Isolation and Characterization of a Novel Vitamin B$_{12}$-Binding Protein Associated with Hepatocellular Carcinoma

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ABSTRACT High levels of a novel vitamin B$_{12}$-binding protein (hepatoma B$_{12}$ BP) have been observed recently in plasma obtained from three adolescent patients with hepatocellular carcinoma. This protein has now been isolated in homogeneous form from the plasma and pleural fluid of two of these patients by the use of affinity chromatography with vitamin B$_{12}$-Sepharose. The hepatoma B$_{12}$ BP belongs to the R-type group of B$_{12}$-binding proteins and is essentially indistinguishable from the recently isolated human milk and saliva R-type proteins in terms of: (a) immunologic properties based on immunodiffusion and immunoprecipitation assays; (b) amino acid composition; (c) molecular weight based on amino acid and carbohydrate content; and (d) absorption spectra. Both hepatoma B$_{12}$ BPs contain more sialic acid and less fucose than the milk and saliva B$_{12}$ BPs. All four proteins contain similar amounts of galactose, mannose, galactosamine, and glucosamine. Differences in sialic acid content appear to account for the differences in electrophoretic mobility that were observed among the four proteins. Differences in total carbohydrate content appear to account for the differences in apparent molecular weight that were observed with both gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Tumor tissue from one of the patients contained 10 times as much R-type protein as did normal liver tissue from the same patient. This suggests, although it does not prove, that synthesis by the tumor is the cause of the high levels of R-type protein found in the plasma of certain patients with hepatocellular carcinoma. Plasma survival studies performed with rabbits indicate that the hepatoma B$_{12}$ BP has a prolonged plasma survival and suggests that this parameter is also of importance.

INTRODUCTION

A recent report (1) described three adolescents who presented with hepatocellular carcinomas, normal leukocyte counts, extraordinary elevations of serum vitamin B$_{12}$ (B$_{12}$),$^{1}$ (15–53 ng/ml, normal 0.3–0.9 ng/ml), and serum unsaturated B$_{12}$-binding capacity, (5–480 ng/ml, normal 0.8–1.4 ng/ml). Studies (2) using sera from two of these patients indicate that the elevations of serum B$_{12}$ and unsaturated B$_{12}$-binding capacity are due to the presence of a B$_{12}$-binding protein (B$_{12}$ BP) that belongs to the R-type$^{2}$ class of immunologically related B$_{12}$ BPs that are normally present in a number of human tissues and body fluids (3). These studies also demonstrated that the hepatoma-related B$_{12}$ BP differs from the R-type B$_{12}$ BP found in increased

$^{1}$Abbreviations used in this paper: B$_{12}$, vitamin B$_{12}$; B$_{12}$ BP, B$_{12}$-binding protein.

$^{2}$This term was originally devised by Gråbeck to denote a vitamin B$_{12}$ BP in human gastric juice that was devoid of intrinsic factor activity. It was designated as protein "R" because of its rapid mobility on electrophoresis. Subsequently, immunologically related vitamin B$_{12}$ BPs were observed in a number of human tissues and body fluids and have been collectively referred to as R-type vitamin B$_{12}$ BPs. The function of the R-type proteins is unknown (3).
amounts in the sera of patients with chronic granulo-
cytic leukemia since the hepatoma B* BP appeared
more acidic during gel electrophoresis and ion-ex-
change chromatography and had a smaller apparent
molecular weight when studied by gel filtration.

To characterize further the nature of the hepatoma
B* BP we have now isolated this protein from the
pleural fluid of one patient and the plasma of a second
patient, and have compared their properties with those of
R-type B* BPs that have been isolated recently (4)
from normal human milk and saliva. Tumor levels of
R-type B* BP have also been determined in the case
of the second patient.

