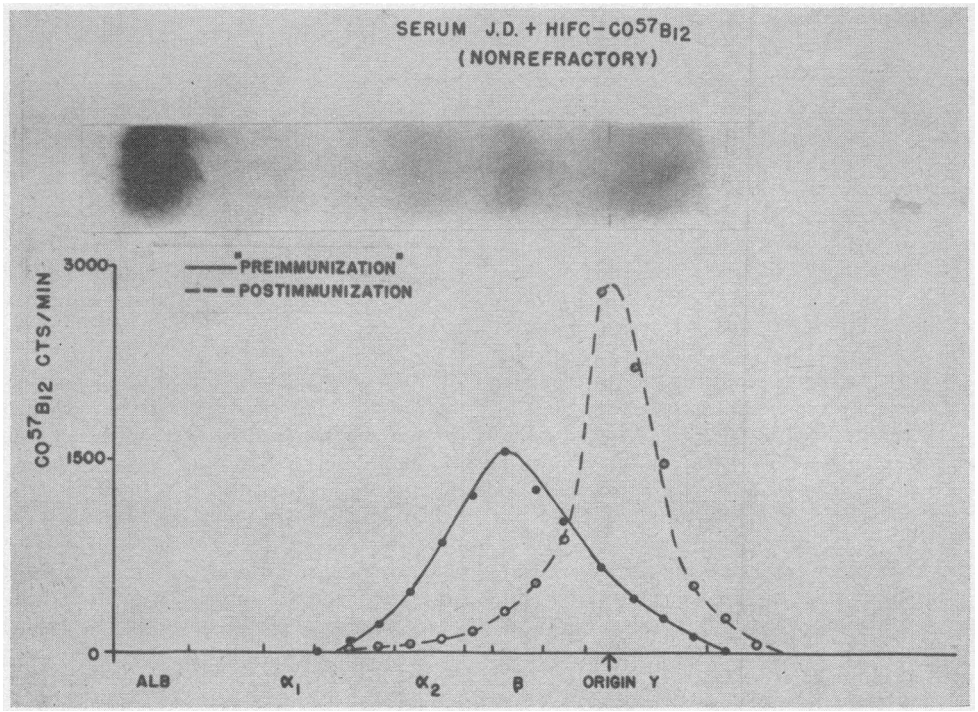

FIG. 4. PAPER ELECTROPHORESIS OF PERNICIOUS ANEMIA SERA (J. D.) WITH Co^{57}-B_{12}-HIFC. The electrophoretic distributions of radioactivity in preimmunization and postimmunization sera are shown. Preimmunization serum was obtained after the patient received the first injection of P-HIFC; this may account for the slurring of the radioactivity peak toward the origin.

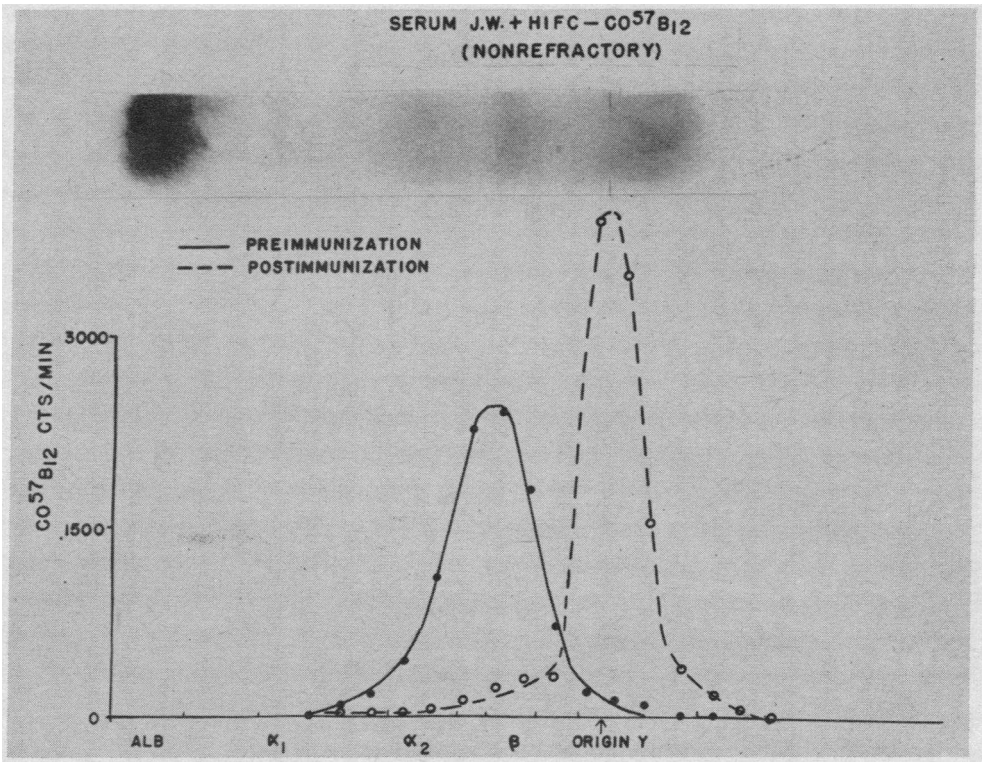

FIG. 5. PAPER ELECTROPHORESIS OF PERNICIOUS ANEMIA SERA (J. W.) WITH Co^{57}-B_{12}-HIFC. The electrophoretic distributions of radioactivity in preimmunization and postimmunization sera are shown.

