Tissue Distribution of Radio-B₁₂ after Intravenous Injection of Gastric Juice, Saliva and Plasma-Bound Co³¹B₁₂

II. In Rats

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We have previously shown in man that vitamin B₁₂ (B₁₂) binders of normal human gastric juice (NHGJ)¹ and pernicious anemia gastric juice (PAGJ)² enhance hepatic uptake and delay blood clearance of an i.v. tracer dose of Co³¹B₁₂. This extended the prior work of others using in vivo and in vitro animal systems which demonstrated that gastrointestinal B₁₂ binders with and without intrinsic factor (IF) activity enhance B₁₂ uptake by liver tissue.

In all in vivo studies, however, precise quantitative comparisons of the effects of different B₁₂ binders on hepatic B₁₂ uptake could not be made because of interference by extrahepatic tissues. The role of the reticuloendothelial system in the removal of bound B₁₂, though questioned,² was also not evaluated. Therefore, in the present investigation, we studied the hepatic uptake of a variety of gastrointestinal B₁₂ binders by means of an in situ rat liver perfusion technic without recirculation of blood which permitted rapid and precise quantitative comparisons. In addition, the effect of depression of reticuloendothelial system activity on hepatic removal of bound B₁₂ was also studied.

The results indicate that, compared with unbound B₁₂, binding of B₁₂ to PAGJ, human saliva, hog intrinsic factor concentrate (HIFC), and boiled HIFC increased B₁₂ uptake by rat liver. On the other hand, B₁₂ bound to NHGJ was removed by rat liver in a manner quantitatively similar to that observed with unbound B₁₂. This result is markedly different from our previous finding in man.¹ Depression of reticuloendothelial system activity induced by india ink did not affect the hepatic removal of HIFC-bound B₁₂.

Methods and Materials

Hepatic uptake was quantitated by means of a rat liver perfusion technic without recirculation.⁹ Perfusions were performed in 48 nonfasting female Sprague-Dawley rats weighing 275-325 Gm. Whole rat blood was obtained by cardiac puncture in a heparinized...
syringe from rats of the same strain and size used for perfusion, filtered through the nylon mesh filter of a standard blood transfusion set to remove clots, and oxygenated with a mixture of 95 per cent O₂ and 5 per cent CO₂. The blood was warmed to 37 C. by passage through a water bath and the entire perfusion system maintained at that temperature by enclosure in a plexiglass constant-temperature box. After pentobarbital anesthesia of the test animal, cannulas were positioned in the inferior vena cava and portal vein, and ligatures were placed to isolate the liver from the rest of the circulation. The oxygenated blood was then perfused for 10 minutes via the portal vein through the liver at a constant rate of 4 ml. per minute maintained by a motor driven syringe. The effluent was collected by means of a cannula positioned opposite the hepatic venous outflow into the inferior vena cava. The cannula was connected to a manifold which permitted separation of the effluent into ten 1-minute samples. Throughout the perfusion, the livers appeared grossly normal and examination by light microscopy at the end of the perfusion demonstrated no abnormalities.

Extraction ratios \( \frac{A-V}{A} \) have been calculated from the perfusate concentration (A) and the effluent concentration (V) during the fourth and tenth minutes. The fourth minute was chosen because intrahepatic mixing was complete at that time as determined in a separate investigation. The tenth minute was the end of the perfusion.

**Material for Perfusion**

1. **Unbound B₁₂ (Table 1)**

The B₁₂ concentration of whole rat blood determined before the addition of radio-B₁₂ in 6 pools, derived from 6 donor rats each, ranged from 300-800 pg./ml. using a radioassay. Co²⁰B₁₂ (or Co⁶⁰B₁₂ in those experiments utilizing higher B₁₂ concentrations) was added to whole rat blood to produce final radio-B₁₂ concentrations ranging from 12 pg./ml. to 13,633 pg./ml. These values do not include endogenous serum B₁₂ since the latter is not completely miscible in vitro with added crystalline B₁₂. In 21 rats the following final radio-B₁₂ concentrations and perfusion rates were obtained:

<table>
<thead>
<tr>
<th>Group</th>
<th>Radio-B₁₂ Concentration (pg./ml.)</th>
<th>Radio-B₁₂ Perfusion Rate (pg./100 Gm. rat/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (6 rats)</td>
<td>12 to 60</td>
<td>15 to 71</td>
</tr>
<tr>
<td>B (7 rats)</td>
<td>111 to 410</td>
<td>140 to 450</td>
</tr>
<tr>
<td>C (4 rats)</td>
<td>1379 to 4301</td>
<td>1672 to 6325</td>
</tr>
<tr>
<td>D (4 rats)</td>
<td>7902 to 13,633</td>
<td>9503 to 16,875</td>
</tr>
</tbody>
</table>

2. **Hog Intrinsic Factor Concentrate-Bound B₁₂**

a. Radio B₁₂ was incubated for 30 minutes at room temperature with 0.1 mg. of purified HIFC (WES 727) in physiologic saline solution. The HIFC-bound B₁₂ was then separated by Sephadex G-25 gel filtration according to the method described in this laboratory.

In 8 perfusions HIFC-bound B₁₂ was added to the perfusate to produce radio-B₁₂ concentrations of 174 to 8907 pg./ml. resulting in radio-B₁₂ perfusion rates ranging from 224 to 11,419 pg./100 Gm. rat/minute.

*We are indebted to Dr. Sheldon Rothenberg for performing these determinations.

Co²⁰B₁₂ (8-18 μCi/μg.) and Co⁶⁰B₁₂ (1 μCi/μg.) were generously supplied by Dr. Elmer Alpert and Dr. Charles Rosenblum of Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania.

Purified hog intrinsic factor concentrate was generously provided by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N.Y. Five mg. of this material gave optimum B₁₂ absorption with the Schilling test.
b. Samples of HIFC-bound $B_{12}$ were heated in a water bath at 100°C. at pH 6 for 30 minutes. Gel filtration of aliquots of this boiled material demonstrated that it was still bound to radio-$B_{12}$. Despite lack of change in binding capacity, this heated material showed markedly reduced ability to enhance $B_{12}$ absorption in a pernicious anemia patient as measured by the Schilling test. This is in accord with previous work\textsuperscript{14-16} showing the inactivation of IF by heating with little effect on $B_{12}$ binding.

In 4 perfusions this heat treated HIFC bound to $Co^{60}B_{12}$ was added to the perfusate to produce final HIFC-bound $Co^{60}B_{12}$ concentrations varying from 12,000 to 15,364 pg./ml, resulting in final perfusion rates of 12,666 to 18,412 pg./100 Gm. rat/minute.

c. In 4 additional perfusions, one hour prior to perfusion with nonheated HIFC-bound $B_{12}$, 16 mg. of india ink in 1 per cent gelatin solution per 100 Gm. rat was injected into the tail vein of the test rat according to the method of Halpern et al.\textsuperscript{17} in order to depress the activity of the reticuloendothelial system in the liver. At the time of the perfusion, the liver was diffusely black and some carbon particles were still in circulation.

3. Human Gastric Juice-Bound $B_{12}$

a. NHGJ was prepared by a modification of the method described by Wallerstein et al.\textsuperscript{18} NHGJ was collected on ice in separate batches following maximal histamine stimulation (0.4 mg./10 Kg.) from 2 duodenal ulcer patients. The juice was allowed to stand for 2½ hours after collection and was then brought to pH 7, centrifuged, and sterilized by Berkefeld V filtration. IF activity in the juice was demonstrated by the guinea pig intestinal mucosal homogenate assay.\textsuperscript{19} This material was added in 4 perfusions to produce final radio-$B_{12}$ concentrations of 86 to 3913 pg./ml., resulting in perfusion rates of 106 to 4797 pg./100 Gm. rat/minute.

b. Aliquots of PAGJ-bound $B_{12}$ prepared for injection in human subjects\textsuperscript{2} were added in 4 perfusions to produce final radio-$B_{12}$ concentrations of 106 to 244 pg./ml., resulting in perfusion rates ranging from 172 to 309 pg./100 Gm. rat/minute. Lower concentrations of radio-$B_{12}$ were used in these experiments, since PAGJ is a poor binder and greater amounts of radio-$B_{12}$ would have required excessive dilution of the blood perfusate.

4. Saliva-Bound $B_{12}$

Four ml. of postprandial saliva obtained from a normal individual was incubated at room temperature for one-half hour with $Co^{57}B_{12}$ or $Co^{60}B_{12}$. Bound $B_{12}$ was separated by Sephadex G-25 gel filtration, as described above. This material was added in 3 perfusions to produce final radio-$B_{12}$ concentrations of 92 to 1247 pg./ml. resulting in final perfusion rates of 129 to 1568 pg./100 Gm. rat/minute.