METHODS

Assay of B*- and B**-binding ability. Solutions of crys-
talline B* (Sigma Chemical Co., St. Louis, Mo.) dissolved
in H2O were assayed by measuring the absorption at 361
and 550 nm (5). Solutions containing [57Co]B* and [57Co]B**
(Amersham/Searle Corp., Arlington Heights, Ill.) were
assayed in a Packard gamma scintillation counter (Packard
Instrument Co., Inc., Downers Grove, Ill.). Assay of the
endogenous B* content of biologic fluids and tissue extracts
was performed by the isotope dilution technique of Lau
et al. (6). Unsaturated B*-binding ability was assayed by
a modification (7) of the charcoal adsorption technique of
Gotlieb et al. (8).

Assay of tissues for R-type B*- BP. Tissue samples
were obtained from patient M. M. 30 min after death and
were stored at −20°C. Control liver tissue was obtained
from a 67-yr-old female 30 min after she died from injuries
suffered in an automobile accident. Tissue samples were
thawed and homogenized (Polytron homogenizer, Brink-
mann Instruments, Inc., Westbury, N. Y.) at 4°C in 15
vol (vol/wt) of 0.01 M potassium phosphate pH 7.5, 0.15 M
NaCl. The homogenates were centrifuged at 10,000 g
for 30 min, and the supernates were assayed for B* content
and B*-binding ability. Aliquots were also assayed after
they had passed over immunoabsorbent columns (0.5 cm in
diameter and 2 cm in height) of rabbit antihuman milk
B*- BP-Sepharose, rabbit antihuman transcobalamin II-
Sepharose, and rabbit control serum-Sepharose. The amount
of R-type BP present in the homogenate supernates was
calculated by assaying the homogenate supernate for the
amount of B* and unsaturated B*-binding activity before
and after passage over the antihuman milk B*- BP-Sepha-
rose column. The difference in the values was taken as
the amount of R-type BP that had been bound to the column.

Preparations of immunoabsorbents. Rabbit antiserum
and rabbit control serum were precipitated with 30% (NH4)2
SO4, and the precipitates were dissolved in potassium
phosphate 0.1 M pH 7.5 and dialyzed at 4°C for 24 h
against 300 vol of this buffer with dialysate changes at 1 h
and at 15 h. These samples were then adjusted to protein
concentrations of 20 mg/ml and were coupled to cyanogen
bromide-activated Sepharose-4B (250 mg cyanogen bromide/ml Sepharose) at pH 7.0 using the method of Cuatre-
casas (9).

Amino acid and carbohydrate analyses. Solutions of
protein saturated with [57Co]B* in distilled H2O were
prepared and analyzed on a Beckman model 120C amino
acid analyzer as described previously (7). Tryptophan
was estimated by the method of Edelhoch (10). Values for
the moles of each amino acid per mole of B* were deter-
mined by assaying an aliquot of the hydrolysate applied to
the amino acid analyzer for radioactivity. Neutral hexoses
and amino sugars were assayed by gas-liquid chromatogra-
phy using a modification (7) of the method of Reinhold (11).
Sialic acid was assayed by the thiobarbiturate method
of Warren (12) after hydrolysis in 1 N HCl for 1 min
at 100°C.

Enzymatic removal of sialic acid. Incubations were
performed at pH 6.5 in 0.025 M Na2HPO4, 0.006 M citric
acid, and 0.002 M CaCl2. Incubation tubes contained bovine
serum albumin (Sigma Chemical Co.), 33 μg/ml; B* BP,
4 μg B*/ml; and Vibrio cholerae neuraminidase (Calbio-
chem, San Diego, Calif.), 12.5 U/ml. Incubations were per-
formed at 22°C for 72 h in a toluene atmosphere. The
amount of sialic acid liberated was determined by assaying
total sialic acid as described above and free sialic acid,
which was measured in the same way except that the hy-
drolysis step was omitted.

