vegetation, and relatively low dissolved oxygen concentrations. Koste (1976) points out that *Trochosphaera* often appears with organisms which are indicators of the beta-saprobic zone such as *Microcystis* and *Melosira*. The diatom *Melosira* and the blue-green alga *Microcystis* were very abundant members of the phytoplankton at Reelfoot Lake on the date when collections of the rotifer were made. Koste (1976) further explained that *Trochosphaera* has been found at depths between 0 and 10 M, and repeatedly between 0 and 3 M, with water temperatures between 20-32°C, conductivity between 35-110 micrombos and a pH of 5.9-7.5. McCullough and Lee (1980) found statistically significant positive correlations between numbers of *T. solsititialis* and total iron, true color, CO_2, ammonia-nitrogen, BOD, and a negative correlation with pH. In their study, an average temperature of 16.8°C was recorded, along with an average dissolved oxygen concentration of 1.8 mg/L, the latter caused by large amounts of decaying leaf litter.

Trochosphaera solstitialis seems to be an organism of shallow, environmentally stressed bodies of water that experience rapid and dramatic variations in physical and chemical parameters, and that possess considerable amounts of decomposing vegetation. These physico-chemical characteristics, which prevail at Reelfoot Lake, either represent the optimum conditions for this species, or act adversely to exclude various predators or competitors that might ordinarily prevent the occurrence of this rotifer.

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The authors wish to express their appreciation to Dr. Wintfred L. Smith and the University of Tennessee at Martin for the use of their quarters on Reelfoot Lake. We would also like to thank the U.S. Fish and Wildlife Service, and especially Mr. Jim Wiggington who guided us through much of the National Wildlife Refuge and helped us in many ways during our stay at Reelfoot Lake.

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PURIFICATION AND CHARACTERIZATION OF THE B-PROTEIN-BINDING PROTEIN COMPLEX

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ABSTRACT

The B-Protein-binding protein complex was purified 78.7-fold from a reaction mixture by trichloroacetic acid precipitation followed by selective resolubilization and chromatography on DEAE-Sephadex, Wheat Germ Lectin-Sepharose, ConA-Sepharose and Affi-Gel Blue followed by preparative polyacrylamide gel electrophoresis. The B-Protein-binding protein complex is rather stable. It is not dissociated by 8M urea 6M quanidine-HCl or 2-merceptoethanol, but can be dissociated by 1% SDS. The sites of interaction on B-Protein (100,000 to 120,000 M.W.) and binding protein (10,000 to 15,000 M.W.) have not been elucidated, however, the SH-group of the 4'-phosphopantetheine prosthetic group of the binding protein is not the primary site of interaction with B-protein in the formation of the complex. Furthermore, the B-Protein-binding protein complex has an approximate molecular weight of 65,000 which is 53,000 less than the sum of the two reacting components. SDS-PAGE indicates that B-Protein is composed of two subunits of approximately 53,000 each. It would appear that formation of the B-Protein-binding protein complex involves the displacement of one subunit of B-Protein.

INTRODUCTION

A protein with unique properties, present in the serum

of individuals with cancer has been designated B-Protein (1,2). One method of detecting this protein is through its interaction with a specific radiolabeled protein which has been named binding protein (1-5). The binding protein, which contains tightly bound 4′-phosphopantetheine, is a substructure of the coenzyme A-synthesizing protein complex (CoA-SPC) of Bakers' yeast (6,7). In an assay mixture, radiolabeled binding protein interacts with B-Protein to form a B-Protein-binding protein complex. The amount of radiolabeled complex formed in this assay is used to determine if cancer is present in the serum donor. In this communication, the purification and properties of the B-Protein binding protein complex are reported.

Materials and Methods

Source of Materials [^{35}S]-L-Cysteine was purchased from Schwarz/Mann and ^{3}H G - CoA was purchased from New England Nuclear; other components used in this study were obtained from Sigma Chemical Company with the following exceptions: DEAE-Sephadex A-50, ConA-Sepharose 4B and Wheat Germ Lectin-Sepharose from Pharmacia Fine Chemicals; Affi-Gel Blue from Bio-Rad Laboratories.

Serum for Study Serum from sixty cancer patients, both male and female, are included in this study. The patients were in the age group of 30 to 70, all cancers were of intrinsic origin and had been diagnosed. The blood was collected in 10 ml silicone coated Vacutainer tubes. No anticoagulant was added. The clot that formed was removed, and the tube was centrifuged. Following centrifugation, the serum was decanted into a separate tube and assayed for B-Protein before pooling (1,2). The pooled serum was stored at -70°.

