Human Plasma R-Type Vitamin $\mathrm{B}_{12}$-binding Proteins

II. THE ROLE OF TRANSCOBALAMIN I, TRANSCOBALAMIN III, AND THE NORMAL GRANULOCYTE VITAMIN $\mathrm{B}_{12}$-BINDING PROTEIN IN THE PLASMA TRANSPORT OF VITAMIN $\mathrm{B}_{12}$

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The normal human granulocyte vitamin $\mathrm{B}_{12}$-binding protein, transcobalamin I, and transcobalamin III, have been labeled with $^{125}$I-labeled N-succinimidyl 3-(4-hydroxyphenyl)propionate and utilized for plasma clearance studies performed with rabbits. Both moieties of $^{125}$I-labeled granulocyte vitamin $\mathrm{B}_{12}$-binding protein-$[^{57}$Co$] \text{vitamin } B_{12}$ were cleared rapidly from the plasma (>90% by 5 min) by the liver. After 80 min, the bulk of the $^{125}$I reappeared in the plasma in small molecular weight (<$1000$) form and was rapidly excreted in the urine. After 60 min the bulk of the $[^{57}$Co$] \text{vitamin } B_{12}$ reappeared in the plasma bound to rabbit transcobalamin II and was subsequently taken up by a variety of tissues. Approximately 15% of the $^{125}$I-labeled transcobalamin II-$[^{57}$Co$] \text{vitamin } B_{12}$ complex was excreted intact into the bile during the period from 10 to 80 min after injection. The hepatic uptake of the protein-vitamin $B_{12}$ complex was blocked by the prior injection of desialyzed fetuin but not by native fetuin. Similar results were obtained with $^{125}$I-labeled transcobalamin III-$[^{57}$Co$] \text{vitamin } B_{12}$. Approximately 90% of both moieties of $^{125}$I-labeled transcobalamin I-$[^{57}$Co$] \text{vitamin } B_{12}$ had prolonged plasma survivals similar to that of $^{125}$I-labeled bovine serum albumin. After treatment with neuraminidase, both moieties of the $^{125}$I-labeled transcobalamin I-$[^{57}$Co$] \text{vitamin } B_{12}$ complex were cleared rapidly from the plasma by the liver in a manner that was indistinguishable from that observed in the case of untreated granulocyte vitamin $B_{12}$-binding protein and transcobalamin III.

These observations indicate that desialyzed transcobalamin I and the native forms of the granulocyte vitamin $B_{12}$-binding protein and transcobalamin III are cleared from plasma by the mechanism elucidated by Ashwell and Morel(I) (Ashwell, G., and Morell, A. G. (1974) Adv. Enzymol. 41, 99-128) that is capable of clearing a wide variety of asialoglycoproteins. These observations have implications concerning the function of the human R-type vitamin $B_{12}$-binding proteins, the nature of the enterohepatic circulation of vitamin $B_{12}$, the biologic significance of the mechanism described by Ashwell and Morell, and the etiology of the increased plasma concentration of human R-type protein that occurs frequently in chronic myelogenous leukemia and occasionally in hepatocellular carcinoma and other solid tumors.

Human plasma contains three vitamin $B_{12}$-binding proteins that are referred to as transcobalamin I, II, and III (1-5). Transcobalamin II is immunologically distinct from the other two proteins, has a molecular weight of 38,000 based on gel filtration (2, 3), and is not a glycoprotein (5). Transcobalamin II facilitates the cellular uptake of vitamin $B_{12}$ by a variety of cells (6-9). Transcobalamin II contains only 10 to 25% of the total plasma vitamin $B_{12}$ (1, 10), but $[^{57}$Co$] \text{vitamin } B_{12}$ bound to this protein is cleared rapidly (11, 12) from human plasma with a $t_{1/2}$ of 5 to 90 min. Recent studies (13) performed in rabbits with $^{125}$I-labeled rabbit and human transcobalamin II-$[^{57}$Co$] \text{vitamin } B_{12}$ indicate that both moieties of the transcobalamin II-vitamin $B_{12}$ complex are cleared from the plasma with a $t_{1/2}$ of 60 to 90 min, that the transcobalamin II moiety is degraded during this process, and that a significant amount of the vitamin $B_{12}$ recirculates. The importance of transcobalamin II in vitamin $B_{12}$ transport has been clearly established by the recent report by Hakami et al. (14) that congenital transcobalamin II deficiency results in a severe megaloblastic anemia that responds only to large, frequent injections of vitamin $B_{12}$.

