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**Research Article**

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Prolonged incubation [...]

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## Intrinsic Factor-mediated Attachment of Vitamin B<sub>12</sub> to Brush Borders and Microvillous Membranes of Hamster Intestine \*

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**Summary.** Hamster intrinsic factor (IF) preparations markedly enhanced the uptake of <sup>57</sup>cobalt-labeled cyanocobalamin (B<sub>12</sub>-<sup>57</sup>Co) by brush borders and microvillous membranes isolated from villous absorptive cells obtained from the distal but not the proximal half of hamster intestine. A similar effect was observed with rat and rabbit IF preparations, but IF preparations obtained from man, dog, and hog were ineffective. After fractionation of hamster IF preparations by gel filtration or ion exchange chromatography, the extent to which each fraction enhanced B<sub>12</sub>-<sup>57</sup>Co uptake by brush borders correlated closely with the vitamin B<sub>12</sub> binding capacity of the fraction. IF-mediated attachment of B<sub>12</sub>-<sup>57</sup>Co to brush borders occurred rapidly, was not diminished by removal of glucose or oxygen from the incubation medium, and was not significantly altered when incubation temperatures were reduced from 37° C to 7° C. Marked reduction in uptake occurred, however, in the absence of divalent cations.

IF enhanced B<sub>12</sub>-<sup>57</sup>Co uptake by brush borders isolated from the proximal half of the intestine when these proximal brush borders were preincubated with supernatant fluid obtained after centrifugation of homogenates of distal intestinal mucosa at 28,500 g. The factor in this supernate responsible for the effect on proximal brush borders was shown to be particulate in nature upon centrifugation at speeds of 54,500 g or greater. The resultant pellet contained ribosomes and membranous fragments.

Prolonged incubation of brush borders with crude saline extracts of hamster gastric mucosa resulted in decreased uptake of B<sub>12</sub>-<sup>57</sup>Co and marked lysis of brush borders with concomitant release of tissue nitrogen. Neither lysis of brush borders nor decreased uptake of B<sub>12</sub>-<sup>57</sup>Co with prolonged incubation was observed when hamster IF was partially purified. Furthermore, uptake of B<sub>12</sub>-<sup>57</sup>Co by brush borders increased with increasing purity of the IF preparation used.

These results demonstrate IF-mediated attachment of B<sub>12</sub>-<sup>57</sup>Co to brush borders and microvillous membranes of hamster intestinal cells and provide further support for the presence of a specific receptor for IF-bound vitamin

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**B<sub>12</sub> at the microvillous surface of the intestinal cell. IF-mediated attachment to the intestinal cell surface appears to be facilitated by divalent cations and to result from adsorption rather than an energy-requiring enzymatic reaction. Crude sources of hamster IF contain a factor which causes lysis of brush borders *in vitro* and which may explain in part the inhibitory effects of IF excess previously observed *in vitro*.**

### Introduction

Intrinsic factor (IF), secreted by the stomach, is required for efficient absorption of physiological quantities of vitamin B<sub>12</sub> from the distal intestine (2). The mechanism by which IF promotes absorption of the vitamin is unknown, but it is generally accepted that vitamin B<sub>12</sub> must be bound by IF to form a macromolecular complex if efficient absorption is to occur (3). IF enhances uptake of vitamin B<sub>12</sub> *in vitro* by everted sacs (4, 5), rings (6), and homogenates (7) of intestine obtained from a variety of mammalian species including man (8). Such *in vitro* studies, however, do not localize the cellular sites of IF action and do not distinguish transfer of the vitamin through the cell membrane from uptake by the membrane itself. Although there is evidence to suggest a specific intestinal receptor for the IF-vitamin B<sub>12</sub> complex (5), such a receptor has not been isolated or localized to the surface of the intestinal absorptive cell.

This report describes studies of the effect of IF on the uptake of <sup>57</sup>cobalt-labeled cyanocobalamin (B<sub>12</sub>-<sup>57</sup>Co) by brush borders and microvillous membranes isolated from absorptive cells of hamster intestine. The results provide further support for the presence of a specific receptor for the IF-vitamin B<sub>12</sub> complex at the microvillous surface of the intestinal cell. In addition, crude sources of hamster IF were shown to contain a factor that causes lysis of isolated brush borders.

### Methods

*Isolation of brush borders and microvillous membranes.* Procedures similar to those previously described by Miller and Crane (9) and Eichholz and Crane (10) were used to isolate brush borders and microvillous membranes. Golden hamsters weighing 100 to 150 g were sacrificed by a blow on the head. The entire small intestine was immediately flushed *in situ* with cold 0.15 M NaCl, excised, divided into proximal and distal halves, opened, and spread out on wet paper towels with the mucosal surface facing upwards. The mucosal surface

was scraped lightly with a glass slide to collect villous cells. Scrapings pooled from either the proximal or distal half of the intestines of six hamsters were placed in 100 ml of cold 5 mM EDTA solution buffered to pH 7.4. Homogenization was carried out for 25 seconds in a Waring blender; the speed of the blender was controlled by a rheostat. Since homogenization was found to be a critical step in preparing satisfactory brush border fractions, several preliminary experiments were necessary to determine the optimal speed and duration of homogenization. The homogenate was then filtered through no. 25 bolting silk<sup>1</sup> to remove mucus and large particles. The entire procedure from the time the animals were sacrificed through recovery of the filtered homogenate was carried out in a cold room at 6° C.

The filtrate was then centrifuged in an International PR-2 refrigerated centrifuge for 10 minutes at 1,500 rpm. Thereafter, the sediment was washed twice with 50 to 60 ml of cold 5 mM EDTA and centrifuged again at 1,500 rpm. The pellet was then suspended in Krebs-Ringer bicarbonate solution (KRB) at pH 7.4, centrifuged at 1,500 rpm, and resuspended in cold KRB. This suspension was centrifuged at 500 rpm for 1 minute, and the sediment containing clumped material and nuclei was discarded. Although this step substantially reduced the yield of brush borders, it provided much purer preparations as evaluated by light microscopy. The supernate was then centrifuged at 2,500 rpm for 10 minutes to yield a pellet of relatively pure brush borders.

Brush borders thus prepared from either the proximal or distal half of the intestine of 6 hamsters (4.0 to 7.5 mg of tissue protein) were further fractionated to isolate microvillous membranes (10). The centrifuged pellet of freshly prepared brush borders was suspended in 5 ml of cold 1.0 M Tris buffer (pH 7.0), shaken vigorously with a Vortex mixer for 3 minutes, and allowed to stand in the cold for 30 to 90 minutes. The resulting suspension was layered at the top of a discontinuous density gradient consisting of 20, 30, 40, 50, and 60% glycerol and centrifuged at 63,000 *g* for 10 minutes in a Spinco model L preparative ultracentrifuge. This consistently resulted in the separation of particles into A, B, C, C', and D bands as described by Eichholz and Crane (10). The C and C' bands containing microvillous membranes were collected by aspiration, washed in an excess of cold KRB, and centrifuged in a Sorvall RC-2 high speed centrifuge at 28,000 *g* for 20 minutes.

Mucosal scrapings pooled from either the proximal or

<sup>1</sup> Obtained from Tobler, Ernst, and Trabler, New York, N. Y.

distal half of 3 hamster small intestines were homogenized for 1 minute in 100 ml of KRB. The whole intestinal mucosal homogenate was then centrifuged at 1,500 rpm for 10 minutes at 4° C, and the pellet was resuspended in KRB.

**Light and electron microscopy.** Light and electron microscopy was used to evaluate the tissue preparations. A drop or two of brush border suspension was frequently examined with the phase microscope to assess the purity of the suspension before its utilization for biochemical studies.