Preparation of the Coenzyme A-Synthesizing Protein Complex (CoA-SPC) The CoA-SPC was prepared from Bakers' yeast (Federal Yeasts Corp., Baltimore, MD) as described previously (6,7)

Determination of Protein Protein concentration was determined by absorbance at 280 nm, using an extinction coefficient of 10^3 ml g^{-1} cm^{-1}, and by the method of Bradford (8).

B-Protein Assay In the standard procedure developed for the B Protein Assay (1), a reaction mixture is prepared which contains 4.70 mM disodium ATP, pH 7.2; 0.5 ml buffer A (100mM Tris acetate; pH 7.2; 20 mM magnesium acetate; 50 mM KCl); 0.5 mM calcium D-pantothenic acid; 0.01 mM [^{35}S]-L-cysteine (approximately 20,000 cpm); 0.05 ml of the purified yeast extract containing the CoA-SPC, and water to a total volume of 1 ml. To this reaction mixture, 0.05 ml of serum is added and the total mixture is incubated at 36° for 2 h.

During the incubation step of the assay, a number of chemical reactions take place to form the [^{35}S]-binding protein, all of which are catalyzed by enzymatic activities of the CoA-SPC (3,6,7). These reactions are shown in Fig. 1. The binding protein radiolabeled with [^{35}S]-4′-phosphopantetheine interacts with B-Protein present in serum of individuals with cancer to yield a B-Protein-[^{35}S]-binding protein complex.

The reaction is terminated by heating the mixture in a water bath at 68° for 5 min. The reaction mixture is then cooled at 22° for 5 min. and centrifuged at approximately 1,000 X g for 5 min. Most of the yeast protein is removed as a precipitate. The supernatant liquid is decanted into another tube, and 2 ml of 10% TCA are added. The resulting protein precipitate, containing the B-Protein-[^{35}S]-binding protein complex, is recovered by filtration using a Millipore filtering apparatus with a vacuum of 1 to 4 mm Hg and a Whatman No. 3 MM paper disc. The protein precipitate collected on the disc is washed 4 times with approximately 2 ml of water per wash. The disc is then dried in an oven at 80°, and the radioactivity measured in a Nuclear Chicago, Mark II liquid scintillation counter.

1. D-PANTOTHENIC ACID + ATP ⟶ 5′-ADP-4′-PANTOTHENIC ACID + Pi
2. 5′-ADP-4′-PANTOTHENIC ACID + [^{35}S]-L-CYSTEINE ⟶ [^{35}S]-5′-ADP-4′-PANTOTHENYLCYSTEINE
3. [^{35}S]-5′-ADP-4′-PANTOTHENYLCYSTEINE ⟶ CO_2 + [^{35}S]-DEPHOSPHO-CoA
4. [^{35}S]-DEPHOSPHO-CoA + ATP ⟶ ADP + [^{35}S]-CoA
5. [^{35}S]-CoA + ATP[a] ⟶ 3′,5′-ADP + [^{35}S]-4′-PHOSPHOPANTETHEINE
6. [^{35}S]-4′-PHOSPHOPANTETHEINE + BINDING PROTEIN[b] ⟶ [^{35}S]-BINDING PROTEIN

FIG. 1. *Sequence of reactions involved in the formation of radiolabeled binding protein. [a]The requirement for ATP in reaction 5 is not understood. [b]Binding protein in reaction 6 is a substructure of the coenzyme A-synthesizing protein complex (6,7).*

An alternate procedure for the B-Protein Assay utilizes [^{3}H-G]-CoA as the substrate in reactions 5 and 6 of Fig. 1 with tritium as the radiolabel instead of ^{35}S. In the presence of ATP the [^{3}H-G]-CoA is hydrolyzed by the CoA-SPC to form [^{3}H]-3′,5′-ADP and [^{3}H]-4′-phosphopantetheine(4). The incubation time is reduced from 2 h to 30 min because CoA does not need to be synthesized, and only one of the catalytic activities of the CoA-SPC is required. Both methods have been used for the preparation of radiolabeled B-Protein-binding protein complex.

