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Research Article

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Isolation and Characterization

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A B S T R A C T 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 Gräsbeck 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 granulocytic leukemia since the hepatoma B_{12} BP appeared more acidic during gel electrophoresis and ion-exchange chromatography and had a smaller apparent molecular weight when studied by gel filtration.

To characterize further the nature of the hepatoma B_{12} 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_{12} BPs that have been isolated recently (4) from normal human milk and saliva. Tumor levels of R-type B_{12} BP have also been determined in the case of the second patient.

METHODS

Assay of B_{12} and B_{12}-binding ability. Solutions of crystalline B_{12} (Sigma Chemical Co., St. Louis. Mo.) dissolved in H_2O were assayed by measuring the absorption at 361 and 550 nm (5). Solutions containing [^{57}Co]B_{12} and [^{58}Co]-B_{12} (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_{12} content of biologic fluids and tissue extracts was performed by the isotope dilution technique of Lau et al (6). Unsaturated B_{12}-binding ability was assayed by a modification (7) of the charcoal adsorption technique of Gottlieb et al. (8).

Assay of tissues for R-type B_{12} 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, Brinkmann 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_{12} content and B_{12}-binding ability. Aliquots were also assayed after they had passed over immunoadsorbent columns (0.5 cm in diameter and 2 cm in height) of rabbit antihuman milk B_{12} 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_{12} and unsaturated B_{12}-binding activity before and after passage over the antihuman milk B_{12} BP-Sepharose column. The difference in the values was taken as the amount of R-type BP that had been bound to the column.

Preparations of immunoadsorbents. Rabbit antisera and rabbit control serum were precipitated with 30% $(NH_4)_2$SO$_4$, and the precipitates were dissolved in potassium phosphate 0.1 M pH 7.5 and dialyzed at 4°C for 24 h against 30 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 Cuatrecasas (9).

Amino acid and carbohydrate analyses. Solutions of protein saturated with [^{57}Co]B_{12} in distilled H_2O 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_{12} were determined 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 chromatography 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 Na_2HPO_4, 0.006 M citric acid, and 0.002 M $CaCl_2$. Incubation tubes contained bovine serum albumin (Sigma Chemical Co.), 33 μg/ml; B_{12} BP, 4 μg B_{12}/ml; and *Vibrio cholorae* neuraminidase (Calbiochem, San Diego, Calif.), 12.5 U/ml. Incubations were performed 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 hydrolysis step was omitted.

Quantitative immunoprecipitation of B_{12} BPs. 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_{12} 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 [^{57}Co]B_{12} bound to B_{12}-BP. Incubations were performed at 22°C for 30 min. The tubes were then placed in an ice bath and 0.25 ml of cold, saturated $(NH_4)_2SO_4$ 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 [^{57}Co]B_{12}. Less than 10% of milk, saliva, or hepatoma B_{12} BP-B_{12} is precipitated under these conditions in the presence of 0.2 ml of rabbit control serum.

Purification of hepatoma B_{12}-BPs. The starting material 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 temperature 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_{12}-Sepharose (0.9 cm in diameter and 3.0 cm in height) containing 1.2 mg of covalently bound B_{12}. 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 containing 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 initial 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 dialysate change at 12 h. The dialyzed sample was subjected to repeat affinity chromatography on B_{12}-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 B_{12}-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 H_2O 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 B_{12}-Sepharose was not performed.

Other methods. Preparation of B_{12}-Sepharose (0.4 mg B_{12}/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) B_{12} BPs were isolated as described previously. Plasma survival studies were performed with rabbits as described elsewhere (16).

RESULTS

Purification of hepatoma B_{12} BPs. The purifications of the hepatoma B_{12}-BPs from patients S. A. and M. M. are summarized in Table I. Both proteins were purified using affinity chromatography on B_{12}-Sepharose as the only purification technique. More than 95% of the unsaturated hepatoma B_{12} BP was adsorbed by B_{12}-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 B_{12} BP. After elution in 7.5 M guanidine HCl, hepatoma B_{12} BP (M. M.) appeared homogenous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis while rechromatography on B_{12}-Sepharose was required to achieve homogeneity in the case of hepatoma B_{12} BP (S. A.).