For detailed evaluation of the purity and morphologic preservation of brush borders, 5 ml of chilled chromosmium tetroxide (11) was added to pellets that had been isolated as described above. After 5 minutes, the pellets were broken into pieces of approximately 1 mm<sup>3</sup> in the fixative solution. After 1 to 2 hours of fixation, the fragments were transferred to glass weighing bottles and fixed again in 10% neutral isotonic formal for 1 hour, rapidly dehydrated in graded strengths of ethyl alcohol, and embedded in epoxy resin by the method of Luft (12). The microvillous membranes were fixed, dehydrated, and embedded in the same fashion, except that the entire procedure including embedment was carried out without disruption of the pellet in the tube originally used for recovery of the membranes. Thus, the orientation of the membrane pellet was maintained. Pieces of the embedded microvillous membrane pellets were cut out of the Epon block and mounted with epoxy cement on short aluminum rods machined to fit the microtome chuck after they had been oriented so that the full thickness of the pellets could be sectioned and systematically studied from top to bottom. Sections of both brush border and microvillous membrane pellets were cut 1  $\mu$  thick with glass knives, mounted on glass slides, and stained for light microscopic study with toluidine blue (13) or the periodic acid-Schiff technique.

Thin sections for electron microscopy were cut with diamond knives, mounted on carbon-coated copper mesh grids, and doubly stained with uranyl acetate (14) and lead citrate (15). Stained sections were studied with an RCA EMU-3G electron microscope.

**Intrinsic factor preparations.** IF preparations were obtained from a variety of animal species. Hamster and rat gastric juice was collected by ligation of the pylorus of lightly anesthetized, fasting animals and instillation of 1 ml of 10% NaHCO<sub>3</sub> through a fine polyethylene tube into the stomach. After 5 hours the animals were sacrificed, the stomachs were opened, and gastric contents of 6 to 12 animals were pooled. The pH of this *in vivo* neutralized gastric juice varied from 7.5 to 8.5. After centrifugation at 2,500 rpm and 4° C for 30 minutes, the supernatant gastric juice was titrated to pH 7.0 with 0.1 N HCl and stored at -20° C until used.

Human gastric juice was collected from fasting subjects after injection of 1.5 mg of histalog per kg body weight. Specimens were collected in iced containers and, in order to inactivate pepsin (16), were immediately titrated first to pH 10 with NaOH, and then to pH 7.0 with HCl.

Saline extracts of hamster, rat, rabbit, and dog gastric mucosa were prepared from mucosal scrapings of stomach obtained immediately after animals had been sacrificed. The scrapings were homogenized in Potter-Elvehjem glass homogenizers, and the homogenate was then centrifuged at 2,500 rpm and 4° C for 10 minutes. The supernate was removed and stored at -20° C until used.

Neutralized hamster gastric juice and saline extracts of hamster gastric mucosa were concentrated by ultrafiltration (17) and fractionated on columns of Sephadex G-200 eluted with 0.05 M phosphate buffer, pH 7.5. In addition, hamster gastric mucosal extracts were chromatographed on columns of Amberlite CG-50 ion exchange resin as previously described by Chosy and Schilling (17). Columns were eluted at 6° C and eluates were collected in 4-ml aliquots by means of an automatic fraction collector.

The vitamin B<sub>12</sub> binding capacity of the various IF preparations was determined by dialysis. To serial dilutions of an aliquot of each preparation was added 10 nanograms (10<sup>-9</sup>g, ng) of B<sub>12</sub>-<sup>60</sup>Co (SA 1 mc per mg). After standing for 30 minutes at room temperature, 2-ml aliquots were dialyzed at 6° C against two changes of 3 L of 0.15 M NaCl for 48 hours in Visking cellophane bags. After dialysis 1 ml of fluid was removed from each bag, and its radioactivity was compared with that of a 1-ml undialyzed aliquot. After the binding capacity had been calculated, the concentration of the IF preparation was

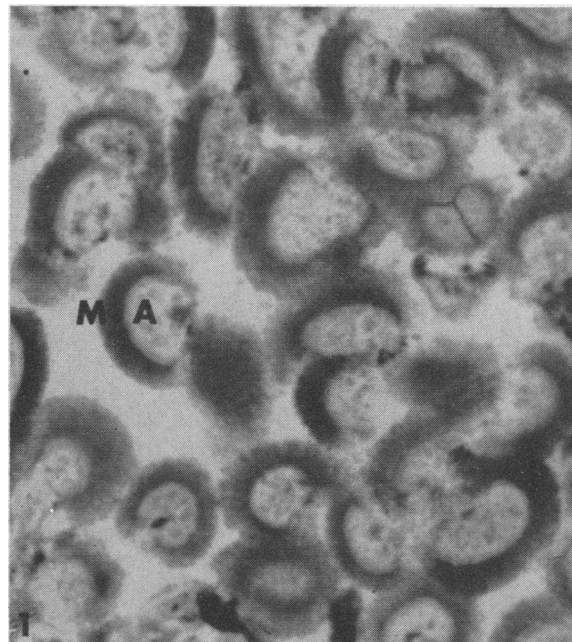


FIG. 1. LIGHT MICROGRAPH OF A SECTIONED PELLET OF HAMSTER BRUSH BORDERS. The preparation consists almost exclusively of brush borders. The microvillous surface (M) and underlying apical cytoplasm (A) can be readily identified. Epon embedment, toluidine blue,  $\times 2,000$ .

diluted to the desired level by the addition of 0.15 M NaCl.

Nitrogen concentrations were determined by a micro-Kjeldahl technique (18), and pepsinogen activity was assayed by the hemoglobin substrate method of Anson and Mirsky (19).

*Uptake of  $B_{12}$ - $^{57}Co$  by brush borders and microvillous membranes.* Freshly prepared pellets containing brush borders or microvillous membranes were resuspended in cold KRB, and 1-ml aliquots of these suspensions were added to 25-ml Erlenmeyer flasks containing 4 ml of KRB, 2 ng of  $B_{12}$ - $^{57}Co$  (SA 13.5 to 20  $\mu\text{C}$  per  $\mu\text{g}$ ), and sufficient IF preparation to bind the 2 ng of cyanocobalamin. In control flasks 0.15 M NaCl was added instead of an IF preparation. Incubations were usually performed with KRB containing 250 mg per 100 ml glucose at 37° C in room air for 60 minutes in a Dubnoff shaking metabolic incubator. Some incubations were carried out in an atmosphere of 95%  $O_2$  and 5%  $CO_2$ ; in other experiments the flasks were stoppered after they had been thoroughly gassed with nitrogen. The brush borders added to each flask usually contained 60 to 800  $\mu\text{g}$  of tissue protein, but readily interpretable results could be obtained with as little as 50 to 100  $\mu\text{g}$  of tissue protein. Microvillous membrane preparations added to incubation mixtures contained 100 to 200  $\mu\text{g}$  of tissue protein.

Immediately after incubation an excess of cold KRB was added and the mixture was centrifuged at 4° C and 2,500 rpm for 10 minutes. The tissue pellet was washed twice more in cold KRB and centrifuged again. Radioactivity remaining in the washed pellet was determined in a Packard Autogamma detector. Background activity did not exceed 50 cpm; each picogram of  $B_{12}$ - $^{57}Co$  yielded 20 to 30 cpm above background. Tissue uptake was calculated as picograms  $B_{12}$ - $^{57}Co$  per milligram tissue nitrogen.

## Results

*Purity and integrity of tissue preparations.* Figure 1 is a light micrograph of a representative field of a sectioned hamster brush border pellet fixed in osmium tetroxide, embedded in Epon, and stained with toluidine blue. The pellets consisted of pure brush borders, except for an occasional isolated nucleus and a small amount of amorphous material presumably derived from epithelial cell cytoplasm. The isolated brush borders could be readily identified by their densely stained, crescent-shaped, microvillous surface and by the paler underlying attached apical cytoplasm.