Polyacrylamide-Gel Electrophoresis-(PAGE) The PAGE was carried out at 4° on 5 X 90 mm gels, 7% gel concentration, at 2 mA per gel for 3 h in 0.18 M Tris, 0.09 M boric acid, 2.5 mM Na_2EDTA pH 8.1 according to the method of Davis (9). SDS-PAGE was run using the method of Weber and Osborn (10). Gels were stained and fixed in 0.04% Serva Blue W (Serva Fine Chemicals, New York, NY) in 20% TCA for 40 min. Gels were destained in 7% acetic acid overnight.

Duplicate gels were assayed for protein content and radioactivity. Stained gels were scanned with a densitometer and the results were expressed by plots of absorbance of light at 573 nm wavelength versus millimeters of gel length measured from the top of the gel. The duplicate gels were sliced into 2 millimeter thick sections with a fixed blade gel slicer. Each gel slice was placed in a scintillation vial with 15 ml of a scintillation cocktail prepared from 6.0 g of PPO (2,5-diphenyloxazole), 1 liter toluene, 20 ml Hyamine Hydroxide (New England Nuclear) and 10 ml of NCS tissue solubilizer (Amersham Corp.) (11). The gel slices were extracted 18 h before counting. Results were expressed as c.p.m. versus millimeter of gel length.

Gel Filtration Estimation of Molecular Weight Gel filtration chromatography was carried out using a column of Sephacryl S-200 (Pharmacia Fine Chemicals), 5 X 91 cm, maintained at 4°. The column was equilibrated with buffer A at a flow rate of 55 ml per hour using a Milton Roy minipump. Samples were eluted with buffer A, the effluent was monitored at 280 nm in a flow-through quartz cuvette, and the absorbance pattern was recorded. The molecular weights of B-Protein and the B-Protein-binding protein complex were estimated using this column and calibration standards of known molecular weight (12). The standards used were: chymotrypsinogen A (molecular weight 25,000) ovalbumin (molecular weight 45,000), human serum albumin (molecular weight 67,000), and gamma globulin (molecular weight 150,000).

DEAE Sephadex A-50 Approximately 7 g of DEAE-Sephadex A-50, particle size 40-140 microns was hydrated and degassed under a vacuum at 5° in 500 ml of buffer B, containing 0.1 M Tris-acetate, pH 7.0, 0.02 M magnesium acetate. Final column dimensions (2.5 X 30 cm) were achieved by pumping approximately two bed volumes of starting buffer through the column. Following dialysis against starting buffer the sample was pumped onto the column. The column effluent was monitored at 280 nm using a flow-through quartz cuvette, 1 cm path length, a Beckman Acta II dual beam spectrophotometer, and a Heath recorder. The unbound material was collected as a single fraction at 4°. The bound material was eluted from the column using buffer C (containing 0.1 M Tris-acetate, pH 7.0, 0.02 M magnesium acetate 0.5 M NaCl). The absorbance of the effluent was monitored at 280 nm as described above and collected in 8 ml fractions in a refrigerated (4°) fraction collector.

Wheat Germ Lectin-Sepharose Affinity chromatography on Wheat Germ Lectin-Sepharose was performed at 4° with a column 0.8 X 9 cm. First, 5 column volumes of the starting buffer which contained 0.15 M NaCl, 0.05 M NaH_2PO_4, 0.02% NaN_3, pH 7.2 were passed through the gel matrix. The sample was then applied to the column and allowed to penetrate the gel. The unbound material was washed through the column by four bed volumes of starting buffer and collected as a single fraction. The flow rate was approximately 5 ml/h. The bound material was eluted from the column with four bed volumes of 0.45 M N-acetyl-D-glucosamine in starting buffer. The effluent was collected as a single fraction at a flow rate of 5 ml/h.

ConA-Sepharose 4B Lectin affinity chromatography on ConA-Sepharose 4B, was performed at 4° using a column (1 X 10 cm) equilibrated with four volumes of starting buffer (0.01 M Tris-HCl, pH 7.0, 0.50 M NaCl, 0.001 M $MgCl_2$, 0.001 M $CaCl_2$ and 0.02% NaN_3). The sample was applied to the column and the flow rate was adjusted to approximately 20 ml/h. After the sample had penetrated the gel, four bed volumes of starting buffer were used to elute the unbound material which was collected as a single fraction. Four bed volumes of 0.25 M 1-0-methyl-α-D-Glucopyranoside in starting buffer, were used to elute the bound proteins. Again the effluent was collected as a single fraction.

