Radioiodination of Human Intrinsic Factor

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ABSTRACT Human intrinsic factor (*IF*) saturated with ^{60}Co-labeled cyanocobalamin ($^{60}CoB_{12}$) was purified and then iodinated with ^{125}I to yield ^{125}I-labeled IF-$^{60}CoB_{12}$ preparations of high specific activity. Sephadex G200 and DEAE-cellulose chromatography of the iodinated IF-$^{60}CoB_{12}$ complex showed coincidence of the major ^{125}I and the ^{60}Co radioactivity peaks. During starch-gel electrophoresis ^{60}Co radioactivity from noniodinated and iodinated complexes migrated to the same extent while ^{125}I radioactivity from the iodinated complex migrated slightly further anodally than did the ^{60}Co radioactivity. After the iodinated complex was mixed with antibody to the IF-B_{12} complex (antibody II) the ^{125}I and ^{60}Co radioactivity were: (*a*) precipitated in similar amounts by antiglobulin serum, (*b*) eluted coincidentally in the 19S region on Sephadex G200, and (*c*) excluded to the same extent from starch gel during electrophoresis. After equilibrium exchange of IF "blocking" antibody (antibody I) for ^{60}Co-vitamin B_{12} on ^{125}I-labeled IF, ^{125}I radioactivity from the IF-antibody I complex: (*a*) was precipitated by antiglobulin serum, (*b*) was eluated in the 19S region on Sephadex G200 gel filtration, and (*c*) migrated slowly towards the anode on starch-gel electrophoresis. Urinary excretion of ^{60}Co radioactivity in pernicious anemia patients after oral administration of ^{60}Co-vitamin B_{12} bound to freshly prepared ^{125}I-labeled IF was similar to that obtained with noniodinated intrinsic factor.

These results show that iodination of IF-$^{60}CoB_{12}$ complex does not markedly alter the chromatographic, electrophoretic, antigenic, or absorption-promoting properties of IF.

INTRODUCTION

Ignorance of the precise role of intrinsic factor (IF) in the physiological absorption of vitamin B_{12} persists despite nearly 40 yr of investigation. Of particular importance in preventing progress in this field is the fact that presently available methods of tracing IF action depend on binding of IF to vitamin B_{12} labeled with a radioactive marker. Dependence on binding to radioactive vitamin B_{12} prevents examination of the metabolism of IF itself, and only an independently labeled IF can overcome this difficulty.

In an attempt to obtain a suitably labeled IF preparation, human IF was first purified by the method of Chosy and Schilling (3) and then iodinated with ^{125}I by the technique of Greenwood, Hunter, and Glover (4). To determine whether iodination had altered the IF-B_{12} complex, the chromatographic, electrophoretic, antigenic, and absorption-promoting properties of purified preparations of IF were compared before and after iodination. The results indicate that ^{125}I-labeled IF-B_{12} is sufficiently similar to unlabeled IF to serve as a tracer of IF.

METHODS

Purification of human intrinsic factor. Human IF possessing a high degree of purity was prepared from human gastric juice by a modification of the method of Chosy and Schilling (3). Gastric juice was obtained from patients with peptic ulcer during routine gastric secretory studies. Specimens were collected in iced containers after intramuscular injection of 1.5 mg of Histalog/kg body weight. The crude juice was filtered through glass wool, raised to pH 10 with NaOH to inactivate pepsin (18), titrated to pH 7.0 with HCl and stored in 2-liter pools at $-20°$C. To 500 ml of pooled gastric juice was added sufficient ^{60}Co-labeled cyanocobalamin ($^{60}CoB_{12}$) to saturate all the vitamin B_{12} binding sites of the gastric juice. The specific activity of the $^{60}CoB_{12}$ was 0.1 mc/mg. After incubation at room temperature, the gastric juice–B_{12} was ultrafiltered through Visking casing (Union Carbide Corp., Visking Div., Chicago, Ill.) to a volume of about 10 ml.

All procedures involved in purification, except radioactivity counting, were performed at 4°C. 4.0-ml fractions were collected from each column directly into radioactivity counting tubes.

As the first purification step, the ultrafiltered sample was applied to a 1.8 × 20 cm column of Amberlite CG-50 cation exchange resin of mesh size 200–400. The resin was prepared

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by the method of Hirs, Moore, and Stein (5), equilibrated and packed in 0.05 M sodium acetate buffer, pH 5.4, and eluted with 0.58 M sodium acetate buffer, pH 5.4. Two protein-bound radioactivity peaks were consistently found, the first comprising about 10% of the total $^{60}CoB_{12}$-binding material. Since Chosy and Schilling (3) had previously shown that only the second of these two peaks has in vivo IF activity, 4.0-ml fractions from this peak were pooled and ultrafiltered.

The second step in the purification process consisted of downward flow Sephadex G200 gel filtration using a 50 × 3.8 cm column and 0.05 M sodium phosphate buffer, pH 7.5. A single protein-bound radioactivity peak was eluted in the albumin region from the ultrafiltered pooled fractions from the Amebrlite column. 4.0-ml fractions from this peak were pooled and concentrated by ultrafiltration.

The final purification step was ion-exchange chromatography on a 1.8 × 18 cm column of DEAE-cellulose prepared by cycling through HCl and NaOH and equilibrated in 0.02 M sodium phosphate buffer, pH 7.5, at 4°C. The concentrated sample obtained from gel filtration was allowed to enter the DEAE-cellulose column which was then washed with 40 ml of the equilibrating buffer. A gradient elution was applied to the column with rising molarity and falling pH from 0.02 M sodium phosphate buffer, pH 7.5, to 0.2 M sodium dihydrogen phosphate, pH 4.3. A single protein-bound radioactivity peak was eluted, and after removal of an aliquot for nitrogen determination (6), the final pooled sample was stored at −20°C until used.