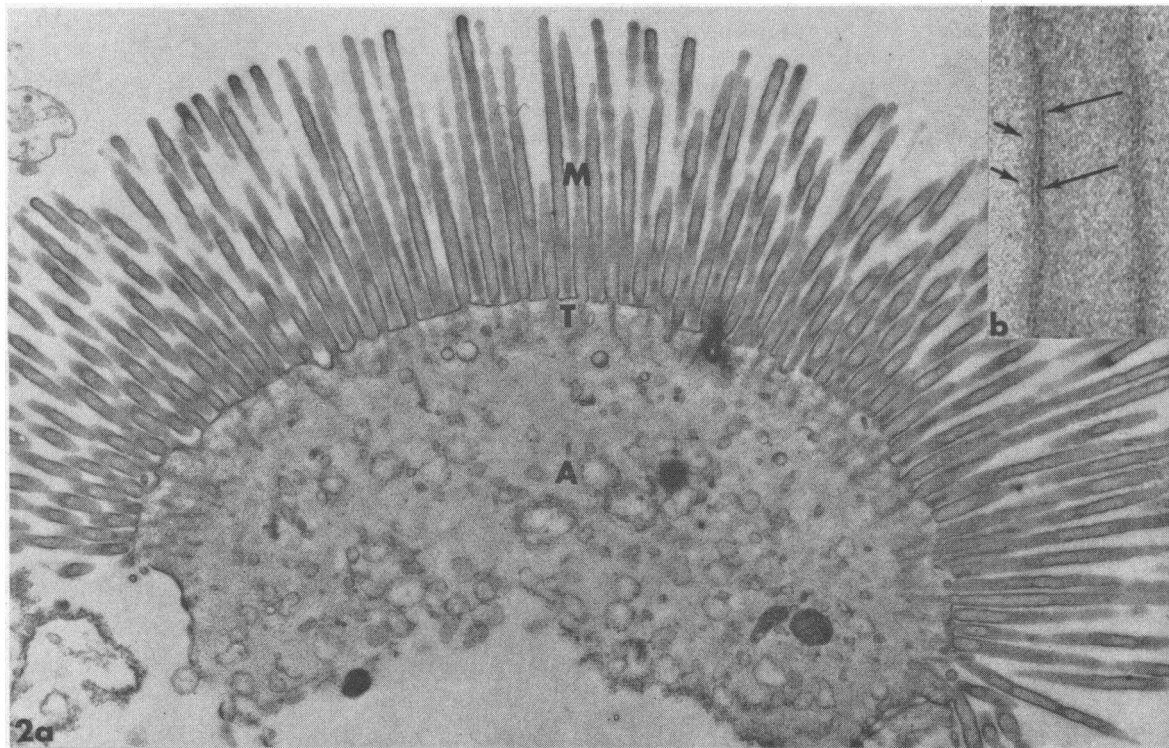


FIG. 2. FINE STRUCTURE OF A TYPICAL HAMSTER BRUSH BORDER. a) Electron micrograph demonstrating good morphologic preservation of the microvilli (M), terminal web region (T), and attached apical cytoplasm (A).  $\times 17,000$ . b) High magnification of a portion of a microvillus from an isolated brush border. The trilaminar structure of the microvillous membrane (long arrows) as well as its surface coat (short arrows) is well preserved.  $\times 110,000$ .

The structural features of the microvilli of isolated hamster brush borders were not greatly altered by the fractionation procedure. Figure 2a is an electron micrograph of a typical isolated brush border. The microvilli, terminal web, and the small portion of attached apical cytoplasm can be readily identified and are well preserved. At higher magnification, it is apparent that the surface coat of the microvillous membrane is more compact than it is in intact, well-preserved hamster epithelium, but the trilaminar unit membrane structure of the apical plasmalemma and the microvillous core are well preserved (Figure 2b).

The microvillous membrane pellets isolated from Tris-disrupted brush borders also appeared remarkably pure and morphologically well preserved (Figure 3a). Both longitudinal and cross sections of the narrow membranous sleeves of the microvilli were seen; some wider membranous profiles that

probably resulted from vesiculation of the membrane during the isolation procedure were also present. The characteristic trilaminar structure of the microvillous membrane with at least a portion of the surface coat still attached to its outer lamina could be regularly identified at higher magnification (Figure 3b).

*Effect of intrinsic factor on B<sub>12</sub>-<sup>57</sup>Co uptake by tissue preparations.* As shown in Figure 4, neutralized hamster gastric juice consistently enhanced attachment of B<sub>12</sub>-<sup>57</sup>Co to brush borders and microvillous membranes obtained from the distal but not the proximal half of hamster small intestine. In the presence of IF uptake by distal brush borders was fivefold greater and by distal microvillous membranes was tenfold greater than was uptake by whole mucosal homogenates. In contrast, uptake of B<sub>12</sub>-<sup>57</sup>Co by whole mucosal homogenates, brush borders, and microvillous membranes ob-

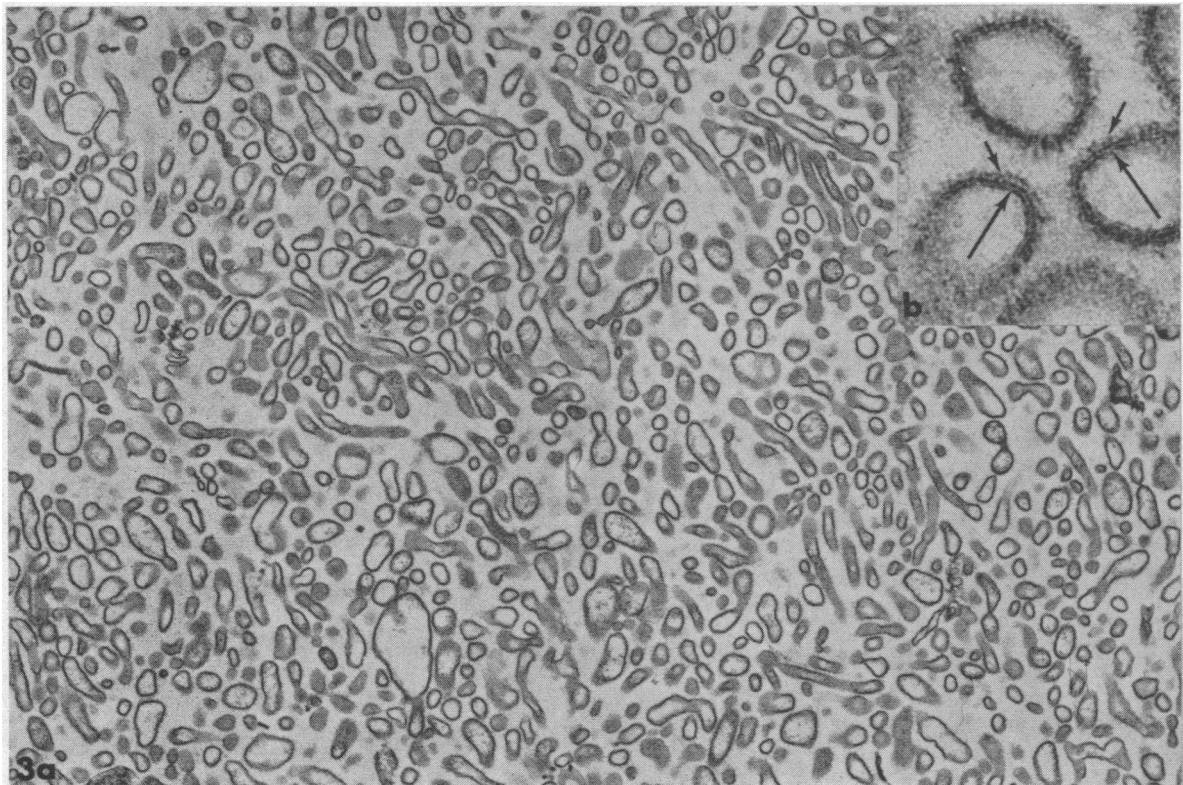


FIG. 3. FINE STRUCTURE OF A TYPICAL PELLET OF ISOLATED MICROVILLOUS MEMBRANES. a) Survey electron micrograph illustrating the purity of the preparation. Longitudinally sectioned, tangentially sectioned, and cross sectioned profiles as seen. Some of the membranous profiles are larger in diameter than well-formed microvilli. This probably represents vesiculation of some of the microvilli during the preparative procedure.  $\times 15,000$ . b) High magnification of isolated microvillous membranes. Their trilaminar membrane (long arrows) and surface coat (short arrows) are readily identified.  $\times 110,000$ .