Results

1. Unbound $B_{12}$

Results obtained in 21 perfusions with unbound $B_{12}$ show (Table 1) that variations in $B_{12}$ concentration of the perfusate did not affect hepatic extraction ratios except at the highest concentrations tested (Group D). This latter group is therefore omitted from all statistical comparisons with bound radio-$B_{12}$. There was no significant difference between the mean extraction ratios over the range of concentrations from 12 to 4301 pg./ml, even though the latter radio-$B_{12}$ concentration is approximately 10 times the normal rat serum $B_{12}$ level. In each group the mean extraction ratio declined significantly ($p<.05$) between 4 and 10 minutes. At the highest perfusate concentration (Group D), mean extraction ratios were significantly lower ($p<.05$) than each of the other groups both at 4 and 10 minutes, with little $B_{12}$ being removed at 10 minutes. At the lowest perfusate concentration (Group A), radio-$B_{12}$ was probably completely bound by serum binders.
Results obtained in 4 perfusions with NHGJ-bound B\textsubscript{12} and 4 others with boiled B\textsubscript{12} bound to boiled HIFC, and HIFC-bound B\textsubscript{12} after injection are shown in Table 1 and Figures 1 and 2.

a. The mean extraction ratio in 8 rats perfused with nonheated HIFC-bound B\textsubscript{12} was much higher than with unbound B\textsubscript{12} (p< .001) at both 4 and 10 minutes. In addition the difference between the 4 and 10 minutes values was not significant.

b. The mean extraction ratios at 4 and 10 minutes in 4 rats perfused with boiled HIFC-bound B\textsubscript{12} (Fig. 1) were also significantly greater (p< .001) than that observed with B\textsubscript{12} alone but not significantly different from that observed with nonheated HIFC.

c. In 4 rats injected with india ink prior to perfusion with nonheated HIFC-bound B\textsubscript{12} (Fig. 2), the mean extraction ratios at 4 and 10 minutes were again significantly greater (p<.001) than that observed with B\textsubscript{12} alone and not significantly different from that observed with nonheated and boiled HIFC in rats without prior india ink injection.

Thus, hepatic removal of B\textsubscript{12} in rats was significantly enhanced by prior binding to HIFC. This enhanced removal was not affected either by impairing intrinsic factor activity by boiling, or by depression of reticuloendothelial system activity. All perfusions with HIFC-bound B\textsubscript{12} were characterized by a lack of significant change in the extraction ratio between the fourth and tenth minutes of the perfusion. This contrasts with the significant decline observed with unbound B\textsubscript{12}.

3. Human Gastric Juice-Bound B\textsubscript{12}

Results obtained in 4 perfusions with NHGJ-bound B\textsubscript{12} and 4 others with...
Fig. 1.—Results obtained in 17 rat liver perfusions with unbound radio-B₁₂ (Groups A, B, and C) compared with results obtained in 8 perfusions with radio-B₁₂ bound to nonheated HIFC and in 4 perfusions with boiled HIFC.

Fig. 2.—Results obtained in 8 rat liver perfusions with B₁₂ bound to nonheated HIFC compared to those in 4 perfusions with nonheated HIFC-bound B₁₂ during depression of reticuloendothelial system activity with India ink.

Fig. 3.—Comparison of results obtained in 17 perfusions with unbound B₁₂ (Groups A, B, and C), 4 perfusions with NHGJ-bound B₁₂, 4 perfusions with PAGJ-bound B₁₂, and 8 perfusions with nonheated HIFC-bound B₁₂.
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PAGJ-bound B12 are shown in Table 1 and Figure 3. For comparison, results obtained with B12 alone and HIFC-bound B12 are also shown.

a. Mean extraction ratios for NHGJ-bound B12 at 4 and 10 minutes were not significantly different from that observed with B12 alone, but significantly less (p<.01) than that observed with HIFC-bound B12.

b. With PAGJ-bound B12 the 4-minute extraction ratio was not significantly greater than that of B12 alone or bound to NHGJ; however, the 10-minute extraction ratio of PAGJ-bound B12 was significantly greater (p<.01) than that of B12 alone or bound to NHGJ. The lack of change in the extraction ratio during the perfusion between 4 and 10 minutes resembled that seen with HIFC-bound B12.