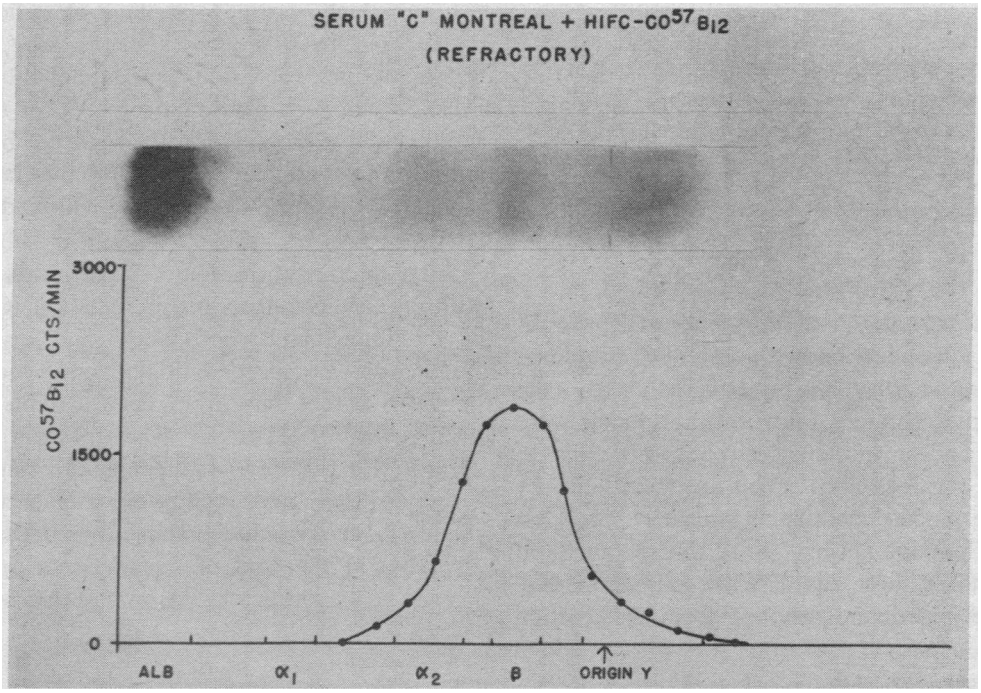

FIG. 6. PAPER ELECTROPHORESIS OF SERUM "C" (PATIENT REFRACTORY) WITH Co^{57}-B_{12}-HIFC. The electrophoretic distribution of radioactivity cannot be distinguished from normal.

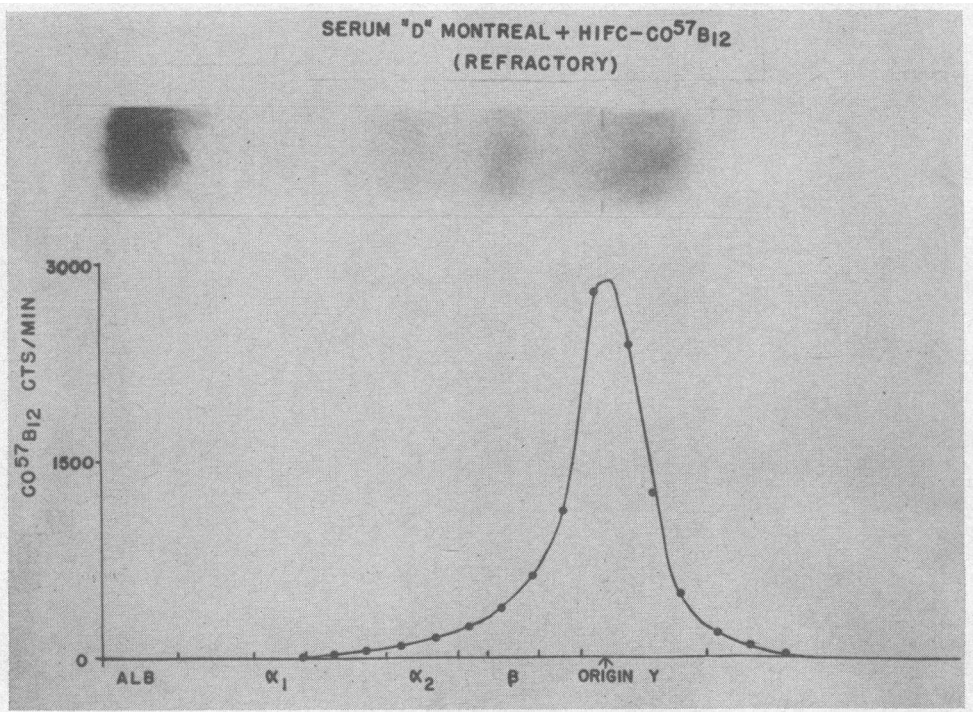

FIG. 7. PAPER ELECTROPHORESIS OF SERUM "D" (PATIENT REFRACTORY) WITH Co^{57}-B_{12}-HIFC. The radioactive material has remained at the origin.

pension mixed thoroughly. A standard volume of the test serum (10 ml of rabbit serum or 40 ml of human serum) was added and the mixture incubated at 37° C for one hour. Precipitate, when formed, was resuspended and the entire mixture fed to the patient. In two studies, 10 ml of rabbit antibovine serum albumin serum and 1 ml of a 400 mg per 100 ml suspension of bovine serum albumin (BSA)[5] (a quantity calculated to result in maximal precipitation) were added to the Co^{60}-B_{12}-HIFC. After incubation at 37° C for one hour, the resultant mixture was fed to the patient.

RESULTS

Serological studies. Serum obtained from rabbits after immunization with P-HIFC contained precipitating antibodies to P-HIFC. By the Ouchterlony technique, four precipitin bands were visualized and on immunoelectrophoresis multiple lines were formed. The rabbit antiserum did not precipitate purified porcine A substance,[6] but did react with porcine plasma [7] to form two precipitin lines. One of these fused, with spur formation, with a precipitin band resulting from the reaction between P-HIFC and rabbit anti-HIFC (i.e., reaction of partial identity).

Figure 1 shows the percentage of radioactivity precipitated from a fixed quantity of Co^{57}-B_{12}-HIFC complex by various amounts of rabbit anti-HIFC serum. A maximum of 78% of the Co^{57} activity was precipitable in the presence of excess antiserum. Possible reasons for the failure to precipitate all of the Co^{57} activity will be discussed later.