Amino acid and carbohydrate analyses. The results of the amino acid analyses are presented in Table II

and indicate that hepatoma B_{12} BP (S. A.) and the human milk and saliva B_{12} BPs do not differ greatly, if at all, in terms of their amino acid compositions. Amino acid analysis was not performed on hepatoma B_{12} BP (M. M.) because of insufficient material.

The results of the carbohydrate analyses are also presented in Table II and reveal that all four B_{12} 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 B_{12} BPs were saturated with [^{57}Co]B_{12} and studied by gel filtration on Sephadex G-150, single symmetrical peaks of radioactivity were observed for all four proteins. Based on the peak elution positions for the individual proteins the following apparent[3] molecular weights were calculated: saliva B_{12} BP, (142,-000); hepatoma B_{12} BP (M. M.), (138,000); hepatoma B_{12} BP (S. A.), (136,000); and milk B_{12} BP, (128,000).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the saliva, milk, and hepatoma B_{12} 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 B_{12} BP, (95,000); hepatoma B_{12} BP (M. M.), (93,000); hepatoma B_{12} BP (S. A.), (92,000); and milk B_{12} BP, (90,000).

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[3] 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
Summary of Purification of Hepatoma B_{12}-Binding Proteins

Item	Volume	B_{12}-binding ability	Protein	Sp act	Purification	Yield
	ml	*μg*	*mg*	*μg B_{12} bound/ mg protein*		*%*
Patient S. A.						
Pleural fluid	275	36.5	17,050	0.0021	1-fold	100
7.5 M guanidine eluate from second B_{12}-Sepharose column after dialysis with [^{57}Co]B_{12}	30.4	12.8*	0.62	20.5	9,760-fold	35.1
Patient M. M.						
Plasma	15.0	6.30	1,200	0.0052	1-fold	100
7.5 M guanidine eluate from B_{12}-Sepharose after dialysis with [^{57}Co]B_{12}	8.70	3.86*	0.19	20.3	3,900-fold	61.3

* Based on the content of [^{57}Co]B_{12}.

TABLE II
Amino Acid and Carbohydrate Analyses

Item	Human saliva B12 BP		Human milk B12 BP		Hepatoma B12 BP	
	First preparation*	Present preparation	First preparation*	Present preparation	S. A.	M. M.
	mol/mol B_{12}					
Amino acid:						
Lysine	19		19	18	18	
Histidine	3		3	4	3	
Arginine	8		8	8	7	
Aspartic	47		49	50	50	
Threonine	21		23	22	20	
Serine	30		33	30	29	
Glutamic	43		45	38	36	
Proline	9		9	10	11	
Glycine	24		25	27	26	
Alanine	21		23	22	21	
Valine	24		26	27	26	
Isoleucine	21		22	24	22	
Leucine	35		37	40	37	
Tyrosine	15		17	17	18	
Phenylalanine	12		13	13	14	
Methionine	8		8	9	8	
Half-cystine	8		8	8	7	
Tryptophan	8		7	8	10	
Total	356		375	375	363	
Mol wt	39,800		41,800	41,800	40,700	
Carbohydrate:						
Fucose	35	36	22	18	10	9
Galactose	47	42	36	38	44	37
Mannose	20	21	20	23	24	27
Galactosamine	4	6	3	2	2	5
Glucosamine	37	44	36	37	38	43
Sialic acid	7	7	6	5	16	19
Total	150	156	123	123	134	140
Mol wt	26,400	27,700	22,000	21,900	25,300	27,000
Total no. residues	506		498	496	497	
Total mol wt	66,200		63,800	63,700	66,000	
Carbohydrate, %	39.9		34.5	34.5	38.3	

* These values have been published previously (4).