Quantitative immunoprecipitation of B*- BP's. Test tubes
contained the following components in a total volume of
0.3 ml: (a) 0.2 ml of rabbit serum consisting of varying
amounts of control and anti-B* BP sera, and (b) 0.1 ml
of 0.05 M potassium phosphate pH 7.5 in 0.75 M NaCl
that contained 500 pg of [57Co]B* bound to B*- BP. Incu-
bations were performed at 22°C for 30 min. The tubes were
then placed in an ice bath and 0.25 ml of cold, saturated
(NH4)2SO4 was added. After standing for an additional 30
min, the tubes were centrifuged at 10,000 g for 15 min
and 0.2 ml of the supernatant solution was removed and
assayed for [57Co]B*. Less than 1% of milk, saliva, or
hepatoma B* BP-B* is precipitated under these conditions
in the presence of 0.2 ml of rabbit control serum.

Purification of hepatoma B*-BP's. The starting mate-
rial from patient S. A. consisted of pleural fluid that was
aspirated approximately 15 min after the death of the
patient and stored at −20°C for 2 wk. The pleural fluid
was thawed and filtered by vacuum suction at room tem-
perature through a Buchner funnel containing a sheet of
S & S glass filter, grade 25, on top of a sheet of S & S filter
paper, grade 520-B (Schleicher & Schuell, Inc.,
Keene, N. H.). The filter was washed with 50 ml of 0.14 M
NaCl, and the 315 ml of recovered filtrate was applied at
room temperature to a column of B*-BP-Sepharose (0.9 cm
in diameter and 3.0 cm in height) containing 1.2 mg of
covalently bound B*. The sample was applied by gravity
at a flow rate of 200 ml/h and eluted with 100 ml of 0.14
M NaCl. The column was eluted subsequently at 4°C with:
(a) 250 ml of 0.1 M glycine-NaOH, pH 10, containing 1.0
M NaCl, (b) 100 ml of 0.1 M potassium phosphate pH 7.5,
and (c) 50 ml of 0.1 M potassium phosphate pH 7.5 con-
taining 5.0 M guanidine- HCl. The final eluting solution
consisted of 0.1 M potassium phosphate pH 7.5 containing
7.5 M guanidine HCl. When 4 ml of this solution had
passed through the column, flow was stopped. After 20 h
an additional 16 ml was collected and pooled with the ini-
tial 4 ml. The 7.5 M guanidine HCl eluate was dialyzed
for 24 h at 4°C against 1.0 liter of 0.01 M potassium
phosphate pH 7.5 containing 0.14 M NaCl with a dia-
sylate change at 12 h. The dialyzed sample was subjected to
repeat affinity chromatography on B*-Sepharose at 4°C. The
procedure was performed essentially as described above
except that the column size and the volumes of the eluting
solutions were reduced by 50%.

The starting material from patient M. M. consisted of
15 ml of pooled serum that had been collected over a 9-mo
period and stored at −20°C for 6-15 mo. The purification
procedure was essentially as described above for patient S. A. except for the following modifications: (a) the column of Bu-Sepharose was 0.9 cm in diameter and 0.75 cm in height; (b) before elution with 0.1 M potassium phosphate pH 7.5 the column was eluted with 40 ml of H2O followed by 50 ml of 0.5 M 3,3′-diaminodipropylamine-HCl, pH 7.5 containing 0.5 M galactose; and (c) repeat affinity chromatography on Bu-Sepharose was not performed.

Other methods. Preparation of Bu-Sepharose (0.4 mg Bu/ml Sepharose) (5), immunization of rabbits (4), adsorption of antisera (13), concentration of protein samples (7), absorption spectra (7), polyacrylamide disk gel electrophoresis (7), sodium dodecyl sulfate polyacrylamide gel electrophoresis (7) protein assays (7), and gel filtration on Sephadex G-150 (7) were performed as described previously. Human intrinsic factor (7), human plasma transcobalamin II (14), and the human milk (4), saliva (4), and granulocyte (15) Bu BPs were isolated as described previously. Plasma survival studies were performed with rabbits as described elsewhere (16).