Affi-Gel Blue Approximately 20 ml of resuspended Affi-Gel Blue, Cibacron Blue 3-A bound to agarose beads, were used to prepare the 1 X 18 cm column. The column, maintained at 4°, was packed using a flow rate of 25 ml/h and approximately 200 ml of starting buffer containing 0.03 M NaH_2PO_4, pH 7.0, and 0.02% NaN_3. At least four volumes of starting buffer were used to equilibrate the column before the sample was applied. After the sample had penetrated the Affi-Gel Blue, the unbound material was washed from the column in four bed volumes of starting buffer. The unbound effluent was collected as a single fraction. The bound material was eluted from the column as a single fraction with four bed volumes of 1.0 M NaSCN, 0.50 M NaCl, in starting buffer.

RESULTS AND DISCUSSION

The radiolabeled B-Protein-binding protein complex used in this study was prepared according to the standard B-Protein Assay procedure described under "Materials and Methods". Following the four 2 ml washes which resolubilized other proteins which interact with the [^{35}S]-binding protein and removes the TCA, the vacuum was interrupted and collection tubes were placed in the vacuum chamber. The vacuum was resumed and the filters were washed an additional 4 times, using 2 ml of water per wash. As shown in Fig. 2 and Table I, once all of the TCA was removed by the previous washes, the B-Protein-binding protein complex was resolubilized and collected in the resulting filtrate. This step in the purification procedure, although cumbersome, was necessary to separate the B-Protein-binding protein complex from most of the other serum proteins which interact with the [^{35}S]-binding protein. Consequently, the B-Protein-binding protein complex can be recovered in a partially purified form by the selective resolubilization procedure. As shown in Table II, the filtration step resulted in a 25.5-fold purification of the complex.

DEAE-Sephadex column chromatography was used to concentrate the complex. The B-Protein-binding protein complex and a high percentage of other proteins present in

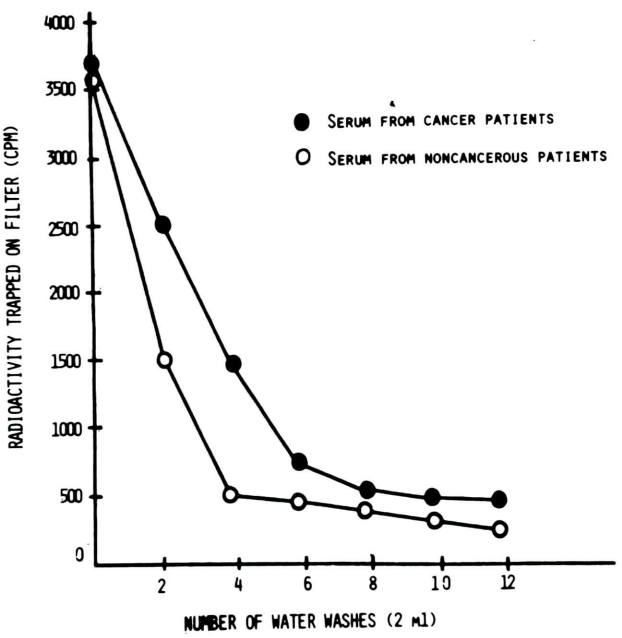

FIG. 2. *Profile of the B-Protein reaction mixture retained on the filter during filtration.*

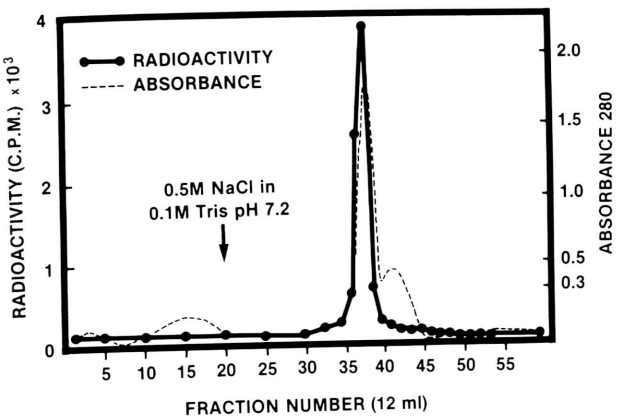

FIG. 3. *DEAE-Sephadex chromatography of the B-Protein-binding protein complex.*

the filtrate bound to the column. As shown in Fig. 3 the radiolabeled complex and most other proteins were eluted with buffer C (0.5 M NaCl in 0.1 M Tris, pH 7 2). In addition to reducing the volume, this step resulted in an 11-fold further purification of the B-Protein-binding protein complex.