Transcobalamin I and transcobalamin III belong to a group of immunologically indistinguishable proteins known as the R-type vitamin $B_{12}$-binding proteins (1-3). R-type proteins contain 33 to 40% carbohydrate and have apparent molecular weights in the range of 120,000 to 150,000 based on gel filtration. Transcobalamin I contains more sialic acid (18...
Particular attention was paid to the role of the liver in clearing binding proteins. Albumin (11, 12). Carmel and Herbert (15) have reported two plasma with a t1/2 of 9 to 12 days that is similar to that of form from granulocytes in vitro after blood is collected. The function of the R-type proteins is unknown. Approximately 80% of the total plasma vitamin B12 is bound to transcobalamin I (1-3) but studies performed in humans indicate that vitamin B12-binding proteins might play some role in the delivery of vitamin B12 to the liver.

In order to elucidate the role of the R-type proteins in the plasma transport of vitamin B12, transcobalamin I, transcobalamin III, and the normal granulocyte vitamin B12-binding protein have been labeled with [125I] saturated with [15Co]vitamin B12, and administered intravenously to rabbits. Particular attention was paid to the role of the liver in clearing these glycoprotein-vitamin B12 complexes, since Ashwell and Morell (16) have described a process in which many asialo-glycoproteins compete for hepatic uptake and subsequent catabolism and because an extensive literature exists (see Glass (17) for a review) that suggests, indirectly, that R-type vitamin B12-binding proteins might play some role in the delivery of vitamin B12 to the liver.

EXPERIMENTAL PROCEDURES

Materials

N-succinimidyl 3-(4-hydroxyphenyl)propionate was obtained from Pierce, Vibrio cholerae neuraminidase from Calbiochem, and fetuin and gelatin, type I, from Sigma. Other materials were obtained as described in the accompanying paper in this series (1) or from commercial sources.

Methods

Labeling of Proteins with [125I] and [131I]—N-succinimidyl 3-(4-hydroxyphenyl)propionate was labeled with [125I] and [131I] by a modification (13) of the method of Bolton and Hunter (18). Proteins were labeled with the iodinated ester as described previously (13). Vitamin B12-binding proteins were present saturated with low specific activity [15Co]vitamin B12 (0.03 pCi/μg) during the labeling procedure. The low specific activity [15Co]vitamin B12 was removed subsequently by dialysis against 7.5 M guanidine HCl and replaced with high specific activity [15Co]vitamin B12 (30 pCi/μg) as described elsewhere (13).

Experiments with Rabbits—Male rabbits (1.5 to 2.5 kg) were fasted for 16 hours before being used for experiments. The techniques utilized have been described elsewhere (13).

Removal of Sialic Acid with Neuraminidase—All incubations were performed at 22° for 72 hours in a toluene atmosphere at pH 6.5 in 0.025 M Na2HPO4, 0.006 M citric acid, and 0.002 M CaCl2. The concentration of Vibrio cholerae neuraminidase was 1.25 units/ml. Total sialic acid, i.e., bound and free, was assayed by the thiobarbituric acid method of Warren (19) after hydrolysis in 1 N HCl for 1 min at 100°. Free sialic acid was assayed in the same way except that the hydrolysis step was omitted.

Fetuin was incubated at a concentration of 50 mg/ml. Greater than 95% of the sialic acid was in the free form at the end of the incubation. Fetuin utilized for control experiments was incubated in the absence of neuraminidase. Fetuin utilized for control experiments was incubated in the absence of neuraminidase. Fetuin utilized for control experiments was incubated in the absence of neuraminidase. Fetuin utilized for control experiments was incubated in the absence of neuraminidase.

125I-Labeled transcobalamin I—[15Co]vitamin B12 was incubated at a concentration of bound [15Co]vitamin B12 of 4 μg/ml (approximately 0.2 mg of protein/ml). The sample was subsequently subjected to gel filtration on Sephadex G-150 as described above. The elution profiles of 125I and 15Co were indistinguishable from those observed with 125I-labeled transcobalamin I—[15Co]vitamin B12 incubated in the absence of neuraminidase, except that the apparent molecular weight of the treated sample (based on both 125I and 15Co) was 138,000 as compared with a value of 150,000 observed with both unincubated and control incubated preparations. Sufficient transcobalamin I was not available for direct measurements of free and bound sialic acid. It has been demonstrated previously (20) that the incubation conditions employed here do result in the release of greater than 90% of the sialic acid bound to the human milk and saliva R-type vitamin B12-binding proteins; less than 10% release was observed when these proteins were incubated in the absence of neuraminidase.

