Separation of Folate Binding Protein from Human Serum by DEAE-Cellulose Column Chromatography

Alfred Zettner and Peggy E. Duly

On diethylaminoethyl-cellulose column chromatography, the folate binding protein in the serum of 21 patients eluted in the early effluents as a single sharply defined peak. The chromatographic behavior of the folate binder remained unchanged whether or not the serum was, before chromatography, complexed with tritium-labeled pteroylglutamic acid ([3H]PGA), dialyzed, or charcoal-adsorbed. Heating to 100 °C for 10 min dissociated the [3H]PGA-binder complex while destroying the folate binding property. The presence or appearance of this folate binder in increased amounts in the serum of patients with various diseases may be related to conditions of increased tissue turnover.

Additional Keyphrases: conditions under which folate binding protein is increased in serum • stoichiometry • identity of binding protein • cow’s milk folate binder

During the past few years, considerable evidence has accumulated to show that human serum contains a protein capable of binding folate derivatives firmly and specifically. Some authors believed this folate binding protein (FBP)1 to be present in sera only in such states as uremia (1) and leukemia (2), and in pregnant women and those taking contraceptives (3); others have found it also in normal sera (4, 5). Zettner and Duly (4) screened over 1000 sera from patients and healthy volunteers and could demonstrate the presence of a binding principle specific for folates in virtually all sera tested. The binding capacity ranged from less than 0.1 to over 8 ng of [3H]PGA per milliliter of serum. Most of the sera bound less than 1 ng/ml, but 20% of them bound more than 2 ng/ml.

Markkanen et al., in a series of publications (6–15), reported on the behavior of endogenous folic acid activity (as determined by L. casei assay) in serum put through Sephadex gel columns, and concluded that folic acid activity was normally carried by proteins in the zones of α2-macroglobulin, transferrin, and albumin; but Jacob and Herbert (16), using a purified preparation, presented evidence against transferrin as a binder of folic acid. Other authors, on the basis of studies with Cohn fractionation (17), equilibrium dialysis (18, 19), electrophoresis, and ultrafiltration (20–22), concluded that folate in serum was bound nonspecifically and weakly to serum proteins, predominantly by albumin. Mantzos et al. (23) showed avid binding of folates by the serum of pigs and some sheep. Waxman (24, 25) found on Sephadex gel filtration that [3H]PGA was bound in serum of folate deficient or uremic patients to proteins of either 50 000 or 200 000 mol wt.

Thus, it appears that folate may be bound to proteins in principally two ways: (a) firmly to specific globulins, with an affinity high enough to withstand charcoal treatment, electrophoresis, and chromatography; and (b) loosely and nonspecifically to albumin, as demonstrable by ultrafiltration and equilibrium dialysis.

In the following, we report on the separation of FBP in human serum by DEAE-cellulose chromatography before and after complexing with [3H]PGA, and after dialysis or charcoal adsorption.

Materials

Tris buffer. 0.2 mol/liter, pH 8.0.

[3',5'-3H]Pteroylglutamic acid, sodium salt ([3H]-PGA, 20 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill. 60005).

Since the purity of this compound, when checked in competitive binding experiments, was considerably less than the 98% stated by the manufacturer, the radiochemical was purified by the column-chromatographic procedure of Nixon and Bertino (26), except that mercaptoethanol was omitted from the eluting buffers. The results were essentially the same as described previously (4). An intermediate stock solution was made in Tris buffer. This stock was kept frozen at −80 °C. Working dilutions were made to contain 10 ng of [3H]PGA per milliliter, in Tris buffer. The concentration of the [3H]PGA in these solutions was determined through competitive binding-inhibition studies with use of milk binder and unlabeled folic acid.

Purified milk folate binder. This was prepared according to the methods of Ford et al. (27) and Rothenberg et al. (28).

Division of Clinical Pathology, Department of Pathology, School of Medicine, University of California, San Diego, Calif.

Address correspondence to University Hospital, 225 W. Dickinson St., San Diego, Calif. 92103.