The radiolabeled complex was purified further by chromatography on Wheat Germ Lectin-Sepharose followed by chromatography on ConA-Sepharose 4B. The complex did not bind to either Wheat Germ Lectin or ConA but certain difficult to remove contaminating proteins did. Furthermore, any B-Protein which failed to form a complex with binding protein was removed by the Wheat Germ Lectin-Sepharose. Polyacrylamide gel electrophoretic patterns of the unbound fractions from Wheat Germ Lectin-Sepharose and ConA-Sepharose are shown in Fig. 4 and 5, respectively. At this stage of the purification procedure the radiolabeled complex had been purified 52-fold (see Table II).

The unbound material eluted from the Wheat Germ Lectin-Sepharose and the ConA-Sepharose columns, which contained the radiolabeled B-Protein-binding protein complex and contaminating proteins, including albumin, was chromatographed on a column of Affi-Gel Blue. The albumin and certain other contaminating proteins bound to the Affi-Gel Blue, whereas the radiolabeled B-Protein-binding protein complex did not bind. As shown in Table II, at this stage in the purification the B-Protein binding protein complex was purified 71-fold. The radiolabeled B-Protein-binding protein complex was further purified by electrophoresis on a large scale preparative polyacrylamide gel electrophoresis apparatus (designed in our laboratory) to remove remaining amounts of contaminating proteins. The radiolabeled complex was collected in a concentrated form and was calculated to be purified 78.7-fold. Both by radioactivity measurements and by protein staining with Serva Blue W, the complex appeared as a single protein band after analytical polyacrylamide gel electrophoresis (Fig. 6).

Properties of the B-Protein-Binding Protein Complex and of its Components Certain physical and chemical properties of the binding protein, B-Protein and the B-Protein-binding protein complex have been determined. The binding ratio between purified binding protein and partially purified B-Protein appears to be 1.0 (5). On this basis, the serum concentration of B-Protein in some patients with advanced cancer, has been estimated to be as

TABLE 1. *Typical Filtration Pattern of B-Protein Reaction Mixture*

Materials Processed	Conditions Following	Reaction Mixture		In Filtrate		On Filter	
		Protein (mg)	Radioactivity (cpm)	Protein (mg)	Radioactivity (cpm)	Protein (mg)	Radioactivity (cpm)
1. Normal Serum Reaction Mixture	Incubation	11.33	6,000	—	—	—	—
2. Cancer Serum Reaction Mixture	Incubation	11.70	6,000	—	—	—	—
3. Normal Serum Reaction Mixture	TCA Step	—	—	0.52	242	—	—
4. Cancer Serum Reaction Mixture	TCA Step	—	—	0.62	186	—	—
5. Normal Serum Retained on Filter	4 H_2O Washes	—	—	2.81	4,560	—	—
6. Cancer Serum Retained on Filter	4 H_2O Washes	—	—	1.78	2,226	—	—
7. Normal Serum Retained on Filter	8 H_2O Washes	—	—	0.48	689	—	—
8. Cancer Serum Retained on Filter	8 H_2O Washes	—	—	1.05	2,977	—	—
9. Normal Serum Retained on Filter	TCA-Step & 8 H_2O Washes	—	—	—	—	7.52	343 (estimate)
10. Cancer Serum Retained on Filter	TCA-Step & 8 H_2O Washes	—	—	—	—	8.35	494 (estimate)

TABLE 2. *Purification of the B-Protein-Binding Protein Complex*

Condition	Total Volume (ml)	Total Protein (mg)	Total Activity (cpm)	Specific Activity (cpm/mg protein)	Yield (%)	Purification (-fold)
Crude Reaction mixture (dialyzed)[a]	38	2977.6	591,006	198	100.00	1.0
Heat denaturation (68°)[b]	34	263.0	583,406	2,218	98.71	11.2
TCA-Precipitation-Selective-Resolubilization[c]	270	23.8	120.586	5,067	20.40	25.5
DEAE-Sephadex	24	7.1	51,240	7,216	8.67	36.4
WGL-Sepharose (unbound)[d]	35	5.4	50,825	9,412	8.60	47.4
ConA-Sepharose (unbound)[e]	70	4.9	50,700	10,347	8.58	52.1
Affi-Gel Blue (unbound)	10	3.2	45,117	14,099	7.63	71.0
Preparative PAGE	1	2.6	40,605	15,617	6.87	78.7

[a]Contained both serum and CoA-SPC of Bakers' yeast. Bound radioactivity was measured after dialysis.
[b]Small amount of the yeast protein and essentially all of the serum protein remained in solution after the 68° heat step.
[c]The mixture was filtered, and the filtrate from washes 5 through 8 contained the resolubilized complex.
[d]WGL-Sepharose removed unreacted B-Protein and small quantities of other contaminating proteins.
[e]ConA Sepharose, although not a very efficient step, removed small quantities of other contaminating proteins which were not removed by the other steps.

high as 6 mg/100 ml of serum.