4. Saliva-Bound B12

The mean extraction ratios obtained in 3 perfusions with saliva-bound B12 (Table 1) were significantly greater (p<.01) than that of B12 alone or bound to NHGJ, but not significantly different from that of PAGJ-bound B12, both at 4 to 10 minutes. It was, however, significantly less (p<.01) than that observed with HIFC-bound B12.

DISCUSSION

The rat liver perfusion system used in this study amplified our investigations in man1,2 and permitted quantitation of hepatic B12 uptake under a variety of conditions.

In comparison with previous studies of others on the influence of various binding materials7 on hepatic B12 uptake, the rat liver perfusion system without recirculation used in this study had the following advantages:

a. The uptake phenomena in our rat liver system reflected activity at the cell membrane, while in liver homogenates3 and slices4,5 uptake may also reflect binding by intracellular binders.

b. The B12 clearance from the blood in our rat liver system was restricted to liver without the participation of nonhepatic tissue in this process, which is not the case in studies using the intact rat.6

c. Because of the constant perfusion rate and timed collection of the entire effluent with no recirculation, removal rates could be accurately determined. In perfusions with recirculation, quantitative determinations of B12 removal may be influenced by variations in perfusion rate, changes in concentration of materials, accumulation of metabolic end products in the perfusate, and difficulties in direct effluent sampling. Recirculation of the effluent furthermore introduces qualitative and quantitative changes of the B12 binders in the perfusate with repeated passage through the liver which modifies the B12 removal process.7 These considerations are eliminated in our perfusion system since there is no recirculation of the perfusate.

We have shown in the present study that the extraction ratio at 4 and 10 minutes for perfused unbound B12 is constant over a wide range of perfusion rates up to 6325 pg./100 Gm. rat/min. Above this rate the extraction ratio decreases significantly, however. This wide range of radio-B12 concentrations was used in order to evaluate whether uptake was different at dosage levels
which were a fraction of the endogenous serum $B_{12}$ level and up to 20 times that level. In the lowest range, the added radio-$B_{12}$ was most probably completely bound to serum binders because of the low $B_{12}$ concentration. Binding of $B_{12}$ to serum therefore did not alter hepatic removal rates. This contrasted with the perfusion experiments using HIFC and boiled HIFC-bound $B_{12}$ where extraction ratios were approximately 3 times greater and did not fall even at perfusion rates as high as 18,412 pg./100 Gm. rat/min. This high clearance of rate of approximately 80 per cent per minute for HIFC-bound $B_{12}$ corroborates the rapid blood clearance of HIFC-bound $B_{12}$ found by others in the intact rat. This rapid clearance was not found, however, in our previous work with homologous IF in man\(^1\) or by others using homologous IF in the rat,\(^3\) indicating that HIFC functions in rat and man in a manner not comparable to homologous IF binders.

The uptake of NHGJ-bound $B_{12}$, in this study, was not significantly greater than that of unbound $B_{12}$, despite the preservation of IF activity of the former as shown in the guinea pig intestinal mucosa homogenate system. This failure of NHGJ to enhance rat liver uptake is in line with the findings of others in the intact rat\(^4\) and rat liver homogenates\(^1\) and slices.\(^2\) It differs, however, from the results obtained with a sequential incubation technic on rat liver slices using human gastric juice.\(^2\)

Some species differences between the responsiveness of rat and human liver to $B_{12}$ binders derived from human and hog stomach exist therefore. The reason for the similarity in clearance of unbound and NHGJ-bound $B_{12}$ is not apparent, especially since uptake of PACJ-bound $B_{12}$ and $B_{12}$ bound to human saliva by rat liver was significantly greater than that of unbound $B_{12}$. Although this might be due to cleavage of the NHGJ-$B_{12}$ complex in the perfusate, this does not seem likely since similarly treated NHGJ-$B_{12}$ was taken up more rapidly by liver and maintained higher blood levels over a 24-hour period than unbound $B_{12}$ after i.v. injection in man. These results suggest, therefore, that species difference is a feature of IF binders in this test system and not of the non-IF binders of PACJ and saliva, which are more likely related to gastric and salivary mucous substances. It is also possible that the divergent behavior of HIFC and homologous human and rat IF may be due to the non-IF binders present in crude HIFC preparations.