1. Nonstandard abbreviations used: FBP, folate binding protein; Tris buffer, tris(hydroxyethyl)methane hydrochloride buffer; [3H]PGA, [3',5'-3H]pteroylglutamate; DEAE, diethylaminoethyl; and PVP, polyvinylpyrrolidone.

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Charcoal. Powder, activated ("Norit A"; CX655; Matheson, Coleman and Bell, Norwood, Ohio 45212).

Polyvinylpyrrolidone (PVP). (Calbiochem, La Jolla, Calif. 92037) of average mol. wt. 40 000 (range, 20 000 to 40 000).

PVP-coated charcoal. A 100 g/liter charcoal suspension in Tris buffer was mixed with an equal volume of a 20 g/liter solution of PVP, so that the mixture contained, per liter, 50 g of charcoal and 10 g of the coating material.

Uncoated charcoal. Suspensions were prepared as above, except that coating material was omitted from the diluting Tris buffer.

Scintillant. This consisted of 5 g of 2,5-diphenyloxazole (Mallinckrodt Chemical Works, St. Louis, Mo. 63160), 100 ml of "Biosolv-BBS-2" (Beckman Instruments, Anaheim, Calif. 92806), 1 liter of toluene.

Liquid scintillation counter. Beckman Model LS-100 C. (Beckman Instruments).

DEAE-cellulose. DE 52 diethylaminoethyl-cellulose, microgranular, preswollen (Whatman Biochemicals/Reeve Angel, 9 Bridewell Place, Clifton, N. J. 07014).

Methods

Preparation of Serum Pool

The unsaturated folate binding capacity of patients' sera obtained routinely by the clinical chemistry laboratory was measured by the addition of [3H]PGA followed by charcoal adsorption. Details of the procedure were as described (4). Those sera that exhibited a binding capacity of approximately 1 ng of [3H]PGA per milliliter were mixed to obtain a pool of about 90 ml and frozen in convenient aliquots at −80 °C. All experiments were done with this serum pool. The endogenous folate content of the pool was 14 μg/liter, as determined by a radioassay developed in our laboratory that involves extraction of bound and free folates through heating and protein precipitation. The sera in the pool came from 21 patients who were hospitalized with the following diagnoses: renal disease with uremia (two patients), coronary arteriosclerosis, alcoholism with liver disease (two patients), torn knee ligament, tuberculosis, retroverted uterus, malignant lymphoma, mitral disease, staphylococcal endocarditis, automobile accident, pulmonary carcinoma (two patients), cerebrovascular accident, bleeding ulcer, cervical carcinoma, broken hip with surgical repair, sarcoma of buttock (mesenchymoma), metastatic carcinoma of prostate, and abdominal stab wound complicated by pneumonia.

Protein Determinations

Protein concentrations in serum samples and the chromatography effluent were determined by the biuret reaction.

Sample Preparation for Chromatography

(a) [3H]PGA. The working solution of [3H]PGA was diluted sixfold with Tris buffer, and 6 ml was applied to the column.

(b) Serum pool as described above. The protein concentration was 60.5 g/liter. Five milliliters was applied to the column.

(c) Serum pool complexed with [3H]PGA. Five parts of serum pool were mixed with one part of [3H]PGA working solution and incubated overnight at 4 °C in the dark, to allow saturation of the unsaturated FBP in the pool. Thus, the total amount of [3H]PGA in the serum exceeded the binding capacity, as verified by PVP-charcoal adsorption, by a factor of two. Six milliliters of this incubation mixture was applied to the column.

(d) Dialyzed serum pool. Ten milliliters of the serum pool was dialyzed against packing buffer for 24 h at 4 °C. Some precipitation of serum proteins occurred (presumably euglobulins, because of the low osmolality of the buffer). The protein concentration after dialysis (including the resuspended precipitate that dissolved instantaneously in the biuret reagent) was 46.5 g/liter. This indicated a dilution factor of 1.3 of the original serum pool. To correspond to 5 ml of the original serum pool, 6.5 ml of the dialyzed and centrifuged serum was applied to the column.