RESULTS

Purification of hepatoma Bu BPs. The purifications of the hepatoma Bu-BPs from patients S. A. and M. M. are summarized in Table I. Both proteins were purified using affinity chromatography on Bu-Sepharose as the only purification technique. More than 95% of the unsaturated hepatoma Bu BP was adsorbed by Bu-Sepharose, and negligible amounts were eluted with 5.0 M guanidine-HCl and the other eluting solutions that were employed to remove contaminating protein before the elution of the hepatoma Bu BP. After elution in 7.5 M guanidine HCl, hepatoma Bu BP (M. M.) appeared homogenous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis while rechromatography on Bu-Sepharose was required to achieve homogeneity in the case of hepatoma Bu BP (S. A.).

Amino acid and carbohydrate analyses. The results of the amino acid analyses are presented in Table II and indicate that hepatoma Bu BP (S. A.) and the human milk and saliva Bu BPs do not differ greatly, if at all, in terms of their amino acid compositions. Amino acid analysis was not performed on hepatoma Bu BP (M. M.) because of insufficient material.

The results of the carbohydrate analyses are also presented in Table II and reveal that all four Bu BPs contain fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid. Differences do exist, however, both in total carbohydrate content and in the content of individual carbohydrates.

Gel filtration on Sephadex G-150. When the saliva, milk, and hepatoma Bu BPs were saturated with [57Co]Bu and studied by gel filtration on Sephadex G-150, single symmetrical peaks of radioactivity were observed for all proteins. Based on the peak elution positions for the individual proteins the following apparent molecular weights were calculated: saliva Bu BP, (142,000); hepatoma Bu BP (M. M.), (138,000); hepatoma Bu BP (S. A.), (136,000); and milk Bu BP, (128,000).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the saliva, milk, and hepatoma Bu BPs were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 1% 2-mercaptoethanol, single protein bands were observed in all four cases. Based on the mobilities of the individual protein bands the following apparent molecular weight were calculated: saliva Bu BP, (95,000); hepatoma Bu BP (M. M.), (93,000); hepatoma Bu BP (S. A.), (92,000); and milk Bu BP, (90,000).

The molecular weight values for R-type proteins obtained by gel filtration and sodium dodecyl sulfate gel electrophoresis appear falsely elevated when compared with values obtained by sedimentation equilibrium and amino acid carbohydrate analyses (4).

### Table I

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<tr>
<th>Item</th>
<th>Volume</th>
<th>Bu-binding ability</th>
<th>Protein</th>
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<td>ml</td>
<td>µg</td>
<td>mg</td>
<td>µg Bu bound/</td>
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<td></td>
<td></td>
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<td>mg protein</td>
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* Based on the content of [57Co]Bu.
TABLE II

Amino Acid and Carbohydrate Analyses

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<th>Item</th>
<th>Human saliva B_{12} BP</th>
<th>Human milk B_{12} BP</th>
<th>Hepatoma B_{12} BP</th>
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<td></td>
<td></td>
<td></td>
<td>M. M.</td>
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<td>Amino acid:</td>
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<td>Carbohydrate, %</td>
<td>39.9</td>
<td>34.5</td>
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</table>

* These values have been published previously (4).

Immunologic studies. Single precipitation lines with patterns of complete identity were observed with saliva B_{a} BP, milk B_{a} BP, granulocyte B_{a} BP, hepatoma B_{a} BP (M. M.), and hepatoma B_{a} BP (S. A.) when these preparations were subjected to immunodiffusion against rabbit antihepatoma B_{a} BP (S. A.) sera. Identical results were also obtained with rabbit antimilk B_{a} BP and antisaliva B_{a} BP sera. None of the three antisera gave precipitation lines with human intrinsic factor or human transcobalamin II.