Radioiodination of human IF. The chloramine T method of Greenwood et al. (4) was used to label the purified IF-$^{60}CoB_{12}$ preparation (IF-$^{60}CoB_{12}$) with radioiodine ^{125}I. The appropriate quantity of chloramine T was selected by estimating the destructive effect of chloramine T on the $^{60}CoB_{12}$ binding capacity of the purified IF. Concurrently, as a basis for comparison between the destructive effect of chloramine T on IF-$^{60}CoB_{12}$ and on crude dialyzed human gastric juice-$^{60}CoB_{12}$ (GJ-$^{60}CoB_{12}$), equal concentrations of chloramine T solution were also added to GJ-$^{60}CoB_{12}$. Equivalence of $^{60}CoB_{12}$ binding by the two preparations was obtained by dilution in saline of the more concentrated IF-$^{60}CoB_{12}$. As a result, both preparations bound 17 ng $^{60}CoB_{12}$/ml, but the nitrogen content of the GJ-$^{60}CoB_{12}$ was more than 300 times greater than that of IF-$^{60}CoB_{12}$.

Serial dilutions containing 80.0–0.009 mg/ml of chloramine T were then added in a final volume of 0.1 ml solution to 1.0 ml either of the GJ-$^{60}CoB_{12}$ or of the IF-$^{60}CoB_{12}$ preparation. This mixture was incubated for 60 min at room temperature. To remove any free $^{60}CoB_{12}$ released by exposure to chloramine T, 1.0 ml of bovine serum albumin-coated charcoal (7) was added to the mixture. After adsorption of free $^{60}CoB_{12}$, the charcoal was separated by centrifugation, and the supernate containing the remaining protein-bound $^{60}CoB_{12}$ was decanted. Radioactivity in the supernate was compared with that of IF-$^{60}CoB_{12}$ and GJ-$^{60}CoB_{12}$ standards which had not been exposed to chloramine T. Table I shows per cent destruction of vitamin B_{12} binding for each amount of chloramine T added. Release of ^{60}Co radioactivity from the GJ-$^{60}CoB_{12}$ complex occurred only with amounts of chloramine T greater than 500 μg. In contrast, 125 μg chloramine T was sufficient to destroy 14% of the $^{60}CoB_{12}$ binding of purified IF. This difference in chloramine T sensitivity of the two preparations may have been related to the difference in their protein concentration, but this possibility was not tested. because of the large quantities of purified material required for such an investigation.

TABLE I

*Per cent Destruction of B_{12} Binding**

Chloramine T added	Purified IF-$^{60}CoB_{12}$ (0.135 μg N)	Crude dialyzed GJ-$^{60}CoB_{12}$ (44 μg N)
μg	%	%
8000	98 ±0.0	92 ±5.0
4000	97 ±1.4	85 ±1.4
1000	96 ±1.4	25 ±11.3
500	73 ±13.5	8 ±2.0
250	28 ±13.5	2 ±5.4
125	14 ±10.6	3 ±2.3
62.5	3 ±3.0	3 ±3.0
31.2	7 ±3.6	1 ±0.5
3.9	5 ±5.0	1 ±1.4
0.9	2 ±2.2	2 ±1.4

* Mean ±SD of two experiments.

Since some denaturation of the IF might occur even with amounts of chloramine T insufficient to cause significant damage to $^{60}CoB_{12}$ binding, 31.2 μg of chloramine T, one-fourth the amount that caused demonstrable destruction, was used for radioiodine labeling.

IF-$^{60}CoB_{12}$ containing 5–8 μg nitrogen and 0.5 μg $^{60}CoB_{12}$ was thawed, transferred to a shortened disposable plastic counting tube, and diluted to 2.0 ml with 0.05 M sodium phosphate buffer, pH 7.5. The tube was then sealed with a rubber cap and placed in a small lead pot in a hood. Through the rubber cap, 1.0 ml of NaI solution containing from 5 μc to 1.0 mc ^{125}I and 31.2 μg of freshly prepared chloramine T in 0.1 ml distilled water was then injected with a tuberculin syringe. 20 min later the reaction was stopped by the injection of 1.0 ml of potassium metabisulphite (96 mg/ml) and 1.0 ml potassium iodide (800 mg/ml). The lead pot containing the tube and reagents was shaken gently after each addition.

To separate the iodinated IF-$^{60}CoB_{12}$ (^{125}I-IF-$^{60}CoB_{12}$) from the iodinating reagents, the contents of the tube were removed through the rubber cap with a long needle attached to a disposable syringe. The syringe was then emptied into a 2 × 40 cm column of Sephadex G50 in bead form, packed in 0.05 M sodium phosphate buffer, pH 7.5. The sample was eluted with the same buffer at a flow rate of 40 ml/hr. The distribution of ^{125}I radioactivity allowed easy identification of radioactivity appearing immediately after the void volume. This protein-bound radioactivity was consistently well separated from free ^{125}I. The ^{125}I-IF-$^{60}CoB_{12}$ was then pooled, ultrafiltered if necessary, and stored at −20°C until use. To ensure complete removal of small amounts of free iodine, ^{125}I-IF-$^{60}CoB_{12}$ was dialyzed against running tap water for 12 hr immediately before use.

Evaluation of the effect of iodination on human intrinsic factor. Since the only difference between ^{125}I-IF-$^{60}CoB_{12}$ and IF-$^{60}CoB_{12}$ was the exposure of the former to the iodination reaction, the chromatographic, electrophoretic, antigenic, and absorption-promoting properties of the two preparations were compared to evaluate the effect of iodination of IF-$^{60}CoB_{12}$.

DEAE-cellulose chromatography was performed by the method already described. Gel filtration was carried out using a reverse flow 2.0 × 103 cm column of Sephadex G200.

Vertical electrophoresis was performed in alkaline starch borate gel by the method of Smithies (8). Electrophoresis was carried out for 18 hr at 130 v either at room temperature or at 4°C. The gel was then cut into 0.5 cm slices and assayed for radioactivity.