(e) Charcoal treatment of serum pool after complexing with [3H]PGA. An aliquot of the sample treated

![Fig. 1. DEAE-cellulose chromatograms](image-url)

(a) Purified [3H]PGA. (b) Serum pool. A single sharp peak of unsaturated FBP is seen, but the maxima of protein and radioactivity do not coincide, that of the radioactivity appearing four fractions later than that of the overlapping protein peak. (c) Serum complexed with [3H]PGA before chromatography. The peak of bound [3H]PGA is identified in position and height to that of the unsaturated FBP in (b). The free [3H]PGA eluted in fractions 268 through 315, identical in position, but not in height with the peak seen in (a). (d) Serum dialyzed against packing buffer before chromatography. Only two peaks of protein are seen, presumably because of precipitation of euglobulins during dialysis. The peak of unsaturated FBP is identical in position and height to that of (b). (e) Serum complexed with [3H]PGA and adsorbed with charcoal before chromatography. The peak of bound [3H]PGA is identical to that in c, but the peak of free [3H]PGA is absent.

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as described under paragraph c was adsorbed with PVP-charcoal. To circumvent the introduction of undesirably large amounts of PVP into the chromatography procedure, 9 ml of the suspension of PVP-charcoal were centrifuged to obtain a sediment button of 450 mg. The supernatant buffer (which contained most of the PVP) was drained off, 9 ml of the sample as described under c was added, and the charcoal was resuspended for 5 min to adsorb the free radioactivity. After centrifugation, 5 ml of the supernate was applied to the column for chromatography.

Column Chromatography

A chromatography column, 25 × 500 mm, was packed with DEAE-cellulose in phosphate buffer (50 mmol/liter, pH 7.0, containing 20 mmol of NaCl per liter). To make quantitative comparisons possible, we chose sample volumes applied to the column to correspond to 5 ml of the original serum pool, allowing for the volume changes as they occurred during complexing with [3H]PGA or dialysis. The volume change caused by the charcoal treatment was less than 5% and therefore could, for the purpose of chromatography, be neglected. Also, a constant 1-ml volume of working solution of [3H]PGA containing 10 ng was used to prepare samples a, c, and e as described above. The samples were chromatographed with 100-ml portions of the eluting buffers consisting of the basic packing solution and increasing the NaCl content in steps from 0.02 to 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, and 0.50 mol/liter. Then, chromatography was continued with 1 liter of buffer with 1.00 mol/liter NaCl until no further radioactive materials eluted. Fractionation was done at 4 °C, maintaining a flow rate of 50 ml/h with a peristaltic pump and collecting 5-ml fractions of the effluent.

Testing for Unsaturated Folate Binding Capacity in Eluates

Portions of 0.5 ml of the eluates were mixed with 100 μl of the working solution of [3H]PGA and incubated for 30 min at room temperature (22 °C). Then 0.5 ml of the PVP-coated charcoal was added and the mixture was incubated for 5 min and centrifuged. The supernate was decanted into 15 ml of scintillant and its radioactivity determined.

Radioactivity in Eluates

Portions of 0.5 ml of the eluted fractions were added to 15 ml of scintillant and the radioactivity measured.

Differentiation of Free and Bound Radioactivity

Portions of 0.5 ml of the fractions that eluted in the regions of the radioactive peaks were adsorbed with 0.5 ml of suspensions of either uncoated or PVP-coated charcoal and after centrifugation the radioactivity of their supernates was determined. Radioactivity adsorbed by uncoated but not by coated charcoals was considered bound (29); radioactivity adsorbed by both charcoals was considered free.

Heat Dissociability

Aliquots of the fractions containing the bound radioactivity were heated for 10 min in a boiling water bath and centrifuged to remove precipitated proteins. The supernate, after cooling to room temperature, was then treated with PVP-coated charcoal to adsorb radioactivity liberated by heating from its complex with protein.