After the third injection of HIFC, both patients with pernicious anemia manifested skin sensitivity of the immediate type (wheal and erythema). Further injections of antigen were necessary, however, before sera from both patients reacted visibly with P-HIFC in agar diffusion systems (Figure 2). Repeated attempts utilizing Ouchterlony, Preer, and immunoelectrophoretic techniques to demonstrate precipitating antibodies to P-HIFC in the sera of five patients previously proved to be refractory to oral HIFC were unsuccessful.

Electrophoretic studies. A Co^{57}-B_{12}-HIFC complex, when added to normal serum or to sera obtained from the pernicious anemia subjects be-

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[6] Knickerbocker Laboratories, New York, N. Y.
[7] Pentex, Inc., Kankakee, Ill.

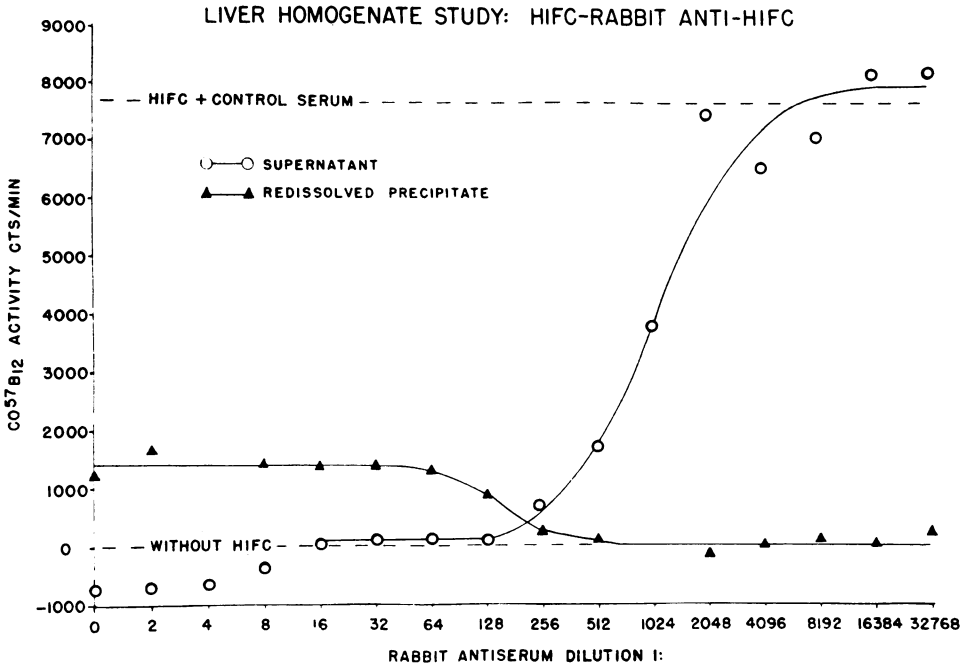

FIG. 8. LIVER HOMOGENATE Co^{57}-B_{12} UPTAKE. To rabbit anti-HIFC serum, serially diluted, was added a standard quantity of HIFC. The products of this reaction (supernatant fluids and redissolved precipitates) were added to liver homogenate, immediately followed by Co^{57}-B_{12}, in the single incubation technique (17).

fore immunization, migrated anodally with the beta globulins on paper electrophoresis (Figures 3–5). In postimmunization sera (containing precipitins) the complex remained fixed at the origin as a homogenous peak (Figures 4 and 5). The electrophoretic mobility of Co^{57}-B_{12}-HIFC in the sera of patients previously proved refractory to oral HIFC was found, in all but one case, to be normal (Table I, Figure 6). In the exceptional serum "D," the Co^{57} activity failed to migrate from the zone of application (Figure 7). It should be noted (Table I) that serum "H," obtained 4 months after serum "D" from the same refractory patient (F. C.) did not impede the migration of the Co^{57}-B_{12}-HIFC complex.

Liver homogenate studies. When the products of the reaction between HIFC and rabbit anti-HIFC serum (i.e., the supernatant fluids and dissolved precipitates) were tested for their capacity to enhance the Co^{57}-B_{12} uptake of rat liver homogenates, curves such as the one depicted in Figure 8 resulted. Supernatant fluids derived from tubes containing antiserum in great excess diminished Co^{57}-B_{12} uptake by the homogenate below that occurring in the absence of HIFC. With progressive dilution of antiserum to 1 : 256, diminishing quantities of precipitate formed; nevertheless, no reciprocal increment in intrinsic factor activity in the supernatant fluid could be demonstrated. At dilutions of antiserum greater than 1 : 256, precipitation was not observed. Supernatant fluids from these tubes did potentiate B_{12} uptake by homogenates. Precipitates formed by the reaction between HIFC and rabbit anti-HIFC, after being dissolved and buffered, enhanced B_{12} uptake by homogenates only 15%, compared to control serum (Figure 8).

TABLE II

Effect of order of addition of reactants on the uptake of Co^{60}-B_{12} by liver homogenate

Substance	Sequence of addition				
HIFC	2	1	2	1	1
Rabbit anti-HIFC		2	1	1	
Normal rabbit serum					1
Saline	1				
Co^{60}-B_{12}	3	3	3	2	2
Counts per minute	3,004	806	2,213	51	4,530