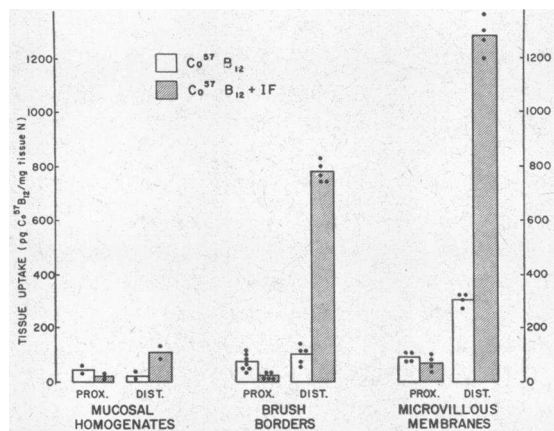


FIG. 4. UPTAKE OF FREE AND INTRINSIC FACTOR (IF)-BOUND  $B_{12}$ - $^{57}Co$  BY VARIOUS PREPARATIONS OF HAMSTER INTESTINE. The same pool of neutralized hamster gastric juice was used as IF for all experiments. Dots represent results of individual experiments; bars indicate mean values. IF stimulated uptake of  $B_{12}$ - $^{57}Co$  by preparations obtained from the distal but not the proximal half of hamster intestine.

tained from proximal intestine was consistently inhibited by the presence of IF.

Table I summarizes the effects of IF and non-IF binders of vitamin  $B_{12}$  obtained from various animal species on  $B_{12}$ - $^{57}Co$  uptake by brush borders isolated from distal hamster intestine. Gastric

TABLE I  
Specificity of intrinsic factor (IF) action on uptake of  $B_{12}$ - $^{57}Co$  by hamster brush borders

| $B_{12}$ -binding protein* | No. experiments | $B_{12}$ - $^{57}Co$ uptake $\pm$ SD |
|----------------------------|-----------------|--------------------------------------|
| None                       | 7               | $88 \pm 15$                          |
| IF preparations            |                 |                                      |
| Hamster gj†                | 5               | $783 \pm 68$                         |
| Hamster gme‡               | 5               | $285 \pm 23$                         |
| Rat gj                     | 3               | $1,064 \pm 89$                       |
| Rat gme                    | 2               | $392 \pm 36$                         |
| Rabbit gme                 | 2               | $175 \pm 14$                         |
| Human gj                   | 4               | $44 \pm 18$                          |
| Hog gme                    | 2               | $87 \pm 6$                           |
| Dog gme                    | 2               | $32 \pm 15$                          |
| Non-IF binders             |                 |                                      |
| Human saliva               | 3               | $45 \pm 4$                           |
| Human serum                | 3               | $28 \pm 3$                           |
| Hamster liver extract      | 2               | $61 \pm 8$                           |
| Hamster kidney extract     | 2               | $81 \pm 11$                          |
| Hamster splenic extract    | 2               | $28 \pm 2$                           |

\* A quantity of each preparation sufficient to bind 2 nanograms ( $10^{-9}$  g, ng)  $B_{12}$ - $^{57}Co$  was added to incubation flask.

† gj = neutralized gastric juice.

‡ gme = saline extract of gastric mucosa.

juice or saline extracts of gastric mucosa or both obtained from hamsters, rats, and rabbits enhanced  $B_{12}$ - $^{57}Co$  uptake by hamster brush borders, whereas human, hog, and dog IF preparations were without effect. Non-IF  $B_{12}$  binders in human saliva and serum and in saline extracts of hamster liver, kidney, and spleen failed to promote uptake of radioactivity by hamster brush borders.

As shown in Figure 5, when hamster gastric juice was subjected to dextran gel filtration on Sephadex G-200, a single peak of vitamin  $B_{12}$  binding activity was observed in the eluted fractions. When 0.1-ml aliquots of these fractions were incubated with distal brush borders, enhancement of tissue uptake of  $B_{12}$ - $^{57}Co$  correlated closely with the vitamin  $B_{12}$  binding capacity of the fractions.

Saline extracts of hamster gastric mucosa fractionated on a column of Amberlite CG-50 (Figure 6) yielded 2 peaks of vitamin  $B_{12}$  binding activity, the second much larger than the first. Marked enhancement of  $B_{12}$ - $^{57}Co$  uptake by hamster brush borders was produced by 0.1-ml aliquots of fractions eluted in the second but not the first peak of vitamin  $B_{12}$  binding activity. Pepsinogen activity of gastric mucosal extracts was readily separated from IF activity by chromatography on Amberlite CG-50.

To determine whether IF could attach to brush borders in the absence of vitamin  $B_{12}$  we performed the experiment summarized in Table II.

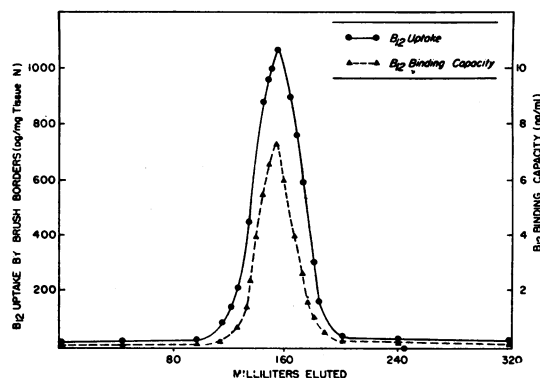


FIG. 5. GEL FILTRATION OF NEUTRALIZED HAMSTER GASTRIC JUICE ON SEPHADEX G-200. Elution with 1 M phosphate buffer, pH 7.4. Eluate collected in 4-ml fractions. Aliquots of the same fraction were tested for  $B_{12}$  binding capacity and for effect on uptake of  $B_{12}$ - $^{57}Co$  by distal brush borders.

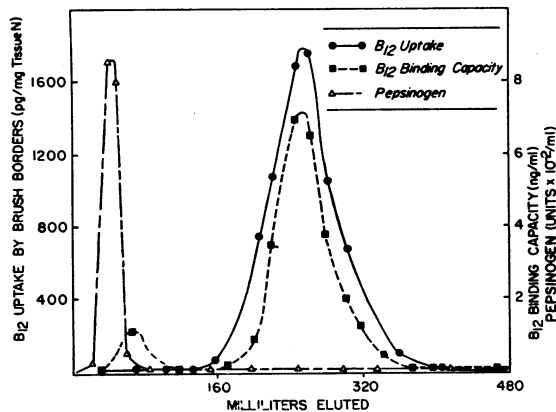


FIG. 6. ION EXCHANGE CHROMATOGRAPHY OF SALINE EXTRACT OF HAMSTER GASTRIC MUCOSA. Elution with 0.58 M acetate buffer, pH 5.4. Aliquots of 4-ml fractions were tested for B<sub>12</sub> binding capacity, effect on B<sub>12</sub> uptake by hamster brush borders, and pepsinogen activity. Ng = nanogram (10<sup>-9</sup> g).