Immunologic studies. Single precipitation lines with patterns of complete identity were observed with saliva B12 BP, milk B12 BP, granulocyte B12 BP, hepatoma B12 BP (M. M.), and hepatoma B12 BP (S. A.) when these preparations were subjected to immunodiffusion against rabbit antihepatoma B12 BP (S. A.) sera. Identical results were also obtained with rabbit anti-milk B12 BP and antisaliva B12 BP sera. None of the three antisera gave precipitation lines with human intrinsic factor or human transcobalamin II.

Saliva B12 BP, milk B12 BP, hepatoma B12 BP (M. M.), and hepatoma B12 BP (S. A.) were precipitated in an equivalent manner when quantitative immunoprecipi-

tation studies were performed with varying amounts of rabbit antihepatoma B12 BP (S. A.) serum as shown in Fig. 1. Detectable immunoprecipitation was observed with as little as 0.003 μl of antihepatoma B12 BP (S. A.) serum. When immunoprecipitation studies were performed with 25 μl of antihepatoma B12 BP (S. A.) serum that had been adsorbed with either saliva B12 BP or milk B12 BP, no detectable immunoprecipitation of saliva B12 BP, milk B12 BP, or hepatoma B12 BP (S. A.) was observed.

Absorption spectra. Absorption spectra of equal concentrations of the milk B12 BP-B12 complex, the hepatoma B12 BP (S. A.)-B12 complex, and free B12 are
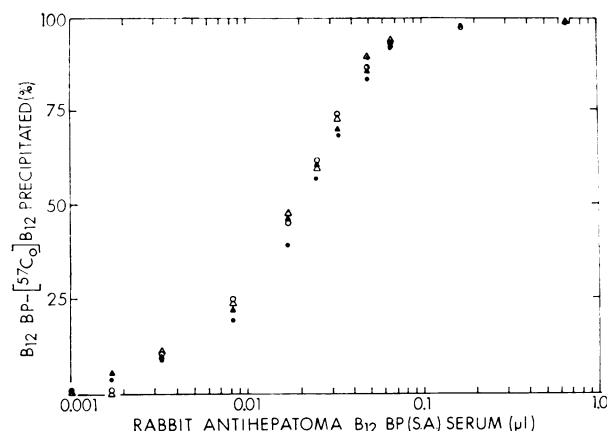
FIGURE 1 Immunoprecipitation assays with rabbit antihepatoma B_{12} BP (S. A.) serum and various R-type B_{12} BP-[^{57}Co]B_{12} preparations. ●, Saliva B_{12} BP-B_{12}; ○, milk B_{12} BP-B_{12}; ▲, hepatoma B_{12} BP (S. A.)-B_{12}; and △, hepatoma B_{12} BP (M. M.)-B_{12}.

FIGURE 3 Polyacrylamide disk gel electrophoresis of the various R-type B_{12} BP-B_{12} 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 B_{12} BP-B_{12}; B, saliva B_{12} BP-B_{12}; C, hepatoma B_{12} BP (S. A.)-B_{12}; D, milk B_{12} BP-B_{12}; E, saliva B_{12} BP-B_{12}; and F, hepatoma B_{12} BP (M. M.)-B_{12}. The arrows indicate the positions of faint protein bands.

presented in Fig. 2. When B_{12} binds to either protein, the spectral maximum for B_{12} 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 B_{12} BP and the hepatoma B_{12} BP (S. A.) is unknown. Previous studies (4) have demonstrated that the milk and saliva B_{12} BPs have essentially identical spectra.

Polyacrylamide disk gel electrophoresis. Single major bands were observed for each of the four B_{12} 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 [^{57}Co]B_{12} as shown in Fig. 4. Fig. 3 reveals that the two hepatoma B_{12} BPs also gave
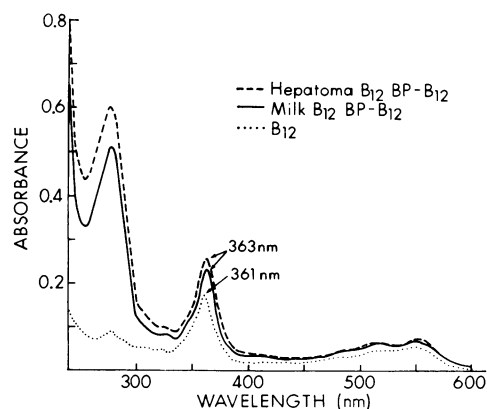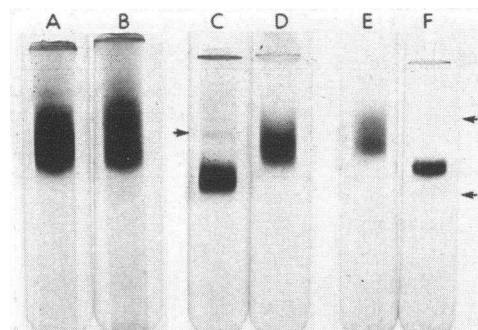