IF antibody serum was obtained from patients with adult type pernicious anemia (P.A.) established by the presence of histamine-fast achlorhydria and abnormal vitamin B_{12} absorption which was corrected by human IF (9). Each serum was tested for antibody I or "blocking" activity by the charcoal test of Gottlieb, Lau, Wasserman, and Herbert (7) and for antibody II or "binding" activity by the antiglobulin coprecipitation technique of Taylor, Roitt, Doniach, Couchman, and Shapland (10) as well as by the electrophoretic retention test of Jeffries, Hoskins, and Sleisenger (11). P.A. sera containing IF antibody II (12) (AbII) precipitated IF-$^{60}CoB_{12}$ with antiglobulin serum and also caused retention of the IF-$^{60}CoB_{12}$ at the origin of the starch gel. Although certain P.A. sera contained only AbI, sera with AbII activity always contained, in addition, AbI.

Reactions between ^{125}I-IF-$^{60}CoB_{12}$ and AbII were demonstrated by three techniques: (*a*) a modification of the antiglobulin coprecipitation technique of Taylor et al. described in detail by Schade, Abels, and Schilling (12), (*b*) the Sephadex G200 gel filtration method of Imrie and Schilling (13), and (*c*) the electrophoretic retention test of Jeffries et al. (11). Reactions between ^{125}I-IF-$^{60}CoB_{12}$ and AbI were demonstrated by incubating the ^{125}I-IF-$^{60}CoB_{12}$ for 24 hr at 37°C with AbI to allow the AbI to exchange (14) with the $^{60}CoB_{12}$ bound to ^{125}I-labeled IF. The same three techniques described above were then used to test for the ^{125}I-IF-AbI complex obtained by this prolonged incubation.

Rabbit anti-human globulin serum obtained from rabbits injected with ethanol-fractionated, DEAE-cellulose-separated, human gammaglobulin was kindly supplied by H. L. Deutsch and R. L. Johnson.

The urine radioactivity test described by Schilling (9) was used to compare absorption of ^{125}I-IF-$^{60}CoB_{12}$ or IF-$^{60}CoB_{12}$ containing 0.5 μg of $^{60}CoB_{12}$ in totally gastrectomized or pernicious anemia patients. When ^{125}I-IF-$^{60}CoB_{12}$ was used, subjects received Lugol's solution of iodine three drops t.i.d. for 3 days before and 7 days after the test to block thyroidal uptake of radioiodine. The dose of ^{125}I administered in these studies never exceeded 1.0 μc, and at least 50% of the ingested ^{125}I radioactivity was excreted in the urine within 24 hr. The tests were performed in the following sequence: free $^{60}CoB_{12}$, IF-$^{60}CoB_{12}$, ^{125}I-IF-$^{60}CoB_{12}$. After the test with IF-bound $^{60}CoB_{12}$, two additional flushing doses of 1000 μg nonradioactive cyanocobalamin were injected intramuscularly and urinary radioactivity was allowed to return to background before the second test with ^{125}I-IF-$^{60}CoB_{12}$ was performed.

Urine radioactivity was determined by counting 500-ml aliquots of urine in a well counter. All other radioactivity was assayed with a Autogamma detector (Packard Instrument Co., Inc., Downers Grove, Ill.) with one channel adjusted to count ^{60}Co radioactivity and another to count ^{125}I radioactivity. With this arrangement ^{60}Co counted approximately 1000 counts/nc above background and ^{125}I contributed to the ^{60}Co channel less than 0.05% of the counts detected in the ^{125}I channel. ^{125}I counted approximately 500 counts/nc above background and to this channel ^{60}Co contributed 5.3% of the counts detected in the ^{60}Co channel. To estimate ^{125}I radioactivity, counts attributable to ^{60}Co were subtracted from the total counts detected in the ^{125}I channel.

RESULTS

IF isolated from human gastric juice by ion-exchange chromatography and Dextran gel filtration bound 1.0 μg $^{60}CoB_{12}$/10–16 μg nitrogen. When compared to crude dialyzed gastric juice, this respresents nearly a 300-fold increase in purity. These relatively pure IF preparations were consistently obtained in yields of 10–20%. Iodination of the purified IF-$^{60}CoB_{12}$ preparation by the modification of the method of Greenwood et al. (4) resulted in ^{125}I-IF-$^{60}CoB_{12}$ preparations with specific activities from 0.4 μc to 50 μc ^{125}I/μg nitrogen. When fresh ^{125}I-labeled NaI was used, 50% of the ^{125}I radioactivity added to the reaction mixture consistently became protein bound. On the other hand, when ^{125}I-NaI that had decayed through two or more half-lives was used, as little as 2% of the added ^{125}I radioactivity became protein bound. The specific activity of the iodinated complex was directly related to the radioactivity of the iodinating reagents. Since virtually all of the ^{60}Co radioactivity present was eluted from the Sephadex G50 column in the protein peak, $^{60}CoB_{12}$ was not released from IF during iodination.

Column chromatography and starch-gel electrophoresis. Chromatography of ^{125}I-IF-$^{60}CoB_{12}$ on DEAE-cellulose resulted in the elution of a major ^{60}Co radioactivity peak that was coincident with the major ^{125}I radioactivity peak (Fig. 1). Immediately following and incompletely separated from the major peak, a second small peak of coincident ^{125}I and ^{60}Co radioactivity was consistently found. With gel filtration on Sephadex G200

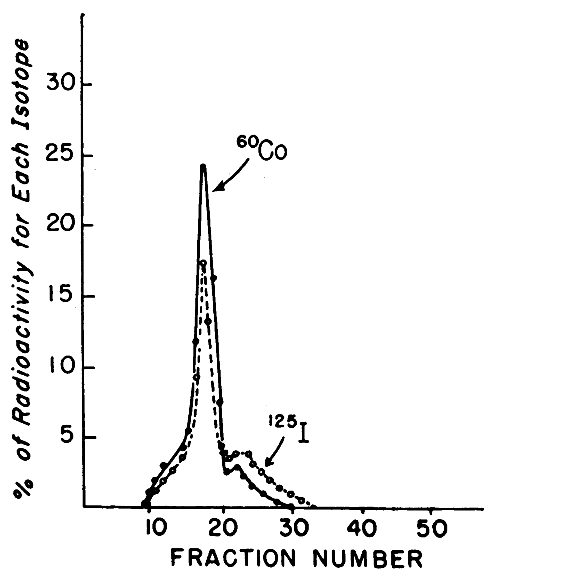

FIGURE 1 Fractionation of ^{125}I-IF-$^{60}CoB_{12}$ on DEAE-cellulose. The major ^{60}Co radioactivity coincides with the major ^{125}I radioactivity peak. A small peak of coincident ^{60}Co and ^{125}I radioactivity follows, and is incompletely separated from the major peak.