B-Protein which contains 7% by weight carbohydrate according to the method of Dubois *et al.* (13) binds to Wheat Germ Lectin-Sepharose (5,14) but the B-Protein-binding protein complex does not. Consequently, it would appear that B-Protein may contain N-acetyl-glucosamine residues which are either lost or masked in some manner, when the B-Protein-binding protein complex is formed. Neither B-Protein nor the B-Protein-binding protein complex binds to ConA-Sepharose or Affi-Gel Blue. Each of these procedures, however, provide a method for removing contaminating proteins.

Binding protein has a high affinity for both B-Protein and CoA-SPC. The affinity between binding protein and B-Protein appears to be higher than the affinity between binding protein and CoA-SPC. Nevertheless the high affinity between the binding protein and CoA-SPC thus far has prevented the recovery of large quantities of radiolabeled binding protein in purified form. Small quantities, however, have been purified. If the purified radiolabeled binding protein is used in place of CoA-SPC in the B-Protein Assay the incubation time can be reduced to only 5 min (14). The interaction between binding protein and B-Protein does not require ATP. It has also been shown that

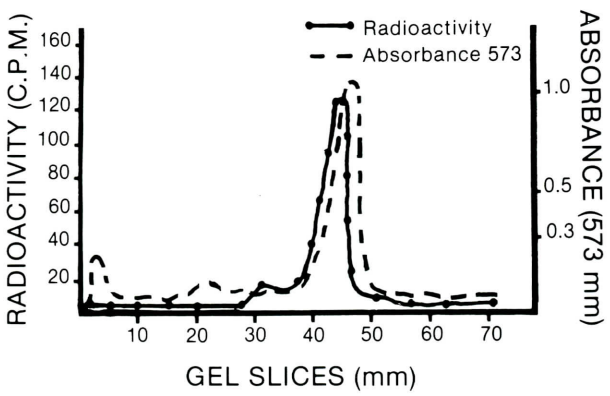

FIG. 4. *PAGE profile of WGL-Sepharose unbound fraction containing the B-Protein-binding protein complex. Shown are the elution profiles of the radiolabeled complex and protein concentration pattern of the unbound fraction.*

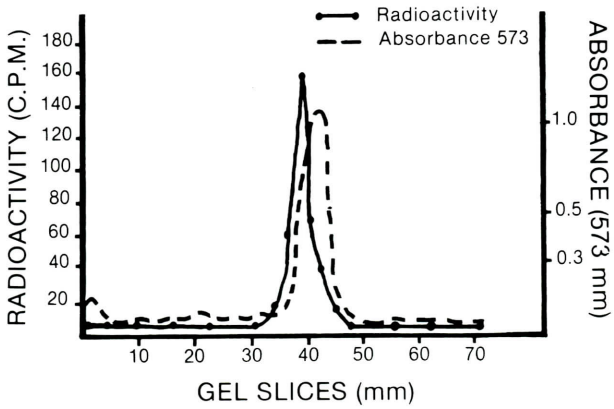

FIG. 5. *PAGE profile of Con A-Sepharose unbound fraction containing the B-Protein-binding protein complex. Shown are the elution profiles of the radiolabeled complex and protein concentration pattern of the unbound fraction.*

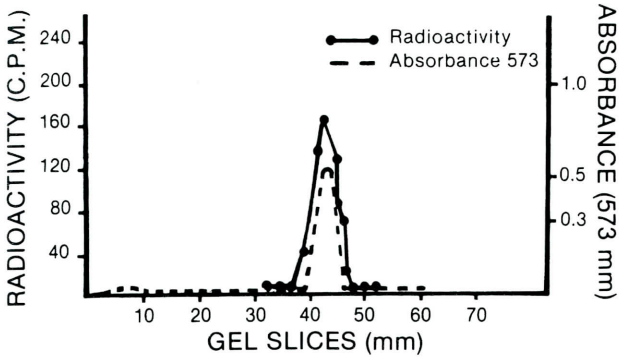

FIG. 6. *PAGE profile of Affi-Gel Blue unbound fraction containing the B-Protein-binding protein complex. Shown are the elution profiles of the radiolabeled complex and protein concentration pattern of the unbound fraction.*

acyl carrier protein of *E. coli,* which has properties similar to the binding protein, can replace binding protein in the B-Protein Assay (14).