Binding of Radioactivity in the Eluates by Milk Binder

Portions of 0.5 ml of the eluted fractions representing the free radioactivity were mixed with 100 μl of a working dilution of the cows' milk folate binder and allowed to complex at room temperature for 30 min. The mixture was then adsorbed with 0.5 ml of the PVP charcoal and after centrifugation the radioactivity of the supernate was determined. The radioactivity remaining in the supernate was taken to indicate the presence of intact [3H]PGA in the eluates inasmuch as the affinity of the milk folate binder is specific for folates.

Portions of 0.5 ml of the supernates of the heat-treated and centrifuged eluates containing the bound radioactivity were tested with milk binder as described above for the free radioactivity. Radioactivity not adsorbed by PVP-coated, but adsorbed by uncoated charcoal, and furthermore, dissociable by heat and bindable again by subsequent addition of milk folate binder was taken to be representative of chemically intact [3H]PGA bound to serum protein in the original eluates.

Results

The results of the column chromatographic studies are summarized in Figure 1.

(a) Purified [3H]PGA. The elution pattern shows one peak of radioactivity, in fractions 288 through 315. The radioactivity in this peak was adsorbable by uncoated and PVP charcoal, was bindable by cows' milk folate binder, and thus reacted as a chemically intact, free folate.

(b) Untreated serum. The serum proteins eluted in three distinct peaks. No protein could be detected beyond fraction 105. All fractions were tested for their ability to bind [3H]PGA. Only one peak of binding, confined to fractions 16 through 25, was seen. This bound radioactivity was adsorbable by uncoated charcoal, but not by PVP-charcoal. The complex was dissociable by heating after which the radioactivity again was bindable by cows' milk binder.

(c) Serum complexed with [3H]PGA. The serum proteins emerged from the column in a pattern identical to that for untreated serum. The radioactivity appeared in two peaks. An early one was present in fractions 16 through 25. The position of this peak coincided exactly with that of unsaturated FBP in the chromatogram of Figure 1b. The material was identified as bound [3H]PGA by charcoal adsorption and heat dissociability. Testing for any residual unsaturated FBP in these el-
utomates resulted only in a 5% increase of the bound radioactivity, indicating that the FBP had eluted from the column at least 95% saturated with \(^3\)H]PGA. The peak of the free \(^3\)H]PGA (fractions 285 through 315) was smaller than the corresponding peak in Figure 1a, as would be expected because a fraction of the total \[^3\]H]PGA in this sample was bound to FBP eluting in the eluates 16 through 25.

(d) Dialyzed serum. The serum proteins emerged in only two peaks. The middle peak of Figures 1b and c was absent, presumably because of the precipitation of globulins during dialysis. A peak of \[^3\]H]PGA binding was seen in fractions 16 through 25, identical to that of Fig. 1b in position and height and in the behavior of its radioactive material in charcoal adsorption and heat dissociability. Dialysis before chromatography apparently failed to uncover folate binding sites occupied by endogenous folates, as otherwise the size of the peak of \[^3\]H]PGA binding should have increased.

(e) Serum complexed with \[^3\]H]PGA and adsorbed with PVP-coated charcoal. The serum proteins eluted in the three peak patterns seen in Figures 1b and c. The peak of bound \[^3\]H]PGA was identical in all characteristics to that seen in Figure 1c, a reflection of the fact that the PVP-coated charcoal did not adsorb the complex of \[^3\]H]PGA with FBP. No free \[^3\]H]PGA was found, indicating that the free \[^3\]H]PGA was removed by the PVP charcoal prior to chromatography.

**Discussion**

These column-chromatographic studies demonstrate that the serum of many patients, with various diseases, contains a substance that can bind \[^3\]H]PGA. This substance emerged from the column with the first peak of serum proteins on DEAE-cellulose chromatography and was destroyed by heating to 100 °C for 10 min. The binding of \[^3\]H]PGA is of such avidity that the complex withstands treatment with charcoal and column chromatography. The FBP eluted in a single, sharply-defined peak occupying a fixed position in the chromatograms, even though the pooled sample of serum was derived from 21 different patients. Moreover, the elution pattern of FBP was the same whether or not it was complexed with \[^3\]H]PGA, or whether the serum was dialyzed or treated with PVP-charcoal before chromatography.