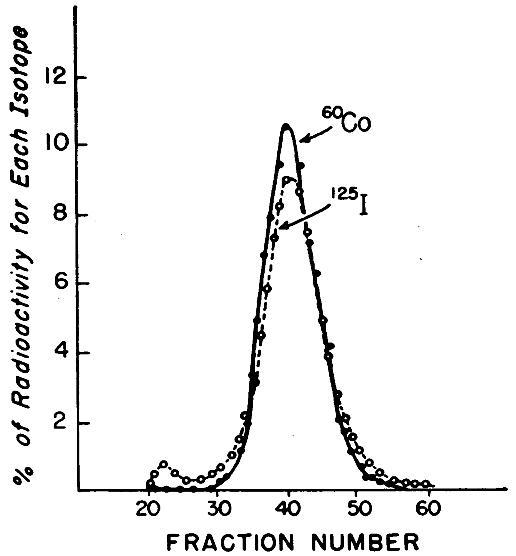

FIGURE 2 Fractionation of ^{125}I–IF-$^{60}CoB_{12}$ complex on Sephadex G200. The ^{60}Co radioactivity peak coincides with the major ^{125}I peak. A small ^{125}I radioactivity peak precedes, and is incompletely separated from, the major peak.

(Fig. 2), coincidence of a single ^{60}Co radioactivity peak with the major ^{125}I radioactivity peak was seen. With this separation a second smaller ^{125}I peak consistently preceded the major ^{125}I peak.

Simultaneous electrophoresis in starch gel of ^{125}I-IF-$^{60}CoB_{12}$ and IF-$^{60}CoB_{12}$ showed coincidence at the same anodal distance of the ^{60}Co radioactivity from both complexes (Fig. 3). Although considerable overlap occurred, the ^{125}I radioactivity peak from ^{125}I-IF-$^{60}CoB_{12}$ consistently migrated 0.5 cm further anodally than did the ^{60}Co radioactivity peak.

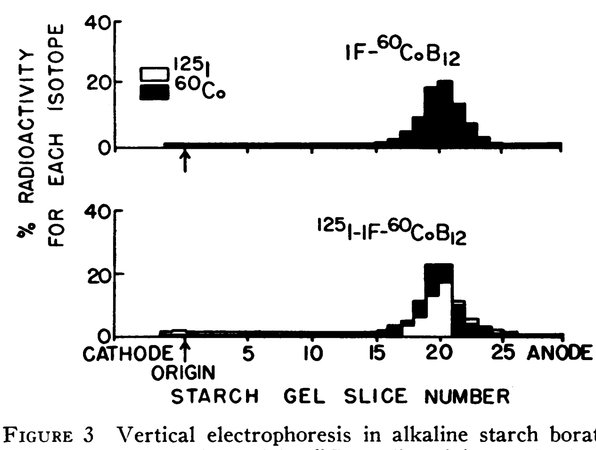

FIGURE 3 Vertical electrophoresis in alkaline starch borate gel for 18 hr at 130 v. The ^{60}Co radioactivity peaks both from noniodinated IF-$^{60}CoB_{12}$ (above) and iodinated IF-$^{60}CoB_{12}$ (below) are coincident at the same anodal distance. The ^{125}I radioactivity is seen one gel slice further towards the anode than the coincident ^{60}Co radioactivity.

Reactions with intrinsic factor antibody II. Incubation of either ^{125}I–IF-$^{60}CoB_{12}$ or IF-$^{60}CoB_{12}$ with normal human serum resulted in precipitation of only a very small proportion of the ^{60}Co radioactivity from each complex when rabbit anti-human globulin serum (antiglobulin serum) was added (Table II). The percentage of ^{125}I radioactivity precipitated, however, was consistently somewhat greater. At present it is not at all clear why in the presence of normal serum, somewhat more ^{125}I radioactivity than ^{60}Co radioactivity should be precipitated by antiglobulin serum. When AbII was incubated with either ^{125}I–IF-$^{60}CoB_{12}$ or IF-$^{60}CoB_{12}$, antiglobulin serum precipitated 20 times more ^{60}Co radioactivity from each preparation than after incubation with normal serum. Although the percentage of ^{125}I radioactivity precipitated by AbII and antiglobulin serum was only 4-fold greater than with normal serum and antiglobulin serum, it was still consistently greater than 50% of the total ^{125}I radioactivity present.

Gel filtration on Sephadex G200 of ^{125}I–IF-$^{60}CoB_{12}$ incubated with AbII consistently showed a strikingly different elution pattern from that obtained after incubation with normal human serum (Fig. 4). Whereas coincident ^{60}Co and major ^{125}I radioactivity peaks were eluted in the albumin region with normal serum (peak *B*, Fig. 4, above), after incubation with AbII, the major ^{60}Co and ^{125}I radioactivity peaks were instead eluted in the 19S region (peak *A*, Fig. 4, below). Moreover, in the albumin region a small peak of ^{125}I radioactivity was eluted alone (peak *B*, Fig. 4, below) and in the third peak only ^{60}Co radioactivity was found (peak *C*, Fig. 4, below). This third peak, which consisted of dialysable free $^{60}CoB_{12}$, was not seen after incubation of ^{125}I–IF-$^{60}CoB_{12}$ with normal serum.

TABLE II

Antigenic Activity of Iodinated Intrinsic Factor–Vitamin B_{12} Complex towards IF Antibody II Serum

	Per cent of total radioactivity of each isotope precipitated by antiglobulin serum*	
	^{125}I	^{60}Co
	%	%
Noniodinated IF-$^{60}CoB_{12}$ + normal serum	—	3.0 ±1.5
Noniodinated IF-$^{60}CoB_{12}$ + IF antibody II serum	—	58.4 ±6.8
Iodinated IF-$^{60}CoB_{12}$ + normal serum	13.3 ±1.9	2.0 ±2.0
Iodinated IF-$^{60}CoB_{12}$ + IF antibody II serum	52.8 ±1.4	54.9 ±1.9

* Mean ±SD of six experiments.