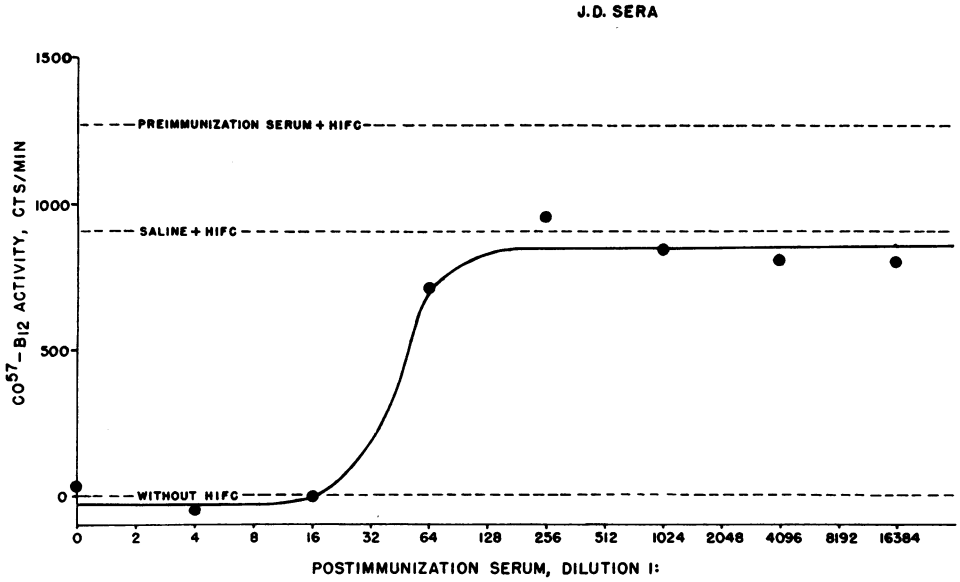

FIG. 9. LIVER HOMOGENATE Co^{57}-B_{12} UPTAKE. Postimmunization serum from J. D. was serially diluted and each dilution incubated with a standard quantity of HIFC. The ability of the latter thereafter to enhance the uptake of Co^{57}-B_{12} by liver homogenates is depicted.

The results of sequential incubation studies performed are depicted in Table II. The data demonstrated that *1*) preincubation of homogenate in rabbit antiserum did not inhibit subsequent vitamin B_{12} binding, *2*) preincubation of HIFC with anti-HIFC totally suppressed the ability of former to facilitate the uptake of vitamin B_{12} by the homogenate, and *3*) the addition of rabbit antiserum to a homogenate previously incubated in HIFC diminished significantly, but not completely, its subsequent uptake of vitamin B_{12}.

The effects of pre- and postimmunization sera

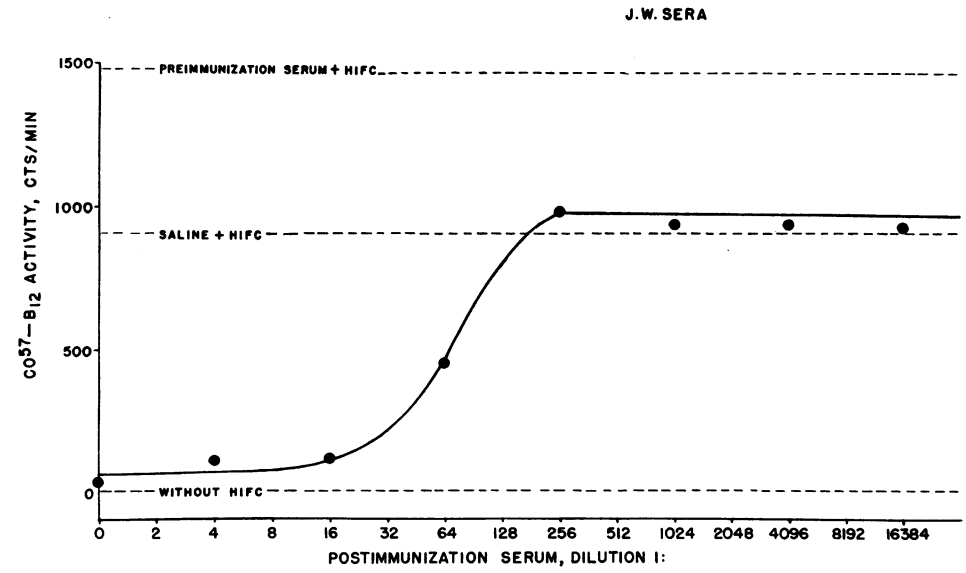

FIG. 10. LIVER HOMOGENATE Co^{57}-B_{12} UPTAKE. Postimmunization serum from J. W. was serially diluted and each dilution incubated with a standard quantity of HIFC. The ability of the latter thereafter to enhance the uptake of Co^{57}-B_{12} by liver homogenate is depicted.

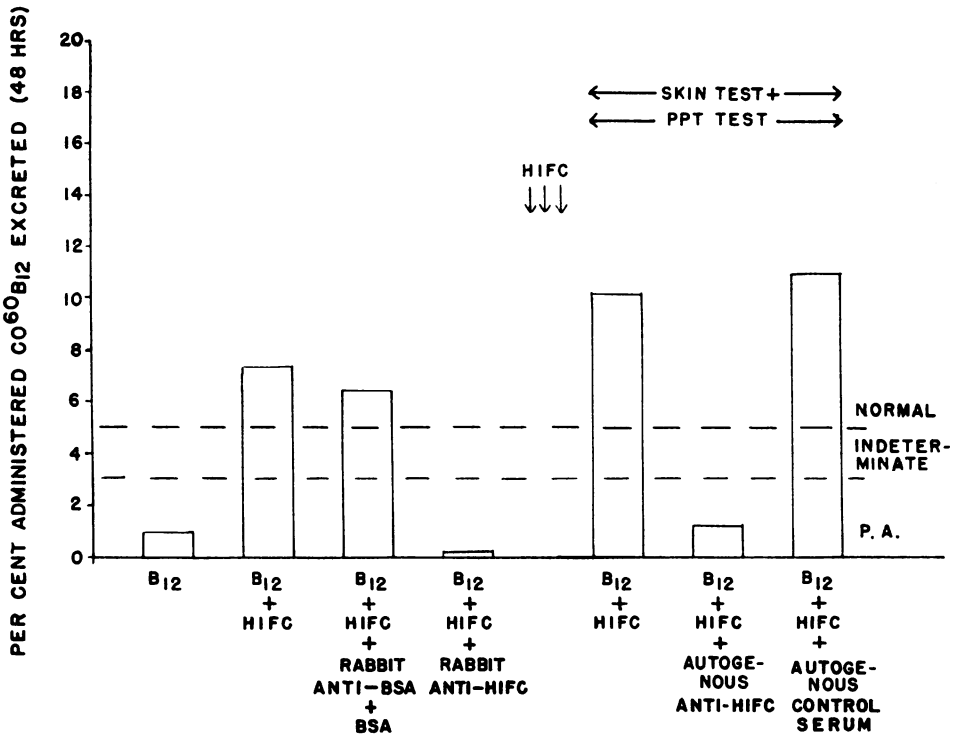

FIG. 11. URINARY EXCRETION OF Co^{60}-B_{12} IN SCHILLING TESTS ON PATIENT J. D. Effects of adding various sera to Co^{60}-B_{12}-HIFC prior to oral administration. In addition, the temporal relationships of P-HIFC injections (HIFC) to subsequent development of skin sensitivity, precipitating antibodies, and Schilling tests are shown.