Other Methods—125I, 15Co, and 131I were assayed on a dual channel Packard Auto-Gramma counter as described elsewhere (13). All other methods were performed as described in the accompanying paper in this series (1).

RESULTS

Labeling of Proteins with 125I and 131I—When the [125I]-labeled human granulocyte vitamin B12-binding protein was saturated with [15Co]vitamin B12 and applied to a column of Sephadex G-150 the elution profile presented in Fig. 1A was obtained. Similar profiles were observed with corresponding preparations.
of transcobalamin I and transcobalamin III (data not presented). Greater than 96% of the 125I and 15Co eluted in an asymmetrical peak that centered on Fraction 28. Greater than 98% of the 125I and 15Co present in Fractions 25 to 29 were adsorbed by rabbit anti-human milk R-type vitamin B12-binding protein-Sepharose; less than 5% were adsorbed by rabbit anti-human vitamin B12-Sepharose or by vitamin B12-Sepharose. Fractions 25 to 29 were pooled and utilized for in vivo experiments. Similar fractions with identical adsorption characteristics were pooled for transcobalamin I and transcobalamin III. The respective molar ratios of the 125I to [15Co]vitamin B12 were 0.18, 0.21, and 0.20 for these preparations of double-labeled granulocyte vitamin B12-binding protein, transcobalamin I, and transcobalamin III. Rechromatography of portions of these preparations on Sephadex G-150 resulted in single symmetrical peaks of radioactivity that contained both the 125I and the [15Co]-vitamin B12 as shown in Fig. 1B for the granulocyte vitamin B12-binding protein. The apparent molecular weight of all three protein-vitamin B12 complexes, based on the 125I and [15Co]-vitamin B12, was 150,000 which is the same value that was observed previously (1) for all three of the noniodinated vitamin B12-binding protein, transcobalamin I, and transcobalamin II-Sepharose; less than 5% were adsorbed by rabbit anti-human milk R-type vitamin B12-binding protein-Sepharose. Neither isotope in this region was adsorbed by vitamin B12-Sepharose or rabbit anti-human transcobalamin II-Sepharose. When Fractions 20 to 29 were rechromatographed on Sephadex G-150, approximately 50% of the [15Co] vitamin B12 eluted as free vitamin B12. The nature of this phenomenon has not been established.

125I-Labeled preparations of apo-human granulocyte vitamin B12-binding protein, apo-transcobalamin I and apo-transcobalamin III eluted from Sephadex G-150 as single symmetrical peaks of radioactivity with apparent molecular weights of 150,000. Greater than 90% of the 125I, but only approximately 50% of the [15Co] vitamin B12, was adsorbed by rabbit anti-human R-type protein-Sepharose. Neither isotope in this region was adsorbed by vitamin B12-Sepharose, or rabbit anti-human transcobalamin II-Sepharose. When Fractions 20 to 29 were rechromatographed on Sephadex G-150, approximately 50% of the [15Co]-vitamin B12 eluted as free vitamin B12. The nature of this phenomenon has not been established.

Hepatic Uptake of 125I-Labeled Vitamin B12-binding Proteins Containing Bound [15Co]Vitamin B12—The data presented in Table I reveals that both moieties of the 125I-labeled granulocyte vitamin B12-binding protein-[15Co]vitamin B12 complex and the 125I-labeled transcobalamin I-[15Co]vitamin B12 complex were cleared rapidly (>80% by 5 min) from rabbit plasma by the liver. The hepatic uptakes of 125I and 15Co were markedly inhibited by the prior injection of desialized fetuin, but not by native fetuin. [15Co]Vitamin B12 bound to the unpurified vitamin B12-binding protein released from granulocytes by incubation with LiCl at 24° (1) was taken up by the liver in a manner (data not presented) that was indistinguishable from that of [15Co]vitamin B12 bound to the 125I-labeled granulocyte vitamin B12-binding protein.