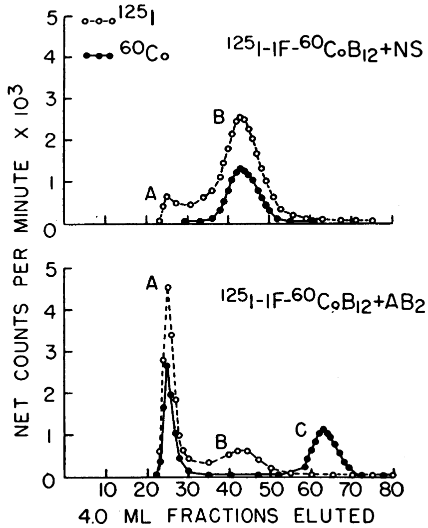

FIGURE 4 Elution pattern from Sephadex G200 of ^{125}I–IF-^{60}CoB$_{12}$ incubated with normal serum (NS, above) and IF antibody II (below). Coincidence of the major ^{125}I peak and the ^{60}Co peak in the region *B* which contains serum albumin and a small ^{125}I peak *A* in the 19S region is seen after incubation with normal serum (above). After incubation with AbII the major coincident ^{125}I and ^{60}Co peak, *A*, is eluted in the 19S region (below). A small ^{125}I peak, *B*, is eluted in the albumin region without coincident ^{60}Co radioactivity and a small ^{60}Co peak, *C*, without coincident ^{125}I is seen in the small molecular region.

Incubation of ^{125}I–IF-^{60}CoB$_{12}$ with AbII serum abolished the anodal electrophoretic migration both of ^{125}I and ^{60}Co radioactivity (Fig. 5, below) that was seen after incubation with normal serum (Fig. 5, above). Cathodal migration of ^{60}Co radioactivity similar to that observed (12, 14) during electrophoresis of free ^{60}CoB$_{12}$ was seen only after incubation of ^{125}I–IF-^{60}CoB$_{12}$ with AbII.

Reactions with intrinsic factor antibody I. Only small amounts of ^{125}I radioactivity were precipitated by antiglobulin serum immediately after mixing ^{125}I–IF-^{60}CoB$_{12}$ either with normal serum or with AbI serum (Table III). Incubation of ^{125}I–IF-^{60}CoB$_{12}$ with normal serum for 24 hr at 37°C produced no change in the ^{125}I radioactivity precipitated by antiglobulin serum. However, incubation of ^{125}I–IF-^{60}CoB$_{12}$ with AbI for 24 hr at 37°C consistently resulted in a marked increase in the ^{125}I radioactivity precipitated. This increase was similar to the precipitation observed when antiglobulin serum was added after AbII was mixed with ^{125}I-IF-^{60}CoB$_{12}$.

Gel filtration on Sepadex G200 immediately after mixing ^{125}I–IF-^{60}CoB$_{12}$ with AbI resulted in the same elution pattern as was seen after mixing with normal serum (Figs. 4, above, and 6, above). After incubation with AbII for 24 hr, ^{125}I and ^{60}Co radioactivity peaks were eluted together in the 19S region (peak *A*, Fig. 4, below). After incubation with AbI, however, the 19S region (peak *A*, Fig. 6, below) contained only ^{125}I radioactivity. The effects of AbII and AbI differed in another important respect. When ^{125}I–IF-^{60}CoB$_{12}$ was incubated with AbII and subjected to gel filtration the albumin region (peak *B*, Fig. 4, below) contained ^{125}I radioactivity only. When AbI was used, however, the albumin region (peak *B*, Fig. 6 below) contained coincident ^{60}Co and ^{125}I radioactivity. In both instances, however, the third peak (peak *C*, Figs. 4, below, and 6, below) contained only ^{60}Co radioactivity.

Starch-gel electrophoresis of ^{125}I–IF-^{60}CoB$_{12}$ immediately after the addition of AbI (Fig. 7, above) showed the same migration of the ^{60}Co and ^{125}I radioactivity towards the anode as was observed when ^{125}I–IF-^{60}CoB$_{12}$ was mixed with normal serum (Fig. 5, above). In addition a small peak of ^{125}I radioactivity was usually retained at the origin, and a small peak of ^{60}Co radioactivity consistently migrated towards the cathode. On

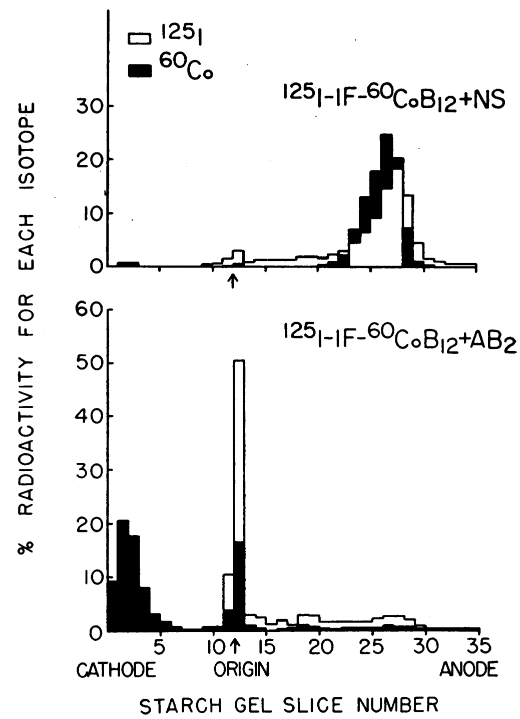

FIGURE 5 Vertical electrophoresis in alkaline starch borate-gel of ^{125}I–IF-^{60}CoB$_{12}$ incubated with normal serum (above) and with IF antibody II (below). The black bars show the per cent of total ^{60}Co radioactivity and the open bars the per cent of total ^{125}I radioactivity in each gel slice. The anodal migration of ^{125}I and ^{60}Co radioactivity peaks seen after incubation with normal serum (above) is abolished after incubation with IF antibody II (below). In addition a peak of ^{60}Co radioactivity migrating towards the cathode is seen after IF antibody II incubation.