from the two patients with pernicious anemia on the vitamin B_{12} uptake of liver homogenates are depicted in Figures 9 and 10. Preimmunization sera enhanced somewhat the B_{12} accumulation by the homogenate in the presence of HIFC. Postimmunization sera produced some turbidity but little precipitate when incubated with HIFC. Nevertheless, they severely curtailed vitamin B_{12} uptake by the liver homogenate. It is noteworthy that, on a volume for volume basis, postimmunization human sera were significantly less inhibitory than rabbit antisera. This presumably reflects a lesser degree of immunization in the human subject. When undiluted serum from five patients previously proved refractory to oral HIFC were tested in the liver homogenate system, only one, serum "D," proved inhibitory (Table I). Serum "H" obtained from the same patient (F. C.) 4 months after serum "D" did not suppress the vitamin B_{12} uptake by the homogenate.

Absorption studies. The results of serial vitamin B_{12} absorption studies performed on the two patients with pernicious anemia (J. W. and J. D.) are shown in Figures 11 and 12. The findings were as follows: *1*) deficient vitamin B_{12} absorption in the absence of an intrinsic factor source; *2*) significant augmentation of B_{12} absorption by HIFC before immunization; *3*) nullification of intrinsic factor activity by rabbit anti-HIFC, but not by rabbit anti-BSA or by the reaction products of BSA and rabbit anti-BSA; *4*) failure of parenteral immunization with P-HIFC to produce refractoriness to orally administered HIFC;[8] and *5*) complete suppression in patient J. D. of vitamin B_{12} absorption when autogenous, postimmunization serum was administered orally with hog intrinsic factor concentrate. In this patient, no inhibition of vitamin B_{12} absorption was induced by

[8] One year after being immunized, patients J. D. and J. W. were again evaluated by means of Schilling tests. At this time, J. D. excreted 11.6% and J. W. 15.4% of the orally administered dose of Co^{60}-B_{12} when it was fed with HIFC, indicating refractoriness had still not developed.

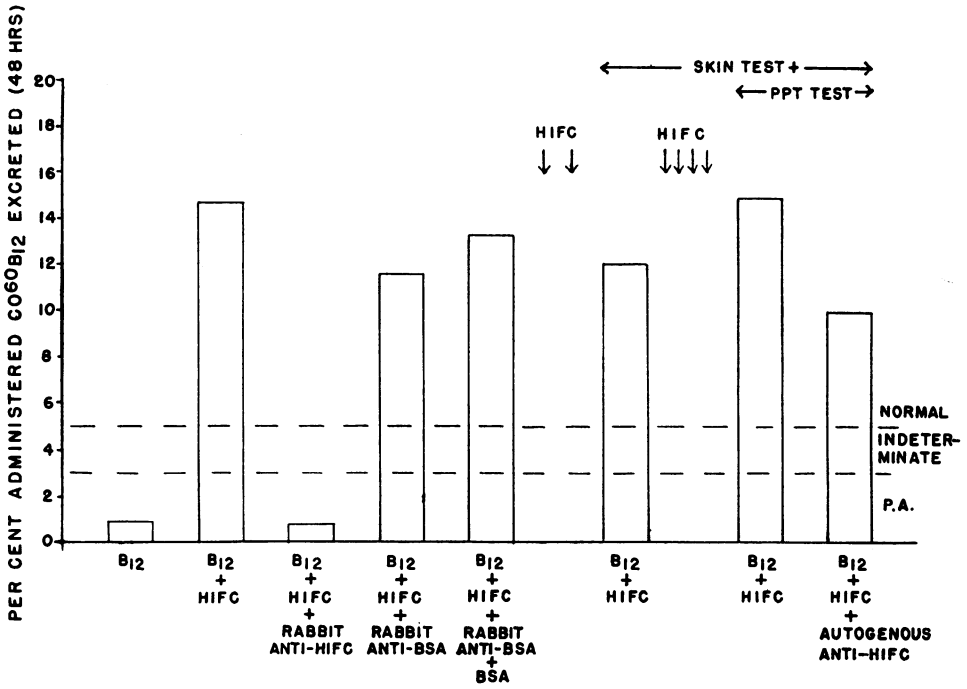

FIG. 12. URINARY EXCRETION OF Co^{60}-B_{12} IN SCHILLING TESTS ON PATIENT J. W. Effects of adding various sera to Co^{60}-B_{12}-HIFC prior to oral administration. In addition, the temporal relationships of P-HIFC injections (HIFC) to subsequent development of skin sensitivity, precipitating antibodies, and Schilling tests are shown.

orally administered, autogenous preimmunization serum. Postimmunization serum from patient J. W. did not clearly inhibit vitamin B_{12} absorption when used in identical fashion to the serum of patient J. D.