Saliva B_{a} BP, milk B_{a} BP, hepatoma B_{a} BP (M. M.), and hepatoma B_{a} BP (S. A.) were precipitated in an equivalent manner when quantitative immunoprecipitation studies were performed with varying amounts of rabbit antihepatoma B_{a} BP (S. A.) serum as shown in Fig. 1. Detectable immunoprecipitation was observed with as little as 0.003 \mu l of antihepatoma B_{a} BP (S. A.) serum. When immunoprecipitation studies were performed with 25 \mu l of antihepatoma B_{a} BP (S. A.) serum that had been adsorbed with either saliva B_{a} BP or milk B_{a} BP, no detectable immunoprecipitation of saliva B_{a} BP, milk B_{a} BP, or hepatoma B_{a} BP (S. A.) was observed.

Absorption spectra. Absorption spectra of equal concentrations of the milk B_{a} BP-B_{a} complex, the hepatoma B_{a} BP (S. A.)-B_{a} complex, and free B_{a} are
presented in Fig. 2. When B₁₂ binds to either protein, the spectral maximum for B₁₂ shifts from 361 to 363 nm and the absolute absorbance at 361 nm increases by approximately 30%. The significance, if any, of the small differences between the spectra of the milk B₁₂ BP and the hepatoma B₁₂ BP (S. A.) is unknown. Previous studies (4) have demonstrated that the milk and saliva B₁₂ BPs have essentially identical spectra.

Polyacrylamide disk gel electrophoresis. Single major bands were observed for each of the four B₁₂ BPs when they were subjected to polyacrylamide disk gel electrophoresis and the gels were stained for protein as shown in Fig. 3 and when unstained gels were sectioned and assayed for [¹⁷Co]B₁₂ as shown in Fig. 4. Fig. 3 reveals that the two hepatoma B₁₂ BPs also gave

RABBIT ANTIEHPATOMA B₁₂ BP (SA) SERUM (µl)

Figure 1 Immunoprecipitation assays with rabbit antihepatoma B₁₂ BP (S. A.) serum and various R-type B₁₂ BP-[¹⁷Co]B₁₂ preparations. ⚫, Saliva B₁₂ BP-B₁₂; ○, milk B₁₂ BP-B₁₂; ▲, hepatoma B₁₂ BP (S. A.)-B₁₂; and Δ, hepatoma B₁₂ BP (M. M.)-B₁₂.

Figure 2 Absorption spectra of equal concentrations (8.4 µg B₁₂/ml) of hepatoma B₁₂ BP (S. A.)-B₁₂, milk B₁₂ BP-B₁₂, and free B₁₂. Spectra were obtained at 22°C in 0.005 M potassium phosphate, pH 7.5.

Figure 3 Polyacrylamide disk gel electrophoresis of the various R-type B₁₂ BP-B₁₂ preparations. Each sample contained 20 µg of protein. Paired gels A and B, C and D, and E and F were subjected to electrophoresis and stained at the same time. A, milk B₁₂ BP-B₁₂; B, saliva B₁₂ BP-B₁₂; C, hepatoma B₁₂ BP (S. A.)-B₁₂; D, milk B₁₂ BP-B₁₂; E, saliva B₁₂ BP-B₁₂; and F, hepatoma B₁₂ BP (M. M.)-B₁₂. The arrows indicate the positions of faint protein bands.