TABLE III

Antigenic Activity of Iodinated Intrinsic Factor–Vitamin B_{12} Complex towards IF Antibody I Serum

	Per cent of total ^{125}I radioactivity precipitated by antiglobulin serum* added:	
	Immediately	After 24 hr incubation
Iodinated IF–B_{12} + normal serum	10.5 ±3.2	16.3 ±1.7
Iodinated IF–B_{12} + IF antibody I	16.1 ±4.1	53.7 ±8.7
Iodinated IF–B_{12} + IF antibody II	51.1 ±6.2	51.9 ±5.5

* Mean ±SD of four experiments.

the other hand, incubation of ^{125}I–IF-$^{60}CoB_{12}$ for 24 hr at 37°C with AbI resulted in an electrophoretic pattern (Fig. 7, below) different from that obtained after similar incubation with either normal serum or AbII (Fig. 5, above and below). Whereas incubation with AbII abolished anodal electrophoretic migration of ^{60}Co and ^{125}I radioactivity, incubation with AbI resulted in the anodal migration of two ^{125}I peaks (Fig. 7, below). The first of these contained only ^{125}I radioactivity and moved only a short distance into the gel; the second contained both ^{60}Co and ^{125}I radioactivity and migrated to the same anodal distance as was observed during electrophoresis immediately after AbI had been mixed with ^{125}I–IF-$^{60}CoB_{12}$. After the 24 hr incubation with AbI, the cathodally migrating ^{60}Co radioactivity peak consisting of free $^{60}CoB_{12}$ increased greatly.

In vivo activity. Urinary excretion of ^{60}Co radioactivity after oral administration of purified but noniodinated IF-$^{60}CoB_{12}$ to patients with pernicious anemia or total gastrectomy was consistently greater than after free $^{60}CoB_{12}$ (Table IV). Enhancement of ^{60}Co urinary radioactivity excretion was also seen after the oral administration of ^{125}I–IF-$^{60}CoB_{12}$. With freshly prepared ^{125}I–IF-$^{60}CoB_{12}$, the urinary excretion of ^{60}Co was not significantly different from that observed with noniodinated IF-$^{60}CoB_{12}$. After storage for 1–2 months, however, the urinary ^{60}Co excretion after ^{125}I–IF-$^{60}CoB_{12}$ fell to less than half of that obtained with noniodinated

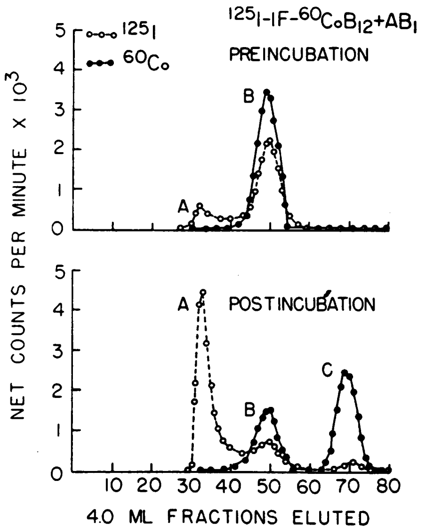

FIGURE 6 Elution pattern from Sephadex G200 of ^{125}I–IF-$^{60}CoB_{12}$ immediately after exposure to IF antibody I (above, preincubation) and after 24 hr incubation at 37° with IF antibody I (below, postincubation). As shown above, the elution pattern after immediate incubation (preincubation) with IF antibody I is similar to that seen after incubation with normal serum. 24 hr incubation with IF antibody I results in a different pattern from that observed both after incubation with normal serum and IF antibody II. As shown below, in the 19S region a single ^{125}I peak (*A*) without coincident ^{60}Co is eluted. In the albumin region a small coincident peak (*B*) of ^{125}I and ^{60}Co is seen. In peak *C*, the small molecular region, only ^{60}Co is found.

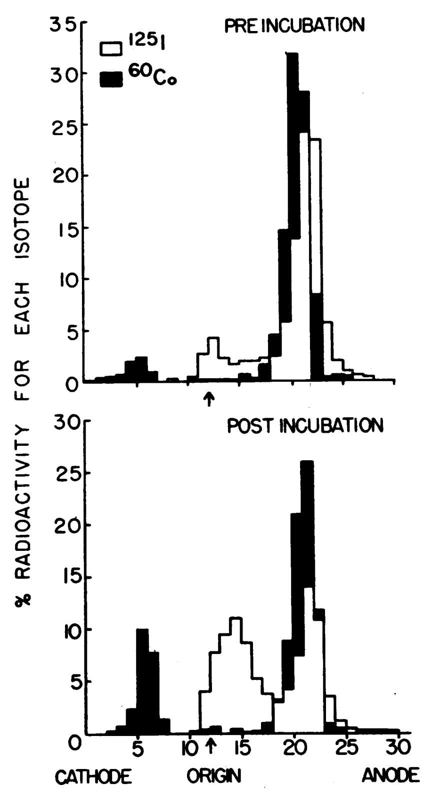

FIGURE 7 Vertical electrophoresis in alkaline starch borate-gel at 4°C of ^{125}I–IF-$^{60}CoB_{12}$ immediately after exposure to AbI (above, preincubation) and after 24 hr incubation at 37°C with AbI (below, postincubation).

TABLE IV
In vivo Activity of Iodinated Intrinsic Factor–Vitamin B_{12} Complex

Subject	H. E.	A. R.	E. C.	B. R.	E. K.
0.5 μg $Co^{60}B_{12}$	1.1	0.7	0.6	1.8	0.5
0.5 μg $Co^{60}B_{12}$–noniodinated IF	13.0	21.1	9.5	16.8*	12.6‡
0.5 μg $Co^{60}B_{12}$–iodinated IF	15.0	14.5	7.4	6.0*	6.2‡

All values expressed in terms of per cent of oral dose excreted in the urine in 24 hr.
* IF preparations stored for 1 month at -20°C.
‡ IF preparations stored for 2 months at -20°C.