Brush borders isolated from the distal half of hamster intestine were preincubated in 4.0 ml of KRB with or without the addition of hamster gastric juice. After incubation for 60 minutes at 37° C the brush borders were washed 3 times in cold KRB and transferred to a second flask containing either free B<sub>12</sub>-<sup>57</sup>Co or B<sub>12</sub>-<sup>57</sup>Co bound to hamster gastric juice. After a second incubation for 60 minutes at 37° C, tissue uptake of radio-

TABLE II  
Effect of preincubation of brush borders with IF on subsequent tissue uptake of B<sub>12</sub>-<sup>57</sup>Co

| Preincubation  | Final incubation                       | B <sub>12</sub> - <sup>57</sup> Co uptake* |
|----------------|--|--|
| NaCl (control) | B <sub>12</sub> - <sup>57</sup> Co     | 123 ± 15                                   |
| IF*            | B <sub>12</sub> - <sup>57</sup> Co     | 227 ± 18                                   |
| NaCl (control) | IF†-B <sub>12</sub> - <sup>57</sup> Co | 1,263 ± 221                                |
| IF*            | IF†-B <sub>12</sub> - <sup>57</sup> Co | 692 ± 154                                  |

\* "IF" = 0.1 ml neutralized hamster gastric juice capable of binding 2.0 ng of cyanocobalamin.

† Mean ± standard deviation of 4 experiments.

activity was determined. Preincubation of brush borders with IF significantly ( $p < 0.01$ ) increased the subsequent uptake of free B<sub>12</sub>-<sup>57</sup>Co and significantly ( $p < 0.01$ ) inhibited subsequent uptake of B<sub>12</sub>-<sup>57</sup>Co bound to IF.

Effect of incubation conditions on IF-mediated uptake of B<sub>12</sub>-<sup>57</sup>Co by distal brush borders (Table III). Removal of glucose from the incubation medium did not alter uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co by brush borders isolated from the distal hamster intestine. On the other hand, uptake was markedly reduced when brush borders were incubated in KRB that contained no calcium or magnesium ions. IF-mediated attachment of B<sub>12</sub>-<sup>57</sup>Co to brush borders was not altered whether incubations were carried out in 95% O<sub>2</sub>, room air, or nitrogen.

TABLE III  
Effect of incubation conditions on IF-mediated uptake of B<sub>12</sub>-<sup>57</sup>Co by brush borders

| Medium* containing     |         |                  |                  | Atmosphere         | Temperature<br>° C | B <sub>12</sub> - <sup>57</sup> Co uptake†<br>pg/mg tissue N |
|------------------------|---------|------------------|------------------|--------------------|--------------------|--|
| KRB                    | Glucose | Ca <sup>++</sup> | Mg <sup>++</sup> |                    |                    |  |
| Changes in medium      |         |                  |                  |                    |                    |  |
| +                      | +       | +                | +                | 95% O <sub>2</sub> | 37                 | 751 ± 52   |
| +                      | 0       | +                | +                | 95% O <sub>2</sub> | 37                 | 754 ± 46   |
| +                      | +       | 0                | 0                | 95% O <sub>2</sub> | 37                 | 253 ± 38   |
| Changes in atmosphere  |         |                  |                  |                    |                    |  |
| +                      | +       | +                | +                | 95% O <sub>2</sub> | 37                 | 751 ± 52   |
| +                      | +       | +                | +                | Air                | 37                 | 783 ± 61   |
| +                      | +       | +                | +                | N <sub>2</sub>     | 37                 | 810 ± 58   |
| Changes in temperature |         |                  |                  |                    |                    |  |
| +                      | +       | +                | +                | Air                | 7                  | 738 ± 43   |
| +                      | +       | +                | +                | Air                | 11                 | 845 ± 37   |
| +                      | +       | +                | +                | Air                | 16                 | 819 ± 29   |
| +                      | +       | +                | +                | Air                | 25                 | 806 ± 46   |
| +                      | +       | +                | +                | Air                | 37                 | 783 ± 61   |

\* To each flask were added 2 ng of B<sub>12</sub>-<sup>57</sup>Co and 0.1 ml of a pool of neutralized hamster gastric juice containing a sufficient quantity of IF to bind all the B<sub>12</sub>-<sup>57</sup>Co present. All incubations carried out for 60 minutes. KRB = Krelis-Ringer buffer.



Similarly the extent of  $B_{12}$ - $^{57}Co$  uptake was not strikingly different over a range of incubation temperatures from 7° C to 37° C.

*Effect of supernate obtained from distal intestinal mucosal homogenates on uptake of IF-bound  $B_{12}$ - $^{57}Co$  by proximal brush borders.* Mucosal scrapings from either the proximal or distal half of hamster intestine were homogenized in 5 mM EDTA and centrifuged at 4° C and 28,500 *g* for 30 minutes in a Sorvall RC-2 high speed centrifuge. To saturate the EDTA, we added 1 ml of 2 M  $CaCl_2$  to 40 ml of the resulting clear supernate. The supernate was then preincubated at room temperature for 60 minutes with 2 ml of brush borders isolated from the proximal half of the intestine.

As shown in Figure 7, IF regularly inhibited  $B_{12}$ - $^{57}Co$  uptake by brush borders isolated from proximal hamster intestine. When these proximal brush borders were preincubated with supernate obtained after EDTA rupture of proximal intestinal mucosal scrapings (proximal extract),  $B_{12}$ - $^{57}Co$  uptake was again inhibited by IF. However, IF consistently enhanced  $B_{12}$ - $^{57}Co$  uptake by proximal brush borders that had been preincubated with supernatant fluid obtained after homogenization of distal mucosal scrapings (distal extract). In contrast to the results observed with proximal brush borders, IF did not enhance  $B_{12}$ - $^{57}Co$  uptake by

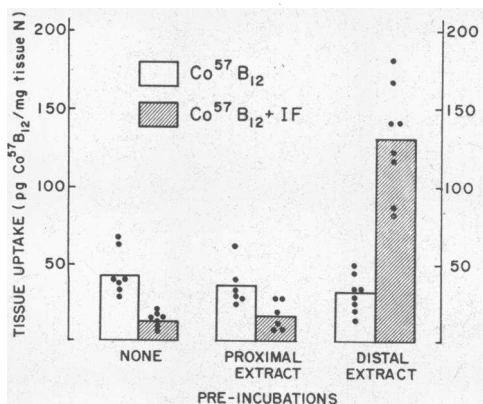


FIG. 7. EFFECT OF PREINCUBATION WITH "EXTRACTS" OF HAMSTER INTESTINE ON IF-MEDIATED UPTAKE OF  $B_{12}$ - $^{57}Co$  BY PROXIMAL BRUSH BORDERS. Bars represent mean values and dots indicate results of individual experiments. The "extracts" consisted of supernatant fluid obtained after mucosal scrapings of either proximal or distal hamster intestine had been ruptured with 5 mM EDTA and centrifuged at 28,500 *g* for 30 minutes.

TABLE IV

*Effect of preincubation with "distal supernate" on subsequent uptake of  $B_{12}$ - $^{57}Co$  by proximal brush borders*

| Distal supernate             | Uptake of $B_{12}$ - $^{57}Co$ * |                           |
|------------------------------|----------------------------------|---------------------------|
|                              | Free $B_{12}$ - $^{57}Co$        | $B_{12}$ - $^{57}Co$ + IF |
|                              | <i>pg/mg tissue N</i>            |                           |
| Untreated                    | 38 ± 12                          | 139 ± 24                  |
| Dialyzed                     | 27 ± 14                          | 165 ± 41                  |
| Heated 60° C for 10 minutes  | 34 ± 12                          | 10 ± 6                    |
| Centrifuged 34,880 <i>g</i>  | 32 ± 8                           | 144 ± 14                  |
| Centrifuged 44,146 <i>g</i>  | 27 ± 9                           | 131 ± 18                  |
| Centrifuged 54,500 <i>g</i>  | 33 ± 10                          | 34 ± 12                   |
| Centrifuged 105,536 <i>g</i> | 26 ± 4                           | 21 ± 7                    |
| Resuspended pellet†          | 29 ± 11                          | 137 ± 38                  |

\* Mean of 3 to 8 experiments ± standard deviation.