Hepatic Release of 125I and 15Co Moieties of 125I-Labeled Vitamin B12-binding Protein-[15Co]Vitamin B12 Complexes—The data presented in Fig. 2A reveal that both moieties of the 125I-labeled granulocyte vitamin B12-binding protein-[15Co]vitamin B12 complex reappeared in the plasma after their initial uptake by the liver. The 125I moiety began to reappear 30 min after injection and was rapidly excreted in the urine. The peak plasma level and the period of maximal urinary excretion of the 125I both occurred between 45 and 75 min. The 15Co moiety did not begin to reappear in the plasma until 60 min after injection and did not reach its peak plasma level until 120 min after injection. The plasma level of 15Co declined slowly after 120 min; only negligible amounts were excreted in the urine. Similar results were observed with the 125I-labeled transcobalamin III-[15Co]vitamin B12 complex (Fig. 2B) and with the desialized 125I-labeled transcobalamin I-[15Co]vitamin B12 complex (Fig. 2C).

The data presented in Fig. 2A reveal that the prior injection of 92 mg of desialized fetuin markedly prolonged the plasma survival of the 125I-labeled granulocyte vitamin B12-binding protein-[15Co]vitamin B12 complex and markedly inhibited the urinary excretion of the 125I moiety. Similar, but less marked inhibition, was observed (Fig. 2B) when 73 mg of desialized fetuin were injected prior to the injection of the 125I-labeled transcobalamin III-[15Co]vitamin B12 complex. The data presented in Fig. 2C reveal that only approximately 10 to 15% of the 125I-labeled transcobalamin I-[15Co]vitamin B12 complex was missing from the plasma 5 min after injection and that approximately 17% of the 125I was excreted in the urine during the subsequent 180 min. The remaining material (85 to 90%) disappeared slowly at a rate equal to that of 125I-labeled bovine serum albumin. This similarity in the clearance rates of bovine serum albumin and transcobalamin I continued for at least 5 days (data not presented).

Properties of 125I-Labeled Material and [15Co]Vitamin B12 Released from Liver—An 80-min plasma sample was obtained from a rabbit that had been administered 125I-labeled granulocyte vitamin B12-binding protein-[15Co]vitamin B12 and the sample was subjected to gel filtration on Sephadex G-150. The elution profile obtained is presented in Fig. 1C. The elution profiles of 125I and [15Co]vitamin B12 both differed markedly from their preinjection profiles (Fig. 1B) and from each other. Less than 2% of either isotope eluted in the 150,000 apparent molecular weight position of intact 125I-labeled granulocyte vitamin B12-binding protein [15Co]vitamin B12. The 125I eluted in two major peaks with apparent molecular weights of 70,000 (30%) and less than 1,000 (70%). None of the 125I present in these positions was adsorbed by rabbit anti-R-type protein-Sepharose or vitamin B12-Sepharose. The nature of the 70,000 apparent molecular weight 125I-labeled material is unknown although the 125I appears to be covalently bound to some component, possibly rabbit albumin, since it is not dialyzable when it is dialyzed against 1% sodium dodecyl sulfate or 7.5 m guanidine HCl in the presence of 1% 2-mercaptoethanol. The
fact that similar material has been observed in experiments in which 125I-labeled rabbit and human transferrin were studied in rabbits (13) suggests that it is formed after the catabolism of a number of similarly 125I-labeled proteins. The nature of the small molecular weight (<1000) 125I is also unknown although free 125I and 125I-labeled N-succinimidyl 3-(4-hydroxyphenyl) propionate, either free or attached to one or a few amino acids, are likely possibilities. All of the 125I excreted in the urine in the experiments shown in Fig. 2 had a similar apparent molecular weight of less than 1000 (data not presented).

All of the 131I vitamin B12 present in the 80-min plasma sample (Fig. 1C) eluted from Sephadex G-150 with an apparent molecular weight of 40,000. This value is the same as the apparent molecular weight of rabbit transcobalamin II which accounts for >80% of the total, and >98% of the unsaturated, vitamin B12-binding protein present in rabbit plasma (13). The 131I vitamin B12 also resembled rabbit transcobalamin II-131I vitamin B12 in that it was precipitated by chicken anti-human transcobalamin II sera but was not precipitated by rabbit anti-human transcobalamin II sera or rabbit anti-human R-type protein sera.