Finally, the possibility has been suggested\(^4\) that the enhancement of hepatic $B_{12}$ uptake when bound to saliva and gastric binders may represent the uptake of foreign substances by the reticuloendothelial system of the liver. The results obtained in this study after reticuloendothelial system depression would speak against this. Using an identical dose of India ink, Benacerraf et al.\(^2\) have shown interference with reticuloendothelial system uptake of a second colloid. Such interference with the uptake of HIFC-bound $B_{12}$ after India ink injection was not observed in the present study. Furthermore, Schwartz et al.\(^2\) have demonstrated that binding of $B_{12}$ to HIFC diminishes splenic $B_{12}$ uptake after intraperitoneal injection in rats. This would not be the case if reticuloendothelial system uptake was responsible for the removal of injected HIFC-bound $B_{12}$. The significant difference observed between the uptake of NHGJ- and PACJ-bound $B_{12}$ also makes this unlikely.
Summary

Hepatic uptake of unbound radio-B₁₂ and radio-B₁₂ bound to various B₁₂ binders was studied in 48 rats using a method of in situ liver perfusion of homologous whole blood without recirculation. Compared with unbound radio-B₁₂, radio-B₁₂ bound to pernicious anemia gastric juice, human saliva, hog intrinsic factor concentrate, and boiled hog intrinsic factor concentrate increased uptake of B₁₂ by rat liver. B₁₂ bound to normal human gastric juice, however, was removed in a manner quantitatively similar to that observed with unbound B₁₂.

Depression of reticuloendothelial system activity by India ink injection prior to perfusion of hog intrinsic factor concentrate-bound B₁₂ did not significantly alter hepatic B₁₂ removal rates. Comparison with results obtained in similar experiments, but without prior reticuloendothelial system depression, suggests that the removal process was accomplished by hepatic parenchymal cells rather than reticuloendothelial cells of the liver.

These results suggest that B₁₂ binders of hog intrinsic factor concentrate, pernicious anemia gastric juice, and saliva may function in the membrane transport of B₁₂ in the liver in a manner both quantitatively and qualitatively different from those of serum. This transport function is obviously not limited to intrinsic factor, since materials with and without intrinsic factor activity can produce this effect.

Summary in Interlingua

Le acceptation hepatic de vitaminsi B₁₂ radioactive non ligate e ligate a varie ligatores de B₁₂ esseva studiate in 48 rattos, utilisante un methodo de perfusion hepatic in sito de homologe sanguine total sin recirculation. In comparation con non-ligate radio-B₁₂, le radio-B₁₂ que es ligate a succo gastric ab anemia perniciose, a saliva human, a concentrato de porcin factor intrinsec, e a bullite concentrato de porcin factor intrinsec augmentava le acceptation de B₁₂ per hepate de ratto. Tamen, B₁₂ ligate a normal succo gastric human eseva eliminate in un maniera quantitativamente simile a illo observate con non-ligate B₁₂.

Le depression del activitate reticuloendothelial per le injection do tinta de China ante le perfusion de B₁₂ ligate a concentrato de porcin factor intrinsec non alterava significativemente le elimination hepatic de B₁₂. Le comparation con resultatos obtenite in simile experimentos, sed sin previe depression del systema reticuloendothelial suggestiona que le processo de elimination eseva compleite per hepatic cellulas parenchymal plus tosto que per hepatic cellulas reticuloendothelial.

Le resultatos del presente studio suggestiona que le ligatores de B₁₂ in concentrato de porcin factor intrinsec, in succo gastric ab anemia perniciose, e in saliva function possibilemente in transporto membranal de B₁₂ in le hepate in un maniera que es quantitativamente e qualitativamente differente ab illo de sero. Iste function de transporto es, per consequente, non restringite a factor intrinsec, proque materiales con e sin activitate de factor intrinsec pote produce le effecto.

References

3. Miller, O. N., and Hunter, F. M.: Stimulation of vitamin B₁₂ uptake in


9. Rothenberg, S.: Radioassay of serum vitamin B\textsubscript{12} by quantitating the competition between Co\textsuperscript{57}B\textsubscript{12} and unlabeled B\textsubscript{12} for the binding sites of intrinsic factor. J. Clin. Invest. 42:1391, 1963.


11. Rothenberg, S.: Radioassay of serum vitamin B\textsubscript{12} by quantitating the competition between Co\textsuperscript{57}B\textsubscript{12} and unlabeled B\textsubscript{12} for the binding sites of intrinsic factor. J. Clin. Invest. 42:1391, 1963.


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