† Pellet obtained after "distal supernate" that had been centrifuged at 54,500 *g* for 30 minutes was resuspended in 40 ml of KRB.

other particles including protein-coated charcoal, calcium phosphate, and homogenates of hamster liver and kidney that had been preincubated with distal supernate.

Proximal brush borders were also preincubated with distal supernates that had previously been dialyzed, heated, or centrifuged at higher speeds (Table IV). Exhaustive dialysis of distal supernate for 48 hours at 6° C against 0.15 M NaCl did not destroy its effect on proximal brush borders. When the distal supernate was heated to 60° C for 10 minutes, however, subsequent uptake of  $B_{12}$ - $^{57}Co$  by proximal brush borders was no longer enhanced by IF. A similar loss of effect was consistently observed when distal supernates were centrifuged in the Spinco ultracentrifuge at 54,500 *g* or 105,000 *g* for 30 minutes.

Distal as well as proximal supernates were centrifuged at 54,500 *g* for 30 minutes, and the pellets obtained were resuspended in KRB. Proximal brush borders preincubated with resuspended distal but not proximal pellets again demonstrated IF enhancement of  $B_{12}$ - $^{57}Co$  uptake. When the resuspended pellets obtained from distal supernates were incubated with  $B_{12}$ - $^{57}Co$  and recentrifuged, it was observed that IF produced a threefold increase in the uptake of  $B_{12}$ - $^{57}Co$  by the pellet itself. On the other hand, IF did not enhance  $B_{12}$ - $^{57}Co$  uptake by pellets obtained after high speed centrifugation of proximal supernates.

Pellets obtained after centrifugation of distal supernates were fixed and embedded in the same manner as pellets of microvillous membranes.

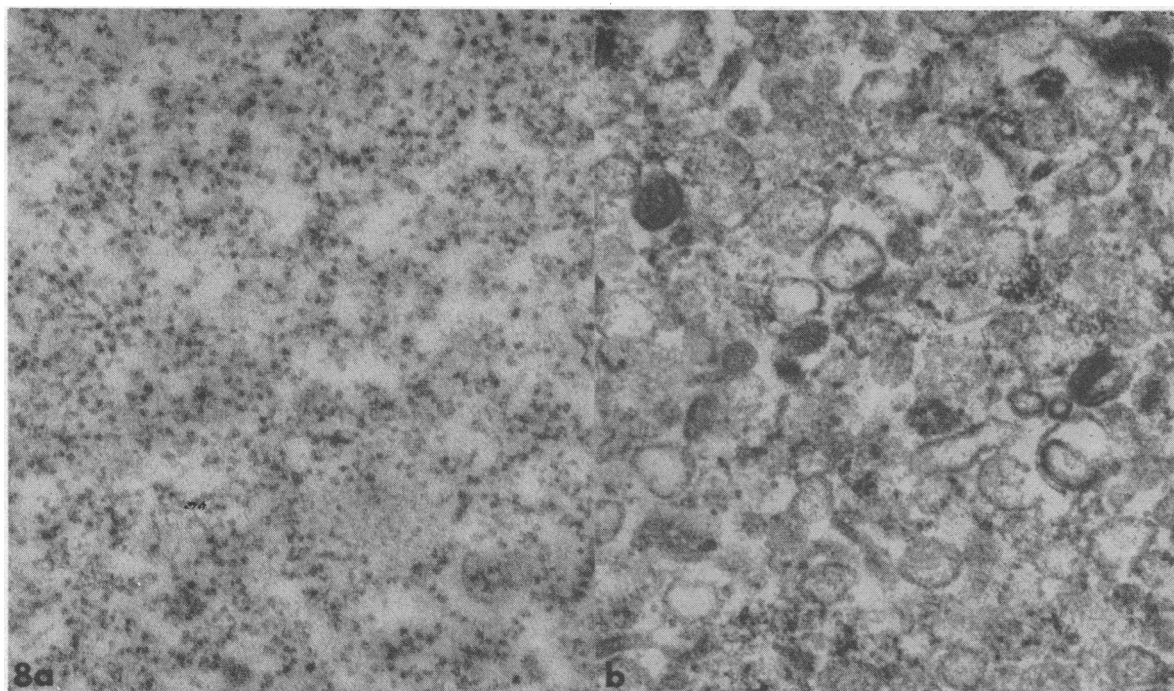


FIG. 8. ELECTRON MICROGRAPHS OF A PELLET OBTAINED AFTER CENTRIFUGATION AT 105,000 G OF SUPERNATE RECOVERED AFTER RUPTURE OF DISTAL INTESTINAL MUCOSAL SCRAPINGS. a) The upper three-fourths of the pellet consists almost exclusively of unattached ribosomes. b) The extreme base contains many membranous fragments in addition to some ribosomes.  $\times 50,000$ .

This allowed systematic top-to-bottom examination with the electron microscope. The upper three-fourths of the pellet consisted almost entirely of particles of approximately 200 A in diameter, which were identified as unattached ribosomes (Figure 8a). The lower fourth of the pellet contained increasing quantities of membranous fragments in addition to many ribosomes. Membranous elements were most abundant at the extreme base of the pellet (Figure 8b). Exact quantitation of the percentage of ribosomes versus membranes in the pellet was not attempted, but it was estimated that approximately 85% of the pellet was composed of ribosomes, whereas 15% consisted of membranes.

The presence of ribosomes in the pellet was supported by studies with ribonuclease A<sup>2</sup> (recrystallized 3 times). Incubation of pellets for 30 minutes at 37° C with increasing concentrations of ribonuclease A resulted in release of increasing quantities of nucleotides as determined by ultraviolet spectrophotometry (20). Treatment of pel-

<sup>2</sup> Sigma Chemical Co., St. Louis, Mo.

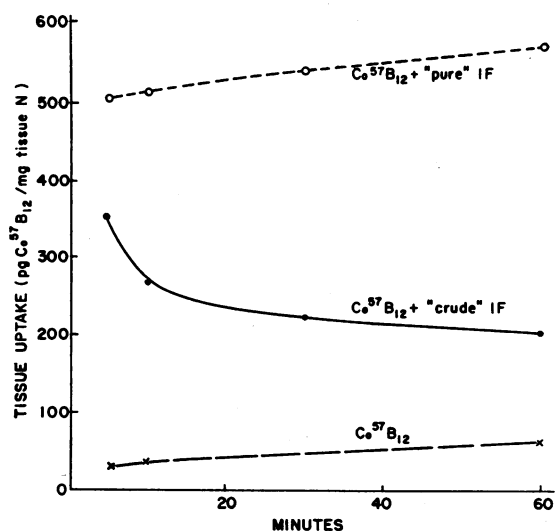


FIG. 9. EFFECT OF DURATION OF INCUBATION ON IF-MEDIATED UPTAKE OF B<sub>12</sub>-<sup>57</sup>Co BY DISTAL BRUSH BORDERS. Each dot represents mean of 2 or 3 experiments. "Crude" IF consisted of saline extract of hamster gastric mucosa. "Purified" IF consisted of peak B<sub>12</sub> binding fraction obtained after gel filtration of crude extract. In each experiment sufficient IF to bind 2 ng of vitamin B<sub>12</sub> was added to incubation flasks. Uptake of B<sub>12</sub>-<sup>57</sup>Co in absence of IF is shown for comparison.

lets with sufficient enzyme to produce maximal nucleotide release, however, did not impair IF-mediated uptake by these pellets.

*Lysis of brush borders by hamster gastric mucosal extract and neutralized hamster gastric juice.* When a crude saline extract of hamster gastric mucosa was used as the source of IF, uptake of  $B_{12}$ - $^{57}Co$  by distal brush borders decreased rather than increased as the duration of incubation was prolonged (Figure 9). No such decrease was observed, however, when this same gastric mucosal extract was used after partial purification by gel filtration on Sephadex G-200. Furthermore, as shown in Figure 10, uptake of  $^{57}Co$  by distal brush borders increased with increasing purity of the IF preparation used. Purity of IF preparations was assessed on the basis of vitamin  $B_{12}$  binding capacity per milligram of nitrogen, and the quantity of each preparation added to the incubation medium was just sufficient to bind 2 ng of  $B_{12}$ - $^{57}Co$ .