DEAE-cellulose chromatography has been used by other workers for the study of FBP's from other sources and we should like to comment here on these reports in comparison with our own findings.

Waxman and Schreiber (24) published a DEAE-cellulose elution pattern of normal and folate deficient sera complexed with \[^3\]H]PGA and charcoal adsorbed prior to chromatography. The radioactivity eluted in two rather broad peaks of different heights, the first with 60 mmol/liter phosphate buffer, the second with 1 mol/liter NaCl. No concomitant protein elution pattern was shown, but they stated that \(\beta\)-globulin accompanied the first and \(\alpha\)-globulin the second peak. It is not clear from their report how much serum was applied to the small 5 ml column, or if the eluates had been tested to establish that the radioactive material was bound. Also, the fact that the elution pattern of the radioactivity in buffer showed two peaks in the same positions as those of the sera (although of different height) is unexplained, since the buffer, run as a control, should not have contained any binding material. Nevertheless, the results do suggest that there was some separation of free and FBP-bound \[^3\]H]PGA in serum.

Fischer et al. (30) chromatographed on DEAE-cellulose the partially purified lysates of chronic myelogenous leukemia cells. FBP, either saturated or unsaturated with \[^3\]H]PGA prior to chromatography, eluted in two peaks, the first with the starting buffer, sodium phosphate (1 mmol/liter, pH 6.0), the second during a linear concentration gradient at 80–100 mmol/liter of sodium phosphate, pH 7.4. On Sephadex filtration, the FBP of the material represented by the first peak had a molecular weight of about 34 500 and the FBP in the second peak a molecular weight of 41 500.

Waxman and Schreiber (31) extracted by affinity chromatography the low-molecular-weight component of human milk FBP, and chromatographed this material on DEAE-cellulose. This FBP eluted in a single peak with a 1 mmol/liter phosphate buffer of pH 6.3. Additional elution with buffers of stepwise increase of ionic strength did not reveal additional FBP components.

The position of the FBP peak in the early fractions of our chromatogram and the demonstration by Fischer et al. (30) and by Waxman and Schreiber (31) that the FBP from chronic myelogenous leukemic cells and that from human milk can be eluted from the DEAE-cellulose columns by such low ionic strength buffers as 1 mmol/liter of course indicates that these FBP's coming from such varied sources seem to share the property of very weak adherence to DEAE-cellulose. This suggested to us that lowering the ionic strength of the starting buffer in our chromatography system might help to resolve the sharp, single peak of FBP into more than one component. Using 100 ml of a starting buffer of 1 mmol/liter sodium phosphate of pH 7.0 followed by a linear gradient to 50 mmol/liter sodium phosphate containing 20 mmol/liter NaCl, we chromatographed a sample prepared as described in paragraph c under **Methods**.

With this buffer system, the first protein peak as shown in panel c of Figure 1 now resolved into two well-separated peaks, the first containing about 70%, the second 30% of the protein. The \[^3\]H]PGA-FBP complex overlapped exactly with the second protein peak; however, no splitting or broadening of the radioactive peak was seen. These preliminary results show that, similar to FBP's from other sources (30, 31), serum FBP can be eluted with buffers of very low ionic strength, and this should prove to be of great advantage in further purification and isolation efforts. The refinement of these DEAE-cellulose separation techniques are under study in our laboratory with the aim.
of isolating sufficient quantities of serum FBP(s) for more definitive characterization that will form the basis of a future report.