Figure 4 The effect of neuraminidase treatment on the electrophoretic mobility of the various R-type B₁₂ BPs. Protein samples were saturated with [¹⁷Co]B₁₂ and incubated for 72 h in the presence or absence of neuraminidase as described in Methods. Samples containing 20 ng of [¹⁷Co]B₁₂ were applied to individual polyacrylamide gels. Electrophoresis was continued until the tracking dye reached the end of the gels. The gels were sectioned into 1-mm slices and assayed for [¹⁷Co]B₁₂. ⚫, neuraminidase absent during incubation; ○, neuraminidase present during incubation. Sialic acid assays were performed on the milk and saliva B₁₂ BPs and revealed that greater than 90% of the sialic acid residues of these proteins were released during the incubation with neuraminidase. Less than 10% of the sialic acid residues were released in the absence of neuraminidase.
single minor protein bands with slower mobilities than their single major bands. These minor bands contained \([^{75}Co]Bi_2\) as shown in Fig. 4, which was due to R-type \(B_u\) BPs since, after elution from sectioned gels, more than 95% of the \([^{75}Co]Bi_2\) could be precipitated with rabbit antimilk \(B_u\) BP sera. Hepatoma \(B_u\) BP (M. M.) also contained a fast-moving minor protein band (see Fig. 3) that did not appear to contain \(B_u\).

Figs. 3 and 4 also reveal that the two hepatoma \(B_u\) BPs gave sharper major bands with greater mobilities than either the saliva or the milk \(B_u\) BPs. After treatment with neuraminidase (Fig. 4) the mobilities of all four proteins were reduced although the reductions were greater in the cases of the two hepatoma \(B_u\) BPs. After neuraminidase treatment, the diffuse nature of the saliva and milk \(B_u\) BP bands was still apparent, although reduced, and the mobility of the bulk of these two protein preparations was still somewhat less than that of the two hepatoma \(B_u\) BPs. These differences may be due to heterogeneity of total carbohydrate residues since molecular sieving does play a role in protein mobility during electrophoresis on 7.5% polyacrylamide gels (17).

**Tissue contents of R-type \(B_u\) BP.** The data presented in Table III demonstrate that metastatic tumor tissue from patient M. M. contained greater than 10 times more R-type \(B_u\) BP than did normal liver tissue from the same patient. R-type \(B_u\) BP could not be detected in liver tissue obtained from a normal subject. The low level of R-type \(B_u\) BP detected in the patient's uninvolved liver may have been due to contamination with plasma since at the time of death the patient's plasma contained 43 ng/ml of \(B_u\) and 423 ng/ml of unsaturated \(B_u\)-binding ability attributable to R-type protein. The failure to detect unsaturated \(B_u\)-binding ability in the patient's uninvolved liver, which should have been present if there was contamination with plasma, could be due to the fact that much of the \(B_u\) present in normal liver appears to be available for binding since it appears as free \(B_u\) when chromatographed on Sephadex G-150 (data not presented).

The unpurified R-type \(B_u\) BP from the tumor extract of patient M. M. was saturated with \([^{75}Co]Bi_2\), mixed with \([^{75}Co]Bi_2\) bound to purified hepatoma \(B_u\) BP (M. M.), and subjected to polyacrylamide disk gel electrophoresis. The gel profile is presented in Fig. 5 and reveals that the bulk of the \([^{75}Co]Bi_2\) migrated slightly slower than the \([^{75}Co]Bi_2\) and that approximately 15% of the \([^{75}Co]Bi_2\) had a slower mobility than that of the major component.

**Plasma survival and hepatic uptake of \([^{75}Co]Bi_2\) bound to R-type proteins.** The data presented in Table IV reveal that 70-80% of \([^{75}Co]Bi_2\) bound to the milk and saliva \(B_u\) BPs was present in the liver 10 min after

---

**Table III**

<table>
<thead>
<tr>
<th>Tissue*</th>
<th>Total Amount bound to R-type (B_u) BP</th>
<th>Total Amount attributable to R-type (B_u) BP</th>
<th>Total (B_u) and (B_u)-binding ability attributable to R-type (B_u) BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (M. M.)</td>
<td>26.9</td>
<td>375</td>
<td>395</td>
</tr>
<tr>
<td>Normal liver (M. M.)</td>
<td>68.7</td>
<td>373</td>
<td>36.9</td>
</tr>
<tr>
<td>Normal liver (control)</td>
<td>313</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Assays were performed on 10,000-g supernates of homogenates that were prepared as described under Methods.
† Determined as the amount adsorbed to rabbit antihuman milk \(B_u\) BP-Sepharose. No \(B_u\) or \(B_u\)-binding ability from any of the tissues was adsorbed by antihuman transcobalamin II or control-Sepharose.
§ Nanograms \(B_u\) or \(B_u\)-binding ability per gram of tissue wet weight.
Table IV
Hepatic Uptake of \(^{125}\text{I}\)-Bovine Albumin and \([^{57}\text{Co}]\)B\(_{12}\) Bound to Human R-Type B\(_{12}\) BPs 10 min after Their Simultaneous Intravenous Injection into Rabbits