DISCUSSION

The evidence that acquired resistance, or refractoriness, to orally administered hog intrinsic factor (HIFC) is the sequel to an immunologic phenomenon may be summarized as follows: *1*) It may be acquired following the ingestion of HIFC preparations (20). *2*) It may be overcome, temporarily at least, by the administration of exceedingly large quantities of HIFC—mass action effect (21–23). *3*) It is species specific—patients refractory to hog intrinsic factor will absorb vitamin B_{12} when the latter is ingested with a nonporcine source of intrinsic factor such as human gastric juice (6, 8, 10, 23). *4*) Sera of refractory patients may inhibit vitamin B_{12} absorption when fed with HIFC and vitamin B_{12} to nonrefractory patients with pernicious anemia (13, 24). *5*) The inhibitory principle in serum has been localized to the globulin fraction (25). *6*) Sera of refractory patients may agglutinate red cells that have been coated with HIFC (13, 25). *7*) Patients refractory to oral HIFC therapy will occasionally, on discontinuation of such therapy, regain partial ability to absorb vitamin B_{12} when it is administered with oral HIFC. When large quantities of HIFC are fed to such individuals, refractoriness reappears very quickly. This has been termed an "anamnestic response" (26).

On the other hand, certain other observations suggest that the problem cannot be resolved so simply. Thus, it has been shown that serum from certain patients with pernicious anemia who have never received hog intrinsic factor preparations, or who, despite such therapy, are not refractory, may suppress vitamin B_{12} absorption when allowed to interact with hog, human, or rat intrinsic factors (25–29). Moreover, although many laboratories have searched for antibodies to hog intrinsic factor in the sera of refractory patients, none has clearly demonstrated precipitating

antibodies, and only one (13) has described a significant correlation between the presence of hemagglutinating antibodies and the refractory state.

Several years ago, Taylor and Morton (30) clearly demonstrated that rabbits immunized with porcine or human intrinsic factor preparations would produce antisera that would, when administered with the appropriate intrinsic factor source, inhibit the absorption of vitamin B_{12} in patients with pernicious anemia. The present studies were undertaken in the hope that systemic immunization with HIFC of nonrefractory patients with pernicious anemia would provoke unequivocal antibody production while, concomitantly, the response of these patients to orally administered HIFC could be ascertained. By means of such observations, it was hoped that certain conclusions could be drawn regarding the relationship of the refractory state to the patient's immunological status. In addition, the serum antibodies resulting from systemic immunization to HIFC could be compared with those found in patients refractory to HIFC.

The studies in rabbits herein reported confirm and extend the findings of Taylor. Rabbits immunized with P-HIFC responded with the production of antibodies capable of precipitating approximately 80% of the Co^{57}-B_{12} bound to a relatively pure preparation of porcine intrinsic factor. Despite the presence of excess antiserum, 20% of the radio-B_{12} activity could not be precipitated. Further studies demonstrated that the nonprecipitable vitamin B_{12} could not be bound by liver homogenates, nor could it be absorbed when fed to patients with pernicious anemia. It was recognized, therefore, that this nonprecipitable vitamin B_{12} was not bound to active intrinsic factor, and probably existed in one of the following forms: *a*) as free unbound vitamin B_{12}, *b*) complexed with nonintrinsic factor-vitamin B_{12} binding substances, *c*) bound to nonfunctioning HIFC. Since, when dialyzed, the supernatant fluid lost no Co^{57} activity, the vitamin B_{12} could not have been free. When the supernatant fluid was subjected to paper electrophoresis as previously described, Co^{57} activity remained fixed at the origin. These findings suggest an immunologic interaction of a vitamin B_{12} complex with rabbit antibody. We feel that the most likely explanation for the incomplete precipitation of Co^{57}-B_{12} in this system is the presence, in rabbit antiserum, of a variety of antibodies, some precipitating and some nonprecipitating. Additional evidence that the rabbit antiserum contains nonprecipitating antibodies to HIFC is derived from the liver homogenate studies. Here the supernatant fluids derived from the reaction between excess rabbit antiserum and HIFC inhibited even the baseline uptake of vitamin B_{12} by the homogenate that occurs in the absence of intrinsic factor. This finding implies the formation of soluble HIFC-antibody complexes that retain their ability to bind vitamin B_{12}, but are incapable of attaching to homogenate receptor sites.

Because of the recognized stability of intrinsic factor at alkaline pH (31), attempts were made to recover intrinsic factor activity from HIFC-rabbit anti-HIFC precipitates by dissolving the latter in weak base. Nevertheless, the yield of intrinsic factor activity, as demonstrated in the liver homogenate system, was unimpressive. Although the reasons for this were not fully investigated, it is probable that most of the intrinsic factor in the solubilized precipitate remains firmly bound to antibody, and is therefore inactive.

The results obtained from the sequential incubation studies may be interpreted as suggesting that the mechanism of action of rabbit antiserum in the liver homogenate system is to bind HIFC in a soluble or precipitated complex and, thereby, to prevent the intrinsic factor from attaching to homogenate receptor sites. The partial suppression by rabbit antiserum of vitamin B_{12} uptake by homogenate previously incubated in HIFC suggests that antibodies can remove HIFC from receptor sites (i.e., that the bond between intrinsic factor and receptor is not so strong as that between intrinsic factor and antibody), or that HIFC, when complexed with antibody, cannot subsequently bind vitamin B_{12} efficiently.

The inhibition by rabbit antiserum of HIFC-facilitated vitamin B_{12} absorption in man is profound. That this is not a function of nonspecific binding of the B_{12}-HIFC complex to antigen-antibody precipitates was demonstrated by the failure of BSA-rabbit anti-BSA precipitates to inhibit vitamin B_{12} absorption.

Further evidence that this inhibition is specifically mediated by the reaction between intrinsic factor and an antiintrinsic factor antibody is the

observation that when 10 ml of rabbit anti-HIFC was incubated with 30 ml normal human gastric juice, it reduced significantly the ability of the latter to facilitate vitamin B_{12} absorption in a patient with pernicious anemia.[9] This patient excreted in her urine 22.8 % of the orally administered 2 μg Co^{60}-B_{12} when given with 30 ml normal human gastric juice, but only 5.45 % of this dose when rabbit anti-HIFC was preincubated with the human gastric juice. The cross reactivity of human intrinsic factor with an antibody to purified porcine intrinsic factor strongly suggests that the physicochemical configuration of that portion of the intrinsic factor molecule which facilitates vitamin B_{12} absorption is similar in the two species.