FIGURE 2 Absorption spectra of equal concentrations (8.4 μg B_{12}/ml) of hepatoma B_{12} BP (S. A.)-B_{12}, milk B_{12} BP-B_{12}, and free B_{12}. Spectra were obtained at 22°C in 0.005 M potassium phosphate, pH 7.5.
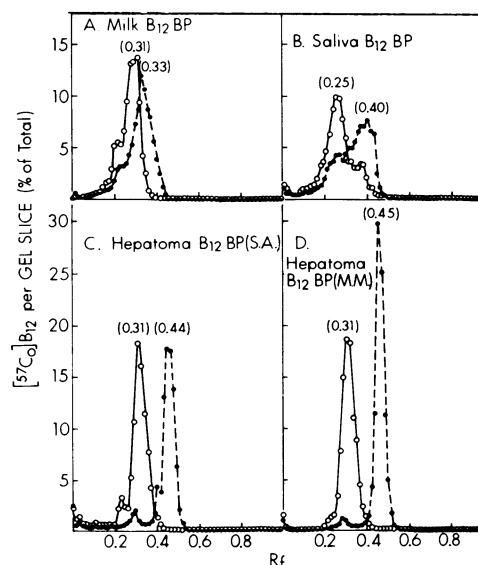

FIGURE 4 The effect of neuraminidase treatment on the electrophoretic mobility of the various R-type B_{12} BPs. Protein samples were saturated with [^{57}Co]B_{12} and incubated for 72 h in the presence or absence of neuraminidase as described in Methods. Samples containing 20 ng of [^{57}Co]B_{12} 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 [^{57}Co]B_{12}. ●, neuraminidase absent during incubation; ○, neuraminidase present during incubation. Sialic acid assays were performed on the milk and saliva B_{12} 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.

TABLE III
Tissue Levels of R-Type B_{12} BP

Tissue*	B_{12}		B_{12}-binding ability		Total B_{12} and B_{12}-binding ability attributable to R-type B_{12} BP
	Total	Amount bound to R-type B_{12} BP‡	Total	Amount attributable to R-type B_{12} BP‡	
			ng/g tissue§		
Tumor (M. M.)	26.9	22.3	375	373	395
Normal liver (M. M.)	68.7	36.9	0		36.9
Normal liver (control)	313	0	0		0

* 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_{12} BP-Sepharose. No B_{12} or B_{12}-binding ability from any of the tissues was adsorbed by antihuman transcobalamin II or control-Sepharose.

§ Nanograms B_{12} or B_{12}-binding ability per gram of tissue wet weight.

single minor protein bands with slower mobilities than their single major bands. These minor bands contained [^{57}Co]B_{12}, as shown in Fig. 4, which was due to R-type B_{12} BPs since, after elution from sectioned gels, more than 95% of the [^{57}Co]B_{12} could be precipitated with rabbit antimilk B_{12} BP sera. Hepatoma B_{12} BP (M. M.) also contained a fast-moving minor protein band (see Fig. 3) that did not appear to contain B_{12}.