The experiment with purified radiolabeled binding protein and radiolabeled acyl carrier protein (ACP) provide some important information which limits the number of possibilities for the mechanism involved in the B-Protein assay. A remote, but possible, explanation for the difference between the amount of labeled protein trapped on the filter when normal serum is used in the assay as compared to cancer serum might be a less efficient labeling of binding protein in the presence of normal serum. One could imagine some component of normal serum interfering with CoA-SPC labeling its binding protein and this interference being absent for cancer serum. The experiments using purified radiolabeled binding protein and purified ACP rule out this possibility because the differences between normal and cancer serum remain upon direct addition of labeled binding protein or ACP.

The B-Protein binding protein complex is not dissociated by treatment with 8 M urea, 6 M quanidine-HCl or 1% 2-mercaptoethanol, however, it is dissociated by dialysis against 1% SDS (3). It is not yet clear whether SDS destabilizes the interaction between B-Protein and binding protein or dissociates the substructure of B-Protein without affecting the site(s) of interaction.

The 4′-phosphopantetheine group of binding protein (6,7) is attached through its 4′-phosphopantetheine acid moiety, not the cysteamine moiety of 4′-phosphopantetheine (see Fig. 1 reaction 2). This was demonstrated through experiments in which the substrate [^{35}S]-L-cysteine was omitted from the reaction mixture and [^{14}C]-D-pantothenic acid was used as the radiolabeled marker resulting in binding protein which contained [^{14}C]-4′-phosphopantethenic acid in place of [^{35}S]-4′-phosphopantetheine. The interaction between B-Protein and the binding protein was not affected by the omission of cysteine (15). Not only do the results of this study indicate that the SH-group of cysteine is not the attachment site of 4′-phosphopantetheine to the binding protein, but it shows that the SH-group is not the principal site of interaction in the formation of the B-Protein-binding protein complex.

Although the 4′-phosphopantetheine moiety was stable to acid treatment, it was released from the binding protein by adjusting the pH to 12.0 with 1N KOH and then heating the mixture at 70° for 1 h. The acid stability and alkaline lability indicate that 4′-phosphopantetheine may be attached to the binding protein through a phosphodiester linkage similar to the attachment of the 4′-phosphopantetheine prosthetic group of acyl carrier protein of *E. coli* (15,16).

The patterns for gel filtration on Sephacryl S-200 of B-Protein and the B-Protein-binding protein complex were compared. As shown in Fig. 7, the elution volumes of the B-Protein-radiolabeled binding protein complex and B-Protein-radiolabeled ACP complex are greater than the elution volume of B-Protein. This pattern indicated that the complex had a lower moleculear weight than B-Protein. It was expected that both complexes would have a larger molecular weight than B-Protein. This discrepancy led to a series of experiments in which B-Protein Assay reaction mixtures, stopped after three different steps in the assay procedure, were added to human serum and chromatographed on Sephacryl S-200. The results of this study are depicted in Fig. 7. As shown in Fig. 7, at each step the B-Protein-binding protein complex had the same elution volume which corresponded to a lower molecular weight than B-Protein except in the case where B-Protein was incubated for one hour at 36° in the absence of binding