Despite the development (with immunization) of skin sensitivity and of precipitating antibodies demonstrable by agar diffusion techniques, two patients with pernicious anemia continued to absorb vitamin B_{12} normally whenever oral HIFC was administered. Nevertheless, postimmunization sera from these patients inhibited hog intrinsic factor activity in liver homogenates, prevented the normal migration of Co^{57}-B_{12}-HIFC complexes on paper electrophoresis, and in one case, when administered orally with HIFC to the serum donor, completely suppressed vitamin B_{12} absorption.

Possible reasons for the inability of postimmunization serum from the other patient (J. W.) to clearly suppress vitamin B_{12} absorption could not be studied adequately because of the limited quantity of serum available.

In most respects, the postimmunization human sera closely resembled the immune antisera produced by rabbits. In contrast, sera from five patients previously proved refractory to oral HIFC in no case contained demonstrable precipitating antibodies to HIFC and, with one exception, did not alter the migration of a Co^{57}-B_{12}-HIFC complex on paper electrophoresis, or inhibit HIFC activity in the liver homogenate system. It is significant that the inhibitory serum "D" was obtained at a time when the patient was receiving oral HIFC. Subsequent sera from this patient were inactive in electrophoretic and homogenate systems. These findings suggest *a*) that perhaps an occasional patient receiving oral HIFC therapy may develop serum antibodies similar to those characteristically induced by systemic immunization, *b*) that these serum antibodies persist only transiently if HIFC is discontinued and that the absence of such antibodies in the sera of the other refractory patients may simply reflect disappearance of antibody subsequent to cessation of oral HIFC therapy, and *c*) that these antibodies are labile and disappear with prolonged storage of serum. At present, none of these possibilities can be dismissed. It should be noted, however, that serum "C" from patient L. W. had a much higher hemagglutination titer than serum "D" from patient F.C. Both patients were receiving oral HIFC when sera "C" and "D" were drawn. Furthermore, these two sera were of approximately the same age when tested. Nevertheless, only serum "D" modified the electrophoretic mobility of HIFC and the ability of the latter to facilitate the uptake of vitamin B_{12} by liver homogenates.

Recent observations (13, 26–29) demonstrate that sera from a fraction of all patients with pernicious anemia, refractory as well as nonrefractory, contain antibody-like substances capable of binding intrinsic factor and of interfering with its ability to facilitate B_{12} absorption. The accumulated evidence to date does not clearly establish that sera from refractory patients are unique either in the quality or quantity of antibodies contained. The studies herein reported demonstrate *1*) that systemic immunization with hog intrinsic factor resulting in the formation of serum antibodies detectable by a variety of techniques does not, of itself, produce refractoriness, *2*) that antibodies to intrinsic factor must be present in the lumen of the gut to prevent B_{12} absorption (a judgment previously voiced by others (6, 29), and it is probable that they do so by preventing the attachment of intrinsic factor to receptor sites (a possibility also previously expressed) (29, 32), and *3*) that the serum of the refractory patient does not act in various *in vitro* systems like that of patients who have undergone systemic immunization with hog intrinsic factor.

These studies, however, do not rule out an immunologic basis for the refractory state. The possibility exists that preferential hyperimmunization of the gastrointestinal tract may result in the

[9] This urinary excretion study was carried out with the aid of Dr. Louis W. Sullivan.

presence of significant quantities of antibody locally within the lumen of the gut with little or no "spill-over" into the systemic circulation. It has been long recognized (33) that the concentration of antibody in the gastrointestinal tract may bear no fixed relationship to its concentration in the serum. An alternative possibility is that refractoriness results from cellular immunity of the tuberculin type unassociated with demonstrable serum antibodies. A third, nonimmunologic mechanism may be conceived: repeated oral administration of heterologous intrinsic factor may, in some manner, induce a change in the mucosal receptors that interferes with the subsequent attachment of such intrinsic factor to receptor sites and, thereby, with its capacity to potentiate vitamin B_{12} absorption. This possibility is quite unlikely, as it would require perpetuation of abnormal receptor sites on rapidly regenerating mucosal cells in the absence of continued ingestion of heterologous intrinsic factor.

SUMMARY

Two patients with pernicious anemia were injected repeatedly with purified hog intrinsic factor concentrate (P-HIFC) until both developed unequivocal evidence of sensitization manifested by skin reactivity of the immediate type and precipitating serum antibodies. Nevertheless, no refractoriness to orally administered hog intrinsic factor was induced.

Postimmunization sera from both patients closely resembled rabbit anti-HIFC serum in their ability to *1*) precipitate P-HIFC in agar diffusion systems, *2*) prevent the electrophoretic migration of a Co^{57}-B_{12}-HIFC complex, *3*) suppress the HIFC-enhanced uptake of vitamin B_{12} by liver homogenates, and *4*) inhibit vitamin B_{12} absorption, when administered orally with HIFC, in one of two subjects with pernicious anemia.

In contrast, sera from five patients previously proved refractory to oral HIFC contained no precipitating antibodies to HIFC and, with one exception, altered neither the electrophoretic migration of a Co^{57}-HIFC complex nor the vitamin B_{12} uptake of liver homogenates.

Since there is no correlation, in the individual patient, between the degree of systemic sensitization to hog intrinsic factor and the ability of the latter to enhance vitamin B_{12} absorption, it is concluded that the refractory state must be determined by local factors within the gastrointestinal tract.

ACKNOWLEDGMENTS

We wish to express our gratitude to Drs. L. Lowenstein and B. Cooper, who sent us many of the sera studied and who generously provided information indispensable for meaningful interpretation of the data herein reported. We are indebted for technical assistance to Mrs. Laurie Dancy and Mrs. Rebecca Fisher Dunn, Misses Brenda Conti and Cornelia Proctor, and Mrs. Nancy Cunneen Boardman.