Gel filtration results similar to those described above were also observed with a plasma sample obtained 180 min after the injection of 125I-labeled granulocyte vitamin B12-binding protein-125I vitamin B12 except that there was less 125I in the small molecular weight region. Indistinguishable results were observed with a plasma sample obtained 180 min after the injection of 125I-labeled transferrin III-125I vitamin B12.

In both experiments it was possible to calculate that less than 2% of the injected 125I and 131I vitamin B12 were still present in the plasma in their preinjection form. In the case of a plasma sample obtained 180 min after the injection of 125I-labeled transferrin I-125I vitamin B12, the elution profile revealed that 91% of the total 125I in the sample had an apparent molecular weight of 150,000, while 3% and 6%, respectively, were in the 70,000 and <1000 apparent molecular weight positions. In the case of 131I vitamin B12, 83% and 17% of the total 131I vitamin B12 eluted with apparent molecular weights of 150,000 and 40,000, respectively. The ratio of 131I to 131I vitamin B12 in the 150,000 apparent molecular weight region was not significantly different from the preinjection ratio and >95% of both isotopes in this region were specifically adsorbed by rabbit anti-human R-type protein-Sepharose. The 125I-labeled bovine serum albumin used in these experiments eluted from Sephadex G-150 in a symmetrical peak, with an apparent molecular weight of approximately 70,000, both before and 180 min after it was administered intravenously to rabbits.

### Table I

Effect of fetuin and desialyzed fetuin on hepatic uptake of 125I-labeled vitamin B12-binding proteins containing bound [131I]vitamin B12

| Experiment | Nonradioactive material injected 5 min prior to injection of radioactive material<sup>a</sup> | Radioactive material injected at time zero<sup>b</sup> | Distribution of radioactive material 5 min after its injection |
|------------|---------------------------------------------------------------------------------|-------------------------------------------------|---------------------------------------------------------------|
|            | 125I-labeled granulocyte vitamin B12-binding protein-131I vitamin B12, 125I-labeled bovine serum albumin | 131I-labeled bovine serum albumin                 | 125I 131I                                                   |
| 1          | None                                                                            | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 2          | Fetuin                                                                         | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 3          | Desialyzed fetuin                                                               | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 4          | None                                                                            | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 5          | Fetuin                                                                         | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 6          | Desialyzed fetuin                                                               | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 7          | None                                                                            | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 8          | Fetuin                                                                         | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |
| 9          | Desialyzed fetuin                                                               | 131I-labeled bovine serum albumin                | 131I-labeled bovine serum albumin                            |

<sup>a</sup>The amount of fetuin or desialyzed fetuin injected was 92 mg.

<sup>b</sup>The amount of I-labeled protein injected was approximately 75 ng. Vitamin B12-binding proteins were saturated with approximately 1.5 ng of vitamin B12.

<sup>c</sup>Per cent of total amount administered.

<sup>d</sup>Assumed to be 100% and used to calculate the plasma volume.
the liver at a faster rate than \(^{57}\text{Co}\) vitamin B\(_2\), and that the \(^{125}\text{I}\) reaches its peak concentration in the kidney at the same time as its maximal excretion in the urine in the form of small molecular weight (\(< 1000\)) fragments (see above). \(^{125}\text{I}\) does not accumulate in the heart or lung at any time period. \(^{57}\text{Co}\)-vitamin B\(_2\) accumulates in the kidney, heart, and lungs, but only after its release from the liver and its subsequent binding to rabbit transcobalamin II in the plasma (see above). This late tissue distribution of \(^{57}\text{Co}\) vitamin B\(_2\) is the same, with the exception of the intestine (see below), as that observed when rabbit transcobalamin II - \(^{57}\text{Co}\) vitamin B\(_2\) is injected intravenously into rabbits (13).