<table>
<thead>
<tr>
<th>Purified R-type B(_{12}) BP</th>
<th>Plasma</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[^{57}\text{Co}]</td>
<td>[^{57}\text{Co}]</td>
</tr>
<tr>
<td>Milk B(_{12}) BP</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>Saliva B(_{12}) BP</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Hepatoma B(_{12}) BP (S. A.)</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Hepatoma B(_{12}) BP (M. M.)</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>Crude extract of tumor from patient M. M.</td>
<td>100</td>
<td>9</td>
</tr>
</tbody>
</table>

* Assumed to be 100% of the total injected and used to calculate the plasma volume.
‡ Percent of total administered. The amount of \(^{125}\text{I}\)-bovine albumin was approximately 100 ng. The amount of \([^{57}\text{Co}]\)B\(_{12}\) injected was approximately 2 ng.

The intravenous injection of these protein \([^{57}\text{Co}]\)B\(_{12}\) complexes into rabbits. Only 14% of the \([^{57}\text{Co}]\)B\(_{12}\) was present in the liver when it was injected bound to hepatoma B\(_{12}\) BP (M. M.). Intermediate amounts (34–35%) were observed in the liver when \([^{57}\text{Co}]\)B\(_{12}\) was injected bound to hepatoma B\(_{12}\) BP (S. A.) and to the R-type protein present in the crude extract of the tumor of patient M. M.

**DISCUSSION**

The human R-type proteins found in various tissues and body fluids give lines of complete identity with each other when studied by immunodiffusion, and yet these proteins often differ from one another in terms of their mobilities during electrophoresis, their elution positions during ion-exchange chromatography, and their apparent molecular weights based on gel filtration. Recent studies with R-type proteins isolated from human milk and saliva (4) indicate that these two proteins are indistinguishable in terms of their total amino acid compositions and in terms of the amino acid sequence of their first 13 amino-terminal residues. Both proteins were found to contain fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid, but the saliva R-type protein contained more fucose and galactose than the milk R-type protein and had a larger apparent molecular weight based on gel filtration and sodium dodecylsulfate gel electrophoresis. On the basis of these studies and the report by Carmel and Herbert (18) of two brothers with apparent congenital deficiencies of all R-type proteins, it has been postulated that all R-type proteins possess the same amino acid sequence in their polypeptide portions and that they differ only in their carbohydrate contents with differences in sialic acid being responsible for differences in behavior during electrophoresis and ion-exchange chromatography and differences in total carbohydrate content being responsible for differences in apparent molecular weight.

The studies reported here confirm the fact that the hepatoma B\(_{12}\) BPs are R-type proteins as well as support the hypothesis outlined above since (a) the two hepatoma B\(_{12}\) BPs are immunologically indistinguishable from the milk and saliva B\(_{12}\) BPs based on immunodiffusion and immunoprecipitation assays; (b) the amino acid composition of hepatoma B\(_{12}\) BP (S. A.) is indistinguishable from those of the milk and saliva B\(_{12}\) BPs; (c) the hepatoma B\(_{12}\) BPs contain more sialic acid and have greater anodic electrophoretic mobilities than the milk and saliva B\(_{12}\) BPs, and these differences in electrophoretic mobility are largely abolished by treatment with neuraminidase; and (d) values for total carbohydrate residues and apparent molecular weight determined for the hepatoma B\(_{12}\) BPs are both intermediate between the corresponding values obtained for the saliva and milk B\(_{12}\) BPs.