IF-$^{60}CoB_{12}$ in the two trials performed. Nevertheless, excretion was still greater than that observed after free $^{60}CoB_{12}$. Nonetheless, simultaneous electrophoresis of these stored preparations of ^{125}I–IF-$^{60}CoB_{12}$ and IF-$^{60}CoB_{12}$ incubated both with normal serum and with AbII demonstrated no change in their electrophoretic or antigenic properties.

DISCUSSION

Radioiodination of IF-$^{60}CoB_{12}$ consistently resulted in preparations of ^{125}I–IF-$^{60}CoB_{12}$ which contained readily measureable quantities of ^{125}I radioactivity. Vitamin B_{12} was not released from the IF-$^{60}CoB_{12}$ complex during iodination. Although the vitamin B_{12}-binding activity of the IF was thus apparently not damaged by the procedure, it was important to determine that other characteristics of the IF molecule had not been altered since many macromolecular substances are able to bind vitamin B_{12}, but unlike IF, do not enhance vitamin B_{12} absorption by the distal small intestine (16) and presumably do not exhibit antigenicity to IF antibody. Since the affinity of vitamin B_{12} for IF (17) differs markedly from the affinity of IF-B_{12} complex for the intestinal cell surface (18), it is likely that at least two distinct active binding sites are present on the IF molecule: (*a*) a vitamin B_{12} binding site present in IF as well as other macromolecules and (*b*) an ileal mucosal receptor binding site and antibody binding sites which are unique to IF. Because of their apparently distinct nature, integrity of one does not necessarily mean integrity of the others. It was therefore essential to determine the effect of the iodination process on the unique antigenic and biologic properties of IF, despite the fact that the vitamin B_{12}-binding activity of IF was not impaired.

The coincidence of the ^{60}Co radioactivity peak with the major ^{125}I radioactivity peak both on DEAE-cellulose and Sephadex G200 indicates that iodination of IF-$^{60}CoB_{12}$ occurs without significant damage to its chromatographic or gel filtration characteristics. Furthermore, the coincidence at the same anodal distance of the ^{60}Co radioactivity peaks from iodinated and noniodinated IF-$^{60}CoB_{12}$ after electrophoresis on starch gel demonstrates the absence of damage to the electrophoretic characteristics of IF-$^{60}CoB_{12}$. During electrophoresis, however, there was a consistent though very slight difference in the migration of ^{60}Co and the ^{125}I radioactivity from iodinated IF-$^{60}CoB_{12}$. Further investigations are necessary to explain this observation.

The chromatographic and electrophoretic behavior of iodinated IF-$^{60}CoB_{12}$ provides a means of testing the effect of iodination on the antigenicity of IF-$^{60}CoB_{12}$. For this purpose one can use the antibody that binds the IF-$^{60}CoB_{12}$ complex (AbII) as well as the antibody that blocks B_{12} binding by IF (AbI). The serum used as the source of AbII activity in these studies always contained AbI activity in addition to AbII activity. The AbI serum used, however, contained only AbI activity.

Several findings indicate that antigenicity of IF-$^{60}CoB_{12}$ is not altered by the iodination procedure: (*a*) the similar precipitation of ^{60}Co radioactivity from iodinated and noniodinated IF-$^{60}CoB_{12}$ by AbII and antiglobulin serum (Table II), (*b*) the transfer of the major ^{60}Co radioactivity peak on Sephadex G200 from the albumin region with normal serum to the 19S region after AbII was mixed with iodinated IF-$^{60}CoB_{12}$ (Fig. 4, above and below), and (*c*) the abolition by AbII of the anodal electrophoretic migration of ^{60}Co radioactivity both from iodinated (Fig. 5, above and below) and noniodinated IF-$^{60}CoB_{12}$. In addition, several observations suggest that ^{125}I radioactivity is attached to the IF-vitamin B_{12} complex rather than to any contaminating substances: (*a*) the ^{125}I radioactivity precipitated by antiglobulin serum from iodinated IF-$^{60}CoB_{12}$ incubated with AbII is four times greater than that precipitated after incubation with normal serum (Table II, (*b*) the ^{125}I and ^{60}Co radioactivity eluted from Sephadex G200 coincided in the albumin region when iodinated IF-$^{60}CoB_{12}$ was incubated with normal serum and in the 19S region when incubated with AbII (Fig. 4, above and below), and (*c*) anodal electrophoretic migration both of ^{60}Co and ^{125}I radioactivity from iodinated IF-$^{60}CoB_{12}$ was abolished by incubating with AbII (Fig. 5, below).

The consistent appearance of cathodally migrating ^{60}Co radioactivity seen on starch-gel electrophoresis (Fig. 5) when iodinated IF-$^{60}CoB_{12}$ was mixed with AbII serum suggests that this serum, which has both AbI and AbII activity, has an effect in freeing $^{60}CoB_{12}$ from its binding to IF. This effect of AbII serum was also seen during gel filtration on Sephadex G200. Free $^{60}CoB_{12}$ was eluted in the small molecular region (Fig. 4). On the other hand, release of $^{60}CoB_{12}$ from IF by serum that contains AbI activity alone occurs only after prolonged incubation (Table III, Fig. 6, above and below,

Fig. 7, above and below). Since release by AbI requires prolonged incubation (14) and since $^{60}CoB_{12}$ was released shortly after IF-$^{60}CoB_{12}$ was mixed with AbII serum, it is possible that this release was an effect of the AbII activity rather than any AbI activity present in the AbII serum. On the other hand, since AbII sera tend to contain high titers of AbI, the observed rapid release might have resulted from the presence of these high titers. To distinguish unequivocally release due to AbI from that due to AbII, serum containing AbII alone would be required, but this type of IF antibody is found very rarely in pernicious anemia patients.