Since these results suggested that a factor which inhibited tissue uptake of  $B_{12}$ - $^{57}Co$  might be present in crude sources of IF, we examined the ef-

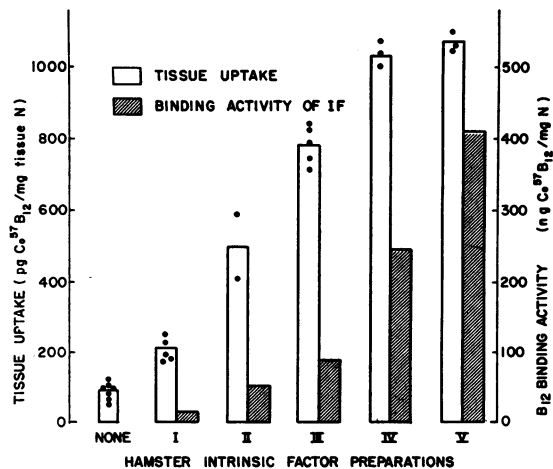


FIG. 10. EFFECT OF PARTIAL PURIFICATION OF HAMSTER IF PREPARATIONS ON UPTAKE OF  $B_{12}$ - $^{57}Co$  UPTAKE BY DISTAL BRUSH BORDERS. In each uptake experiment the quantity of IF added was just sufficient to bind 2 ng of  $B_{12}$ - $^{57}Co$ . Hatched bars indicate relative purity of IF preparations in terms of  $B_{12}$  binding capacity per milligram of nitrogen. Hamster IF preparations included saline extract of gastric mucosa (I), *in vivo* neutralized gastric juice (III), and various fractions (II, IV, V) obtained after gel filtration of crude sources.

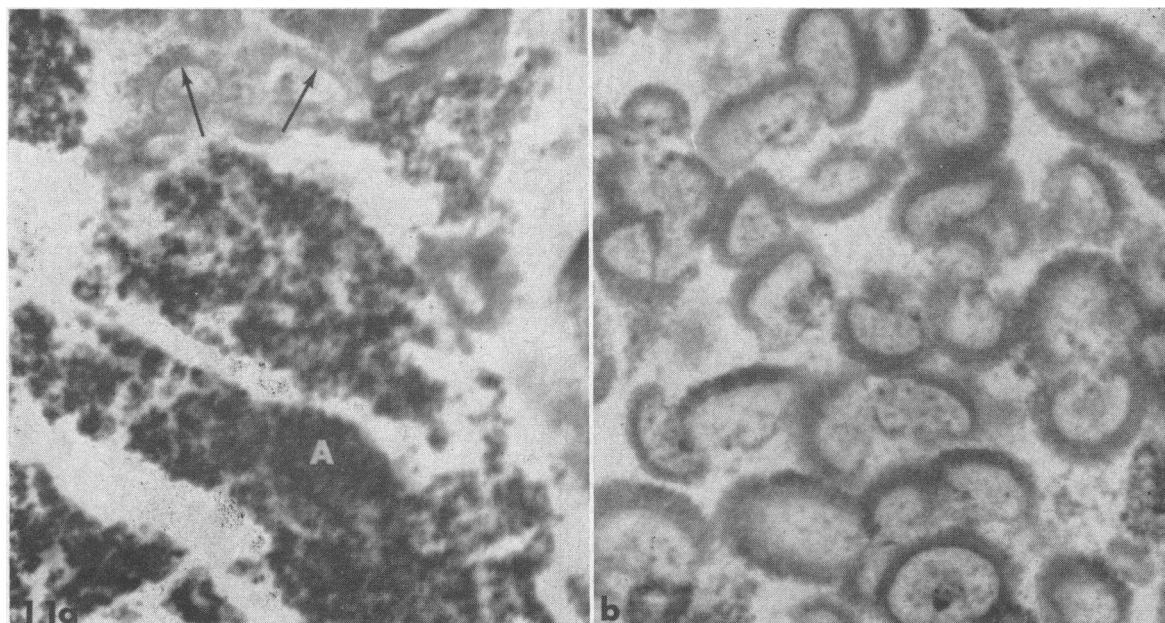


FIG. 11. LIGHT MICROGRAPHS OF PELLETS OF BRUSH BORDERS INCUBATED WITH CRUDE AND PARTIALLY PURIFIED HAMSTER IF. Incubations carried out at  $37^{\circ}C$  for 10 minutes with saline extract of gastric mucosa (crude IF) and same extract after gel filtration on Sephadex G-200 (partially purified IF). a) After incubation with crude IF, large amounts of amorphous material (A) are seen. Only a few, pale-staining, degenerating brush borders (arrows) can be identified. Epon embedment, toluidine blue,  $\times 2,000$ . b) After incubation with partially purified IF, the brush borders remain well preserved and appear comparable to pellets of unincubated brush borders (compare to Figure 1). Epon embedment, toluidine blue,  $\times 2,000$ .

fect of crude and partially purified IF on the morphologic appearance of brush borders. Figure 11a is a light micrograph of a representative field of a pellet of brush borders incubated with a crude saline extract of hamster gastric mucosa for 10 minutes at 37° C. Large amounts of amorphous material, never seen in control pellets, are present. A few faintly stained degenerating but still recognizable brush borders can be identified. Figure 11b is a light micrograph of a representative field of a pellet of brush borders that had been incubated with a partially purified saline extract of hamster gastric mucosa for 10 minutes at 37° C. In contrast to those in Figure 11a, the brush borders are still intact after incubation and appear quite comparable to those seen in unincubated preparations (compare with Figure 1). Similar but less marked effects were observed with *in vivo* neutralized hamster gastric juice. Further support for the destructive effect of crude sources of IF was derived from the observation that addition of increasing quantities of gastric mucosal extract resulted in increased release of tissue nitrogen into the incubation medium.

### Discussion

Intrinsic factor consistently enhanced uptake of <sup>57</sup>Co-labeled cyanocobalamin by brush borders and microvillous membranes isolated from the villous cells of the distal half of hamster small intestine. This observation, together with the fact that uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co by microvillous membranes was tenfold greater per milligram of tissue nitrogen than was uptake by homogenates of whole intestinal mucosa, suggests localization of IF action *in vitro* to the apical surface of the intestinal cell. The highly developed mucopolysaccharide surface coat that is applied to the outer leaflet of the microvillous unit membrane was retained in these preparations of brush borders and microvillous membranes (Figures 2 and 3). There is now good evidence (21) that this surface coat is an integral part of the apical plasma membrane rather than simply adsorbed extraneous material. The present studies, however, do not demonstrate whether IF promoted vitamin B<sub>12</sub> uptake by the unit membrane itself or by its surface coat.

Several observations establish the specificity of the observed IF-mediated attachment of B<sub>12</sub>-<sup>57</sup>Co

to the apical cell membrane isolated from the villous absorptive cell surface and suggest that the process is of physiological significance. Enhancement of tissue uptake of B<sub>12</sub>-<sup>57</sup>Co by IF was observed only with brush borders and microvillous membranes isolated from the distal half of the intestine. An effect of IF on vitamin B<sub>12</sub> uptake by distal but not proximal intestine has previously been observed with everted sacs (22) and homogenates (23) of intestine. In addition, IF-mediated absorption of the vitamin *in vivo* appears to occur at the distal rather than the proximal intestine of man (24). Only IF from hamsters, rats, and rabbits promoted B<sub>12</sub>-<sup>57</sup>Co uptake by hamster brush borders; human, hog, and dog IF preparations were without effect. Except that hog IF was slightly active, this same species specificity was observed with everted sacs of hamster intestine (25). Finally, a direct relation was demonstrated between enhancement of tissue uptake of B<sub>12</sub>-<sup>57</sup>Co and the vitamin B<sub>12</sub> binding activity of hamster gastric juice or hamster gastric mucosal extract after fractionation of these sources of IF by gel filtration (Figure 5) or ion exchange chromatography (Figure 6).