The time course of appearance and distribution of \(^{125}\text{I}\) and \(^{57}\text{Co}\) vitamin B\(_2\) in the small intestine is distinct from that of the other organs (see Fig. 3). Both moieties appear in equal amounts in the proximal small intestine between 5 and 30 min after injection and reach their maximal values by 60 min. At later time periods (120 to 180 min) \(^{57}\text{Co}\) vitamin B\(_2\) is present in excess of \(^{125}\text{I}\) and the bulk of both moieties are present in the distal small intestine, which is where vitamin B\(_2\) appears to be absorbed from the gastrointestinal tract in rabbits (22). The \(^{125}\text{I}\) and \(^{57}\text{Co}\) vitamin B\(_2\) enter the intestine via the bile, since when the common bile duct was cannulated in rabbits the level of both moieties in the intestine at 60 and 180 min fell by over 90%. The time course of appearance of \(^{125}\text{I}\) and \(^{57}\text{Co}\) vitamin B\(_2\) in the bile is shown in Fig. 4. When a pooled sample of bile collected from 0 to 180 min after injection was subjected to gel filtration on Sephadex G-150, greater than 90% of the \(^{125}\text{I}\) and \(^{57}\text{Co}\) vitamin B\(_2\) had a molecular weight of approximately 150,000 (data not presented). Greater than 90% of both moieties were selectively adsorbed by rabbit anti-human R-type protein-Sepharose.

Results indistinguishable from those described in the preceding paragraphs for the granulocyte protein were also obtained with \(^{125}\text{I}\)-labeled transcobalamin III - \(^{57}\text{Co}\) vitamin B\(_2\) (data not presented). The results of similar early time period experiments performed with \(^{125}\text{I}\)-labeled transcobalamin I - \(^{57}\text{Co}\) vitamin B\(_2\) were consistent with the prolonged plasma survival of this complex. After 4 days approximately 50% of the \(^{125}\text{I}\) had been excreted in the urine and the \(^{57}\text{Co}\) vitamin B\(_2\) had a tissue distribution similar to that observed 4 days after the injection of rabbit transcobalamin II - \(^{57}\text{Co}\) vitamin B\(_2\) (13). Experiments performed at intermediate time periods did not indicate a particular tissue site of uptake for transcobalamin I - vitamin B\(_2\).

**Plasma Survivals of Apo and Holo-R-type Proteins**—The presence of bound vitamin B\(_2\) had no effect on the hepatic uptake of the human granulocyte vitamin B\(_2\)-binding protein and transcobalamin III. The \(^{125}\text{I}\) label originally present on these apo-proteins was excreted slightly more rapidly in the urine than was the \(^{125}\text{I}\) label originally present on the holo-proteins. The significance of this difference has not been determined although it could reflect a difference in susceptibility to lysosomal proteases within the hepatocyte. Apo-transcobalamin I was cleared from plasma twice as fast as holo-transcobalamin I, although both forms were cleared much more slowly than their granulocyte vitamin B\(_2\)-binding protein and transcobalamin III counterparts. It should be noted that all of the differences observed between the apo- and holo-forms of these three proteins could be related to the fact that 30 to 40% of each apo-protein preparation appears to be present in a denatured state since it does not bind to vitamin B\(_{12}\)-Sepharose (see above).

**DISCUSSION**

Ashwell and Morell and their associates (23) have described and elucidated a mechanism by which a large number of asialoglycoproteins, including the asialo forms of orosomucoid, fetuin, ceruloplasmin, haptoglobin, \(\alpha-2\)-macroglobulin, thyroglobulin, chorionic gonadotropin, follicle-stimulating hormone, and luteinizing hormone are cleared from plasma and catabolized by the liver. (For a review see Ashwell and Morell (16).)
tein binding to common receptors that are present on hepa-

tocytes intact, presumably by pinocytosis, and are degraded by

membrane receptors, asialoglycoproteins appear to enter hepatocytes intact, presumably by pinocytosis, and are degraded by lysosomal enzymes over the ensuing 30 to 90 min. The biological function of this phenomenon has been difficult to elucidate since all of the proteins listed above appear to be present normally in plasma in their fully sialated forms. It has been postulated that sialic acid is slowly released from these proteins in vivo by neuraminidase but this has not been demonstrated.

The studies presented here demonstrate that transcobalamin I is included among those glycoproteins whose desialyzed forms are cleared rapidly from plasma by the mechanism of Ashwell and Morell. The studies concerning the granulocyte vitamin B₁₂-binding protein and transcobalamin III provide additional evidence for the concept that the latter protein is secreted by granulocytes in unaltered form (1, 4, 24). This appears to be the first example of a glycoprotein that is secreted from cells in a form such that the native protein is cleared rapidly from plasma by the mechanism of Ashwell and Morell. The observation that R-type proteins vary markedly in terms of their plasma survival suggests that this parameter may be important with respect to the etiology of the increased levels of plasma R-type protein that are observed frequently in chronic myelogenous leukemia (2, 3) and occasionally in hepatocellular carcinoma (25) and other solid tumors (26).