REFERENCES

1. Castle, W. B. Development of knowledge concerning the gastric intrinsic factor and its relation to pernicious anemia. New Engl. J. Med. 1953, **249**, 603.
2. Sturgis, C. C., and R. Isaacs. Desiccated stomach in treatment of pernicious anemia. J. Amer. med. Ass. 1929, **93**, 747.
3. Sharp, E. A. Antianemic factor in desiccated stomach. J. Amer. med. Ass. 1929, **93**, 749.
4. Mollin, D. L., and G. I. M. Ross. Vitamin B_{12} deficiency in the megaloblastic anaemias. Proc. roy. Soc. Med. 1954, **47**, 428.
5. Blackburn, E. K., H. Cohen, and G. M. Wilson. Oral treatment of pernicious anaemia with combined vitamin B_{12} and intrinsic factor preparation. Brit. med. J. 1955, **2**, 461.
6. Schwartz, M., P. Lous, and E. Meulengracht. Reduced effect of heterologous intrinsic factor after prolonged oral treatment in pernicious anaemia. Lancet 1957, **1**, 751.
7. Lowenstein, L., L. Brunton, L. Shapiro, N. DeLeeuw, and M. Dufresne. Maintenance therapy of pernicious anemia with oral administration of intrinsic factor and vitamin B_{12}. Canad. med. Ass. J. 1957, **77**, 923.
8. Berlin, R., H. Berlin, G. Brante, and S-G. Sjöberg. Failures in long-term oral treatment of pernicious anemia with B_{12}-intrinsic factor preparations. Acta med. scand. 1958, **161**, 143.
9. Killander, A. Oral treatment of pernicious anemia with vitamin B_{12} and purified intrinsic factor. I. The value of serial estimation of the vitamin B_{12} level of serum. Acta med. scand. 1958, **160**, 339.
10. Killander, A. Oral treatment of pernicious anemia with vitamin B_{12} and purified intrinsic factor. II. Studies on the reduced effect of prolonged treatment. Acta Soc. Med. upsalien. 1958, **63**, 1.
11. Bethell, F. H., W. B. Castle, C. L. Conley, and I. M. London. Present status of treatment of pernicious anemia. J. Amer. med. Ass. 1959, **171**, 2092.

12. Kaplan, M. E., R. Zalusky, J. Remington, and V. Herbert. Immunological studies with intrinsic factor in man. J. clin. Invest. 1962, **41**, 1370.
13. Lowenstein, L., B. A. Cooper, L. Brunton, and S. Gartha. An immunologic basis for acquired resistance to oral administration of hog intrinsic factor and vitamin B_{12} in pernicious anemia. J. clin. Invest. 1961, **40**, 1656.
14. Ouchterlony, Ö. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta path. microbiol. scand. 1953, **32**, 231.
15. Preer, J. R., Jr. A quantitative study of a technique of double diffusion in agar. J. Immunol. 1956, **77**, 52.
16. Scheidegger, J. J. Une microméthode de l'immuno-électrophorèse. Int. Arch. Allergy 1955, **7**, 103.
17. Herbert, V., Z. Castro, and L. R. Wasserman. Stoichiometric relation between liver-receptor, intrinsic factor and vitamin B_{12}. Proc. Soc. exp. Biol. (N. Y.) 1960, **104**, 160.
18. Ellenbogen, L., and W. L. Williams. Quantitative assay of intrinsic factor by urinary excretion of radioactive vitamin B_{12}. Blood 1958, **13**, 582.
19. Schilling, R. F. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. J. Lab. clin. Med. 1953, **42**, 860.
20. Schwartz, M., and P. Lous. Acquired resistance to intrinsic factor *in* Proc. 7th Cong. Europ. Soc. Haemat., London, 1959. 1960, part II, pp. 2–8.
21. Stokes, J. B., and W. R. Pitney. Pernicious anaemia treated orally with "Bifacton." Refractoriness to potent animal intrinsic factor. Brit. med. J. 1958, 1, 322.
22. Berlin, R., H. Berlin, G. Brante, and S-G. Sjöberg. Refractoriness to intrinsic factor-B_{12} preparations abolished by massive doses of intrinsic factor. Acta. med. scand. 1958, **162**, 317.
23. Schwartz, M., P. Lous, and E. Muelengracht. Absorption of vitamin B_{12} in pernicious anemia. Defective absorption induced by prolonged oral treatment. Lancet 1958, **2**, 1200.
24. Schwartz, M. Intrinsic-factor-inhibiting substance in serum of orally treated patients with pernicious anaemia. Lancet 1958, **2**, 61.
25. Schwartz, M. Intrinsic factor antibody in serum from patients with pernicious anaemia. Lancet 1960, **2**, 1263.
26. Schwartz, M. Acquired resistance to intrinsic factor *in* Vitamin B_{12} and Intrinsic Factor, 2nd European Symposium, Hamburg, 1961. Stuttgart, Ferdinand Enke, 1961, p. 613.
27. Taylor, K. B. Inhibition of intrinsic factor by pernicious anaemia serum. Lancet 1959, **2**, 106.
28. Abels, J., A. Jansz, M. G. Woldring, A. Arends, and H. O. Nieweg. Experiments on the nature of the intrinsic factor inhibiting substance in serum of patients with pernicious anaemia *in* Vitamin B_{12} and Intrinsic Factor, 2nd European Symposium, Hamburg, 1961. Stuttgart, Ferdinand Enke, 1961, p. 623.
29. Jeffries, G. H., D. W. Haskins, and M. H. Sleisenger. Antibody to intrinsic factor in serum from patients with pernicious anemia. J. clin. Invest. 1962, **41**, 1106.
30. Taylor, K. B., and J. A. Morton. An antibody to Castle's intrinsic factor. J. Path. Bact. 1959, **77**, 117.
31. Gräsbeck, R. Studies on the vitamin B_{12}-binding principle and other biocolloids of human gastric juice. Acta. med. scand. 1956, suppl. 314.
32. Herbert, V. D. The Megaloblastic Anemias. New York, Grune & Stratton, 1959, p. 107.
33. Koshland, M. E., and Burrows, W. Quantitative studies of the relationship between fecal and serum antibody. J. Immunol. 1950, **65**, 93.