The latter observation is consistent with previous studies which suggest that binding of vitamin B<sub>12</sub> in a macromolecular complex plays an important role in IF-mediated absorption of the vitamin *in vivo* (3). When both free and IF-bound vitamin B<sub>12</sub> are present in man (26, 27) and in rats (27), the IF-bound vitamin is preferentially absorbed. When human gastric juice is subjected to ion exchange chromatography with Amberlite CG-50, two peaks of vitamin B<sub>12</sub> binding activity are obtained, but only the second and larger peak is able to promote vitamin B<sub>12</sub> absorption in patients lacking IF (17). When hamster gastric mucosal extract was similarly fractionated, only the second peak significantly enhanced B<sub>12</sub>-<sup>57</sup>Co uptake by brush borders isolated from hamster intestine. It should be emphasized, however, that not all substances that bind vitamin B<sub>12</sub> promote its absorption (3), and in the present study non-IF binders of vitamin B<sub>12</sub> in serum, saliva, and tissue extracts failed to stimulate B<sub>12</sub>-<sup>57</sup>Co uptake by brush borders (Table I).

On the basis of studies of IF action *in vitro*, Herbert (5, 28) postulated a specific receptor for the IF-vitamin B<sub>12</sub> complex located on the surface

of intestinal cells and suggested that the IF molecule contains two active sites, one which binds vitamin B<sub>12</sub> in a macromolecular complex and one which attaches to the intestinal receptor. The present studies with brush borders and microvillous membranes support the concept that this postulated receptor is located on the apical cell membrane. In addition, sequential incubation of brush borders provided results consistent with those previously described in rat liver slices (28) and everted intestinal sacs (5). In our studies, brush borders, preincubated with IF alone, subsequently took up more free B<sub>12</sub>-<sup>57</sup>Co than did control preparations, whereas uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co was inhibited when brush borders were preincubated with IF (Table II). These observations suggest that 1) IF in the absence of vitamin B<sub>12</sub> is able to attach to the postulated intestinal receptor, 2) free B<sub>12</sub>-<sup>57</sup>Co subsequently becomes bound to receptor-attached IF, and 3) uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co is reduced after preincubation with IF alone because receptor sites have been "covered." Since preincubation of IF with brush borders produced only partial enhancement of free B<sub>12</sub>-<sup>57</sup>Co uptake and only partial inhibition of uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co, it is possible that, under these experimental conditions, the intestinal receptor more readily accepts the IF-B<sub>12</sub>-<sup>57</sup>Co complex than IF alone. It should be pointed out, however, that in some experiments with everted intestinal sacs (4, 22), preincubation of tissue with IF failed to stimulate subsequent uptake of vitamin B<sub>12</sub>.

IF enhancement of B<sub>12</sub>-<sup>57</sup>Co uptake was not diminished when brush borders were incubated in the absence of glucose or oxygen and was not significantly altered by changes in incubation temperature from 7° C to 37° C (Table III). This suggests that the observed attachment of IF-bound B<sub>12</sub>-<sup>57</sup>Co to brush borders, although specific, resulted from adsorption and did not depend upon an energy-requiring enzymatic process. The observation that prolonged incubation did not significantly increase uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co (Figure 9) supports this view. A role for divalent cations in the attachment process is suggested by the decreased uptake of IF-bound B<sub>12</sub> in incubation mixtures devoid of calcium and magnesium ions (Table III). These results agree

with the observations of Sullivan, Herbert, and Castle (7), who showed that IF enhancement of vitamin B<sub>12</sub> uptake by guinea pig intestinal mucosal homogenates occurs in the absence of glucose or oxygen, is not markedly affected by temperature changes or time, and requires the presence of divalent cations. Everted sacs of intestine may constitute a more complex system, however, since IF-mediated uptake of vitamin B<sub>12</sub> by this tissue preparation is markedly inhibited by anaerobic conditions, absence of glucose, and low incubation temperatures (22). It is possible that uptake by the relatively intact intestinal mucosa of everted sacs may include an energy-dependent step that is lost when mucosal cells are homogenized. In any case, direct demonstration of an IF effect upon intestinal brush borders and microvillous membranes makes feasible isolated examination of the initial attachment phase of vitamin B<sub>12</sub> absorption as well as more precise localization and characterization of an intestinal receptor for the IF-vitamin B<sub>12</sub> complex.

The possibility that other subcellular fractions may also affect intestinal brush border uptake of IF-bound B<sub>12</sub>-<sup>57</sup>Co was raised by experiments in which proximal brush borders were preincubated with the supernatant fluid ("distal supernate") obtained from homogenization of distal intestinal mucosa (Figure 7). After preincubation with this distal supernate, proximal brush borders appeared to behave like distal brush borders in that IF clearly enhanced uptake of B<sub>12</sub>-<sup>57</sup>Co. Preincubation with supernatant fluid obtained after homogenization of proximal intestinal mucosa did not produce this effect.

Since the supernatant fluid was obtained after centrifugation at 28,500 *g* and appeared to be perfectly clear, our initial interpretation of this experiment (1) was that a soluble "receptor" for the IF-vitamin B<sub>12</sub> complex had been transferred from the distal mucosa to proximal brush borders. This view is no longer tenable, however, since it was subsequently demonstrated that centrifugation at higher speeds (greater than 54,500 *g*) yields a small pellet, and that the resultant supernate then has no effect on proximal brush borders. Since, in addition, the resuspended pellet obtained after high speed centrifugation of distal supernate was capable of causing subsequent IF enhancement

of B<sub>12</sub>-<sup>57</sup>Co uptake by proximal brush borders, it would appear that the active factor in distal supernate is present in a particulate form. IF also promoted enhanced uptake of B<sub>12</sub>-<sup>57</sup>Co by the pellet itself.

This pellet was found to consist largely of unattached ribosomes together with a relatively small quantity of membranous fragments (Figure 8). Further studies are required to determine the exact source of the membranous fragments, but it seems likely that some are derived from intracytoplasmic membranous organelles, whereas others represent fragments of plasma membrane. The mechanism by which these subcellular particles result in IF enhancement of B<sub>12</sub>-<sup>57</sup>Co uptake by proximal brush borders is under investigation. Whether the ribosomes or the membranes are responsible and how they mediate IF enhancement of B<sub>12</sub> uptake remain to be determined.

Incubation of brush borders with crude hamster IF preparations resulted in marked destruction of normal architecture (Figure 11). Partial purification of these IF preparations by gel filtration eliminated this destructive effect (Figure 11b). Brush border destruction was accompanied by release of tissue nitrogen into the incubation medium, suggesting that a proteolytic enzyme was responsible. The effect cannot be attributed to peptic activity since incubations were carried out at pH 7.4. The observations that 1) prolonged incubation of brush borders with crude hamster IF resulted in decreased rather than increased uptake of B<sub>12</sub>-<sup>57</sup>Co and, 2) partially purified IF preparations were more effective than crude preparations in promoting B<sub>12</sub>-<sup>57</sup>Co uptake suggest that the destructive action of crude IF sources interfered with uptake of B<sub>12</sub>-<sup>57</sup>Co by brush borders. Studies with guinea pig intestinal mucosal homogenates (7, 23) have shown that tissue uptake of vitamin B<sub>12</sub> is impaired in the presence of excessive quantities of neutralized human gastric juice. This inhibitory effect of crude IF excess may be explained in part by the presence of a factor that lyses brush borders *in vitro*. Further studies are required, however, to determine whether the inhibition caused by excessive quantities of crude IF may also be due in part to covering of intestinal receptor sites by IF molecules to which no vitamin B<sub>12</sub> is bound (5).

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