On Sephadex gel filtration (24, 25), [3H]PGA, when complexed with the serum of uremic or some folate-deficient patients, eluted as a sharp peak associated with proteins of 50,000 mol wt. This material appears to correspond well with the FBP found in our studies, especially in view of the fact that our serum pool also contained sera from two patients with uremia. Waxman, however, also showed that an FBP of estimated molecular weight 200,000 was present in the serum of a folic acid-deficient patient. We examined the DEAE-cellulose eluted fractions of our material containing the FBP by Sephadex filtration and found only a single peak of bound [3H]PGA, associated with the proteins of approximately 50,000 mol wt.

Our findings are somewhat at variance with the conclusions presented by Markkanen et al. (9, 10), who claimed to have identified three serum proteins that are binders of endogenous folic acid: α2-macroglobulin, transferrin, and albumin. Their chromatograms of Sephadex filtration show distribution of folic acid activity almost over the entire protein region with maxima of activity coinciding with protein maxima. It is not clear in their reports whether they tested to ascertain that the folate which eluted with the protein peaks was indeed bound. However, studies of folate binding in serum based either on the addition of extraneous [3H]PGA or on the estimation of endogenous folate are not entirely equatable, since they are aimed to detect different substances, the unsaturated FBP in the former, and the naturally occurring folate in the latter case. The endogenous folate content of our serum pool was 14 μg/liter as-à-vis an unsaturated FBP of 1 μg/liter. In view of the high, albeit differing, affinity of serum FBP for its ligands (4), one must assume that the endogenous folate in the serum pool was bound. In our experiments dialysis before chromatography failed to uncover additional binding sites on the FBP. The relationship between FBP and endogenous folates still remains unclear, and we are now studying the problem.

Folate binding proteins are widely distributed in mammalian tissues; they have been found to be present in such varied locations as leukocytes from patients with chronic myelogenous leukemia (2, 30), the brush border membranes of small intestinal epithelial cells of rats (32), hog kidney (33), rat liver cells (34), and human (31) and cows’ milk (27). FBP appears to be consistently present in high concentrations in these locations. In contrast to this, the concentrations of FBP in human serum vary considerably (4). Some normal persons seem entirely to lack FBP in their serum. It is somewhat difficult to visualize a distinct physiological function for FBP in serum in view of such great quantitative variability. Waxman (25) has suggested that FBP in serum may be the result of increased cellular synthesis and its release in conditions such as leukemia or folate deficiency. We should like to add the possibility that FBP may be released into the blood stream at increased rates in conditions that are characterized by increased turnover, destruction, or wasting of FBP-containing tissues. These general conditions appear to have been present in the patients whose sera we selected for our study on the basis of their increased content of FBP: malignancies, uremia, pneumonia, injuries, intestinal bleeding, surgery, liver disease, and infarction. Thus, FBP in serum may not have an essential physiological role, but may be incidental to tissue turnover.

Finally, we should like to make the following quantitative considerations. The binding capacity of a given amount of FBP is fixed and saturable, with an affinity that is highly specific (4). These characteristics, of course, imply a stoichiometric relationship between folate and FBP. If one assumes that one molecule of serum FBP binds one molecule of folate (and the binding data in (31) seem to support this concept for human milk FBP), then the molar concentration of FBP in serum should approximate that of serum folates, which ranges from 5 to 50 nmol/liter. Consequently, with a 50,000 mol wt, the concentration of FBP in serum should then range from 0.25 to 2.5 mg/liter, and with a 200,000 mol wt from 1 to 10 mg/liter. These quantitative relationships would seem to exclude major serum proteins that are known to be homogeneous entities from considerations as specific folate binders, that is to say, proteins such as transferrin or albumin (but not necessarily the heterogeneous α2-macroglobulin), the molar concentrations of which in serum typically exceed those of folates by two to three orders of magnitude. For purified transferrin, experimental evidence has been presented (16) indicating that it lacks folate binding properties.

As has also been pointed out by others (5, 25) the investigation of the FBP's is only at its beginning. It is to be hoped that the interest recently awakened by several groups of workers will eventually result in the elucidation of the many questions that the discovery of the FBP's has raised.

References