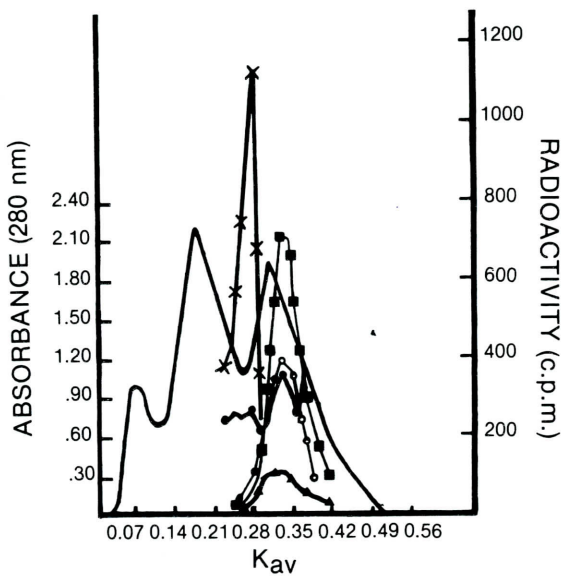

FIG. 7. *Sephacryl S-200 fractionation demonstrating a difference in the elution volume between B-Protein and the B-Protein-binding protein complex.* (x——x), *B-Protein Assay reaction mixture minus binding protein after 1 h incubation;* (o——o), *B-Protein Assay reaction mixture containing ACP in place of binding protein after 1 h incubation;* (•——•), *complete B Protein Assay reaction mixture, step prior to 68° heating;* (■——■), *complete reaction mixture, step prior to TCA precipitation;* (▲——▲), *purified B-Protein binding protein complex. Solid line with no symbols is the absorbance pattern for serum. The serum protein peak with the greatest elution volume is serum albumin (M. W. 68,000)*

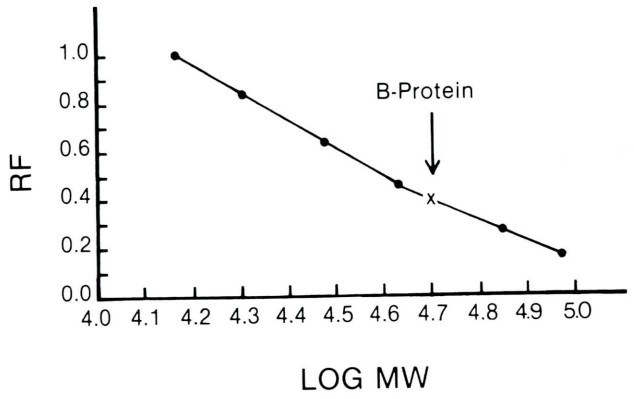

FIG. 8. *SDS-Polyacrylamide gel electrophoresis determination of molecular weight. The standard proteins used to calculate the molecular weight were: phosphorylase B (molecular weight 94,000), bovine serum albumin (molecular weight 68,000), ovalbumin (molecular weight 45,000), carbonic anhydrase (molecular weight 30,000), soybean trypsin inhibitor (molecular weight 20,100) and lactalbumin (molecular weight 14,400).*

protein or ACP. These results support the interpretation that either a conformational change took place or a subunit of B-Protein was displaced when the B-Protein-binding protein complex was formed.

SDS-PAGE studies of B-Protein conducted according to the method of Weber and Osborn(10) indicate that B-Protein is a dimer. Each subunit having a molecular weight of 52,000-53,000 (Fig. 8).

Sedimentation velocity studies conducted on B-Protein prior to and following treatment with SDS, support the dimer character of B-Protein. Electrophoretically homogeneous B-Protein was studied in a sedimentation velocity experiment. Two protein peaks were observed, the major peak (approximately 70% of total protein) moving faster than the minor peak (approximately 30% of total protein). Since the sample appeared to be pure and homogeneous by all other criteria this finding suggested the possibility of an association-dissociation equilibrium. The sample was dialyzed versus 0.1% SDS and the sedimentation velocity experiment was repeated. The results of this experiment showed a shift of the equilibrium so that only about 10% of the total protein remained in the faster moving peak while the slower peak now contained about 90% of the total protein.

Thus, B-Protein appears to be a dimer with subunits of equivalent molecular weight. This conclusion is based on the apparent molecular weight of 100,000-120,000 obtained by gel filtration studies and following SDS-polyacrylamide gel electrophoresis a single protein band with a molecular weight of 52,000 was obtained. The molecular weight of the B-Protein-binding protein complex is approximately 65,000 as determined by gel filtration chromatography (Fig. 7, compare Kav values for the complex and human serum albumin peaks). This suggests that when the binding protein interacts with B-Protein one of the subunits of B-Protein is displaced because the molecular weight of binding protein (10,000-15,000) plus the molecular weight of one subunit of B-Protein (52,000) equals approximately 65,000. The finding that B-Protein will bind to a Wheat Germ lectin column and that the B-Protein-binding protein complex does not, would be consistent with the proposed mechanism that a subunit of B-Protein containing the N-acetyl-glucosamine residues has been displaced when binding protein interacts with it. Finally, when acyl carrier protein (approximate M.W. 9,000) interacts with B-Protein, a complex is formed which also has a molecular weight of approximately 65,000. Physical evidence that B-Protein exists as a dimer was also obtained from sedimentation velocity experiments.

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