One might expect that when an antiglobulin coprecipitation test was performed with purified IF-$^{60}CoB_{12}$ and AbII serum, all or nearly all of the IF-bound ^{60}Co radioactivity would have been precipitated. Instead, the amount of $^{60}CoB_{12}$ bound to purified IF, precipitated (Table II), was similar to that found by other workers using radioactive vitamin B_{12} bound to crude gastric juice (10). Crude gastric juice contains, in addition to IF, non-IF vitamin B_{12}-binding macromolecules that presumably are not antigenic to AbII and thus are not precipitated by the addition of AbII and antiglobulin serum. Purified IF-$^{60}CoB_{12}$ does not contain these non-IF vitamin B_{12}-binding macromolecules (15) so that the presence of nonantigenic vitamin B_{12} binders in the supernate after antiglobulin precipitation cannot account for the unprecipitated vitamin B_{12} radioactivity. The fact that some $^{60}CoB_{12}$ is released from the IF-$^{60}CoB_{12}$ complex in the presence of AbII serum may explain why in the test performed with relatively pure IF-$^{60}CoB_{12}$ all of the $^{60}CoB_{12}$ was not precipitated by AbII and antiglobulin serum. Unfortunately, the amounts of radioactivity used in these experiments were insufficient to determine accurately whether this unprecipitated $^{60}CoB_{12}$ was in fact free vitamin which had been released from IF.

The results of gel filtration and electrophoresis studies indicated that prolonged incubation of AbI with ^{125}I–IF-$^{60}CoB_{12}$ resulted in an exchange of AbI for $^{60}CoB_{12}$ on ^{125}I-IF. As a result of this exchange free $^{60}CoB_{12}$ was released and ^{125}I–IF-AbI complex was formed. Several observations support the occurrence of this exchange and indicate that the ^{125}I radioactivity was attached to IF rather than to cyanocobalamin: (*a*) after exchange a 4-fold greater precipitation of ^{125}I radioactivity occurred when antiglobulin serum was added to the reactants than occurred before exchange (Table III); (*b*) ^{125}I radioactivity was eluted from Sephadex G200 as a peak in the albumin region coincident with ^{60}Co radioactivity (Figs. 4 above, and 6 above), and after exchange, as a single peak in the 19S region without coincident ^{60}Co radioactivity (Fig. 6, below); and (*c*) only after exchange were two anodally migrating peaks of ^{125}I radioactivity, one of which was associated with ^{60}Co radioactivity, consistently seen on starch-gel electrophoresis (Fig. 7, below).

The results of gel filtration and electrophoresis after prolonged incubation of ^{125}I–IF-$^{60}CoB_{12}$ with AbI are best explained on the basis of an equilibrium (14) between ^{125}I–IF-AbI and ^{125}I–IF-$^{60}CoB_{12}$. The ^{125}I–IF-AbI is eluted in the 19S region on Sephadex G200 (peak *B*, Fig. 4, below) and migrates a short distance into the gel on starch-gel electrophoresis (Fig. 7, below). The ^{125}I–IF-$^{60}CoB_{12}$ is eluted in the albumin region on Sephadex G200 (peak *B*, Fig. 6, below) and migrates into the starch gel to the same anodal distance as noniodinated IF-$^{60}CoB_{12}$ (Fig. 7, below). The free $^{60}CoB_{12}$ displaced by AbI appears in the third radioactivity peak from Sephadex G200 (peak *C*, Fig. 4, below) and migrates cathodally in the starch gel (Fig. 7, below). The equilibrium reaction may be written as follows:

$$\begin{array}{ccc} & & \text{AbI} \\ & & + \\ {}^{125}\text{I–IF-}{}^{60}\text{CoB}_{12} & \rightleftarrows & {}^{125}\text{I–IF} + {}^{60}\text{CoB}_{12} \\ & & \downarrow\uparrow \\ & & {}^{125}\text{I–IF-AbI} \end{array}$$

These studies provide additional evidence that AbI and AbII are distinct entities as proposed by Schade, Feick, Imrie, and Schilling (14). In contrast to AbII which precipitated similar percentages of ^{60}Co and ^{125}I radioactivity from iodinated IF-$^{60}CoB_{12}$ in the presence of antiglobulin serum, AbI precipitated no ^{60}Co radioactivity and ^{125}I radioactivity only after exchange had taken place (Table III). Furthermore, AbII shifted the coincident ^{125}I and ^{60}Co radioactivity peak obtained in the albumin region on gel filtration of iodinated IF-$^{60}CoB_{12}$ (Figs. 2, 4, and 6 above) to another coincident peak in the 19S region (Fig. 4, below), while AbI moved only the ^{125}I radioactivity peak to the 19S region (Fig. 6, above). Finally, AbII blocked anodal electrophoretic migration of ^{125}I as well as ^{60}Co radioactivity (Fig. 5, below), and AbI after exchange resulted in two anodally migrating ^{125}I radioactivity peaks, only one of which was associated with ^{60}Co radioactivity (Fig. 7, below).

The results of this study indicate that the iodinated complex is not entirely homogeneous. Iodination of nitrogen-containing impurities and/or aggregation and denaturation of some IF molecules that are still bound to $^{60}CoB_{12}$ during the iodination process may explain the small but persistent ^{125}I radioactivity peaks eluted without coincident ^{60}Co radioactivity from Sephadex G200 (Fig. 2, above, and 6, above) and with coincident (Figs. 2, 4, above, and 6, above) and with coincident ^{60}Co radioactivity from DEAE-cellulose (Fig. 1). The incomplete transfer of ^{125}I radioactivity from the albumin region to the 19S region on Sephadex G200 by AbII

(Fig. 4) also suggests that either iodinated protein is present as a contaminant or that some IF molecules have been altered and have lost antigenic activity. Nevertheless, the weight of evidence from studies with three complementary techniques using two distinct IF antibodies indicates that the predominant ^{125}I-labeled molecule in this heterogenous mixture is in fact IF.

The in vivo trials of iodinated IF-$^{60}CoB_{12}$ tested the absorption-promoting activity of IF. The three studies with freshly prepared material suggest that the iodination procedure did not alter the IF-$^{60}CoB_{12}$ complex from its readily absorbable form. Absorption was decreased, however, after prolonged storage although no alteration in antigenicity to AbII could be demonstrated by starch-gel electrophoresis. Nonetheless, absorption was still greater than after the administration of free $^{60}CoB_{12}$.

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