Figs. 3 and 4 also reveal that the two hepatoma B_{12} BPs gave sharper major bands with greater mobilities than either the saliva or the milk B_{12} 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_{12} BPs. After neuraminidase treatment, the diffuse nature of the saliva and milk B_{12} 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_{12} 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_{12} 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_{12} BP than did normal liver tissue from the same patient. R-type B_{12} BP could not be detected in liver tissue obtained from a normal subject. The low level of R-type B_{12} 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_{12} and 423 ng/ml of unsaturated B_{12}-binding ability attributable to R-type protein. The failure to detect unsaturated B_{12}-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_{12} present in normal liver appears to be available for binding since it appears as free B_{12} when chromatographed on Sephadex G-150 (data not presented).

The unpurified R-type B_{12} BP from the tumor extract of patient M. M. was saturated with [^{58}Co]B_{12}, mixed with [^{57}Co]B_{12} bound to purified hepatoma B_{12} 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 [^{58}Co]B_{12} migrated slightly slower than the [^{57}Co]B_{12} and that approximately 15% of the [^{58}Co]B_{12} had a slower mobility than that of the major component.

Plasma survival and hepatic uptake of [^{57}Co]B_{12} bound to R-type proteins. The data presented in Table IV reveal that 70–80% of [^{57}Co]B_{12} bound to the milk and saliva B_{12} BPs was present in the liver 10 min after
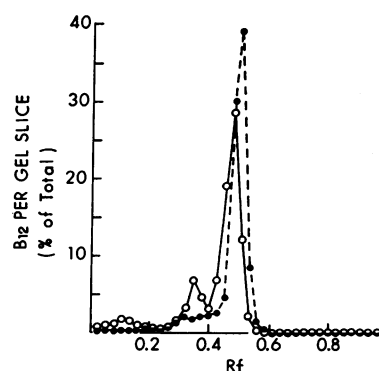

FIGURE 5 Simultaneous disk gel electrophoresis of [^{57}Co]-B_{12} bound to hepatoma B_{12} BP isolated from the plasma of patient M. M. and [^{58}Co]B_{12} bound to the unpurified hepatoma B_{12} BP in the tumor extract of patient M. M. The amount of B_{12} bound to each protein was approximately 2 ng. ●, [^{57}Co]B_{12}; ○, [^{58}Co]B_{12}.

TABLE IV

Hepatic Uptake of ^{125}I-Bovine Albumin and $[^{57}Co]B_{12}$ Bound to Human R-Type B_{12} BPs 10 min after Their Simultaneous Intravenous Injection into Rabbits

Purified R-type B_{12} BP injected	Plasma		Liver	
	^{125}I	^{57}Co	^{125}I	^{57}Co
	%*‡	%‡	%‡	%‡
Milk B_{12} BP	100	21	14	83
Saliva B_{12} BP	100	33	10	71
Hepatoma B_{12} BP (S. A.)	100	69	6	34
Hepatoma B_{12} BP (M. M.)	100	92	7	14
Crude extract of tumor from patient M. M.	100	71	9	35

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

the intravenous injection of these protein $[^{57}Co]B_{12}$ complexes into rabbits. Only 14% of the $[^{57}Co]B_{12}$ 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 $[^{57}Co]B_{12}$ 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 differ-

ences 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 III binds weakly to DEAE, has β-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 α-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 maximums 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_{12} BP found in certain patients with hepatomas must result from either increased secretion of R-type B_{12} 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_{12} BP-[^{57}Co]B_{12} and ^{125}I-labeled native transcobalamin III-[^{57}Co]B_{12} it has been shown that both radioactive moieties are cleared rapidly ($t\frac{1}{2} < 5$ min) from rabbit plasma by the liver by the process originally described by Ashwell and Morell (23) which is capable of clearing and catabolizing a variety of glycoproteins when they are present in their asialo-forms. In the case of native ^{125}I-labeled transcobalamin I-[^{57}Co]B_{12}, both radioactive moieties have prolonged plasma survivals ($t\frac{1}{2} \backsim 10$ days) and rapid hepatic clearance occurs only after the transcobalamin I-B_{12} 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 it to raise the plasma R-type protein concentration to a marked degree. The observations that the majority of [^{57}Co]B_{12} bound to hepatoma B_{12} BP (M. M.) and to hepatoma B_{12} BP (S. A.) is not cleared rapidly from rabbit plasma by the liver supports this hypothesis.

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