Approximately 80 to 90% of the [³⁵Co]vitamin B₁₂ that enters the hepatocyte bound to R-type protein has only a transient stay within this cell. During a several-hour period, that begins approximately 1 hour after uptake, 60 to 70% of the [³⁵Co]-vitamin B₁₂ re-enters the plasma where it is observed bound to rabbit transcobalamin II. This phenomenon remains to be elucidated. Approximately 10 to 20% of the [³⁵Co]vitamin B₁₂ taken up by the hepatocyte is rapidly excreted in the bile during a 1 hour period that begins approximately 10 min after uptake. In this case the R-type protein-vitamin B₁₂ complex appears to enter, transverse, and exit from the hepatocyte in intact form, although the mechanism by which this occurs also remains to be determined. It is not known whether other glycoproteins are excreted intact into the bile after being taken up by hepatocytes but if this is a general phenomenon it appears that an examination of the glycoprotein composition of bile could provide important information concerning the kinds and amounts of naturally occurring glycoprotein that are cleared from plasma in vivo by the mechanism of Ashwell and Morell (16).

The plasma survival data presented here appear relevant to what occurs in humans since significant species differences have not been observed in the process described by Ashwell and Morell (16). This view is supported by the fact that [³⁵Co]vitamin B₁₂ bound to transcobalamin I has a prolonged (t½ = 9 to 12 days) plasma survival in normal human subjects (12). Direct evidence that some human R-type proteins are cleared rapidly from human plasma by the liver is not available although several studies (27, 28) can be interpreted in a way that supports this possibility. The fact that human bile contains 3 to 9 µg of vitamin B₁₂ per day (29) and the fact that the unsaturated vitamin B₁₂-binding protein in human bile is an R-type protein (30) indicate that significant amounts of R-type protein-vitamin B₁₂ may be cleared from human plasma by the liver in vivo. Much if not all of the R-type protein could be derived from granulocytes since the granulocytes produce daily in man (31) contain enough R-type protein to bind 100 to 150 µg of vitamin B₁₂ (32).

The function of the human R-type proteins has been difficult to define although recent studies by Gilbert (33) suggest that the granulocyte vitamin B₁₂-binding protein may play an active role in the handling of intra- and extracellular bacteria by leaching vitamin B₁₂. A similar antibacterial function might also explain the presence of high levels of R-type vitamin B₁₂-binding protein in human secretions since R-type proteins might serve to bind vitamin B₁₂ and prevent its utilization by bacteria that require the vitamin for growth. A similar function has been well documented for the iron-binding protein lactoferrin (34, 35) which is present in granulocytes (36) and a number of secretions. The significance of our observation that the
granulocyte vitamin B₁₂-binding protein is cleared rapidly from plasma by the liver has not been established although it indicates the presence of a mechanism by which vitamin B₁₂ present in areas of cell necrosis and infection within the body can be delivered exclusively to the liver rather than to cells throughout the body as would occur if such vitamin B₁₂ were bound by transcobalamin II (13). This mechanism could be important if the liver controls the amount or coenzyme form of vitamin B₁₂ bound to transcobalamin II (13). This mechanism could be important if the liver controls the amount or coenzyme form of vitamin B₁₂ bound to transcobalamin II in plasma or if the liver contains a mechanism for distinguishing native vitamin B₁₂ from vitamin B₁₂ analogs which are synthesized by bacteria (37) and might be harmful to certain cells within the body. The latter mechanism is suggested by the fact that R-type proteins contain a mechanism for preferentially secreting them into the bile. The fact that intrinsic factor binds a much narrower range of vitamin B₁₂ analogs (38, 39) indicates that many such analogs would not be reabsorbed from the intestine. It is of interest, in regard to this type of protective mechanism, that one of the two brothers with congenital R-type protein deficiency has a poorly defined neurologic illness that is clinically similar to multiple sclerosis (15).

The existence of a granulocyte-mediated mechanism for the transport of vitamin B₁₂ exclusively to the liver suggests a possible biologic function for the process described by Ashwell and Morell and suggests that similar mechanisms may exist for iron and for other vitamins and metals. Transport of this kind could serve to regulate various metabolic systems within the liver as well as serve a scavenger function.

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