It is not possible to compare the properties of the hepatoma B\(_{12}\) BP with its normal counterpart since it has not been possible to demonstrate that liver from normal subjects contains an R-type B\(_{12}\) BP or that normal plasma contains a liver-derived R-type B\(_{12}\) BP. Normal plasma appears to contain two R-type B\(_{12}\) BPs that have been referred to as transcobalamin I and transcobalamin III (19, 20). Transcobalamin I binds weakly to DEAE, has \(\beta\)-mobility on serum electrophoresis, is essentially unsaturated with B\(_{12}\), and appears to be released in large part from granulocytes in vitro after blood is collected (20). Transcobalamin I binds tightly to DEAE, has \(\alpha\)-mobility on serum electrophoresis, is 70–100% saturated with B\(_{12}\), and does not appear to be released from granulocytes in vitro in significant amounts (21). Recent studies (22) have demonstrated that normal plasma transcobalamin I contains more sialic acid and less fucose than transcobalamin III and that transcobalamin I has a carbohydrate composition that is essentially identical to those of the two hepatoma B\(_{12}\) BPs. Transcobalamin I does differ from the hepatoma B\(_{12}\) BPs, however, in its spectral properties since transcobalamin I, like transcobalamin III and the granulocyte R-type B\(_{12}\) BP, has a spectral maximum at 361 nm (22) while the hepatoma B\(_{12}\) BPs, like the milk and saliva R-type B\(_{12}\) BPs, have their spectral maxima at 363 nm. The basis for these spectral differences is not known, but they could be due to differences in the position of carbohydrate side-chain attachment or to differences in carbohydrate sequence, neither of which would be detected by analyses of total carbohydrate composition.

The elevated plasma levels of R-type B$_2$ BP found in certain patients with hepatomas must result from either increased secretion of R-type B$_2$ BP into plasma or to decreased clearance of R-type protein from plasma. The observation that tumor tissue from patient M. M. contained 10 times more R-type protein than did normal liver tissue from this patient indicates, although it does not prove, that increased synthesis and secretion of R-type protein by the tumor itself is an important factor in the elevated plasma levels of R-type protein.

The plasma survival of R-type protein also appears to be an important factor in the plasma levels of these proteins, however, since recent studies (16) have demonstrated that human R-type proteins vary by three to four orders of magnitude in terms of their rate of clearance from rabbit plasma. In the case of $^{125}$I-labeled native normal granulocyte B$_2$ BP-[FeCo]B$_2$ and $^{125}$I-labeled native transcobalamin III-[FeCo]B$_2$ it has been shown that both radioactive moieties are cleared rapidly (t$\leq$5 min) from rabbit plasma by the liver in the process originally described by Ashwell and Morell (23) which is capable of clearing and catalyzing a variety of glycoproteins when they are present in their asialo-forms. In the case of native $^{125}$I-labeled transcobalamin I-[FeCo]B$_2$, both radioactive moieties have prolonged plasma survivals (t$\approx$10 days) and rapid hepatic clearance occurs only after the transcobalamin I-B$_2$ complex, which contains more sialic acid than the granulocyte protein and transcobalamin III, has been treated with neuraminidase to remove sialic acid. On the basis of these studies it has been postulated (24) that an R-type protein must have a prolonged plasma survival if it to raise the plasma R-type protein concentration to a marked degree. The observations that the majority of [FeCo]B$_2$ bound to hepatoma B$_2$ BP (M. M.) and to hepatoma B$_2$ BP (S. A.) is not cleared rapidly from rabbit plasma by the liver supports this hypothesis.

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REFERENCES


Isolation and Characterization of a Novel Vitamin B$_2$-Binding Protein

