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Purification of folate binding factor in normal umbilical cord serum

(folate binder/neonate)

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ABSTRACT **Human umbilical cord serum was found to contain both free folate and folate complexed to a high-molecular-weight factor. The complexed folate was bound to a very high affinity binder and was present in concentrations equivalent to as much as 60 ng of 5-methyltetrahydrofolic acid per ml of serum. Acidification of the serum caused disassociation of the folate–binder complex. Released folates were separated from binder by Sephadex gel filtration, zonal centrifugation through sucrose gradients, or adsorption onto activated charcoal. The separated binding factor, either saturated or unsaturated with folate, had a molecular weight of about 40,000 on Sephadex G-200 chromatography. Binding of [^{3}H]pteroylglutamic acid was rapid and, as in the original endogenous folate–binder complex, was essentially irreversible at neutral pH. The affinity and specificity of the binder were examined by competition experiments using [^{3}H]pteroylglutamic acid and nonradioactive folate derivatives. Oxidized folates were bound in preference to reduced derivatives, but only three to four times more unlabeled 5-methyltetrahydrofolic acid than pteroylglutamic acid was required to produce an equal level of competition. The strong affinity for 5-methyltetrahydrofolic acid, the main serum folate, suggests that the binder could be part of a mechanism by which the fetus concentrates maternally supplied folate for its growth and development.**

Recent studies using a radiochemical ligand binding procedure demonstrated that most sera contain a pool of bound folate (1). In general, the amount of bound folate is small, less than 5 ng of 5-methyltetrahydrofolic acid equivalents/ml of serum. This limitation in supply has thwarted systematic study of serum folate binding factors except in a few pathologic conditions in which "unsaturated binder" was found in the serum (2, 3). Measurements in our laboratory on matched maternal and neonatal (or umbilical cord) serum samples showed that the levels of free folate in the matched samples were similar, whereas the level of bound folate in the neonatal circulation was four to five times greater than that in the maternal serum (Table 1). This source of folate–binder complex has provided an adequate amount of material for preparation of unsaturated binding factor. This paper reports on the disassociation of endogenous folate–binder complex and the separation of folate from the binder. Also included are our preliminary findings on some physical and chemical properties of the binding factor.

MATERIALS AND METHODS

Umbilical cord blood was drawn from the umbilical vein immediately after delivery of the placenta of full term neonates (i.e., those with a birth weight of at least 2500 g). Maternal blood was obtained at random upon admission of expectant mothers to the hospital for the purpose of childbirth. Serum samples were prepared and assayed for folate as described previously (1). Individual samples were then pooled and stored at −40° until used. Folate values are expressed as 5-methyltetrahydrofolic acid equivalents unless otherwise stated.

Endogenous bound folate was dissociated from the natural folate–binder complex by treatment with acid pH. The freed folate was then adsorbed onto charcoal, thus leaving the unsaturated binder in solution. All steps were carried out at 4° unless otherwise stated. Specifically, the pH of the serum was adjusted slowly to 3–3.2 with 1 M citric acid; then 5 mg of Norit A (Matheson, Coleman, and Bell) were added to each ml of serum (starting volume). The suspension was stirred slowly overnight and then centrifuged for 15 min at 12,000 × *g* to pellet the charcoal. The supernatant fluid was slowly adjusted to pH 7.4 with 1 N NaOH. Any precipitate was removed by centrifugation as above and discarded. Aliquots of the neutralized supernatant fluid were then analyzed for remaining folate and for their capacity to bind [^{3}H]pteroylglutamic acid (PteGlu; Amersham/Searle: [3′,5′,9(n)-^{3}H]pteroylmonoglutamic acid, specific activity 26 Ci/mmol) using methods previously reported (ref. 4; see also Fig. 1).

Using the general approach reported by Salter *et al.* (5) for milk and modified by Selhub and Grossowicz (6), the separated binder was concentrated and purified approximately 10,000-fold by affinity chromatography and Sephadex G-100 filtration. Neutralized extracts of serum containing the now unsaturated binder were passed through a 1 × 7 cm column of AH-Sepharose 4B (Pharmacia) to which methotrexate (2-amino, 4-amino, 4-deoxy-10-methyl-pteroylglutamic acid; Lederele) had been covalently linked according to methods presented by Newbold and Harding (7) and that had been equilibrated with 0.05 M potassium phosphate buffer, pH 7.6. More than 90% of the PteGlu binding activity adhered to the column on the first passage. After washing the column with 0.05 M potassium phosphate buffer until no detectable material absorbing at 280 nm was eluted, binder was eluted in about 90 ml of 0.2 N acetic acid. The eluant was then concentrated to about 10 ml by lyophilization and 5 ml aliquots were filtered through a 2.5 × 90 cm Sephadex G-100 column equilibrated in 0.1 M ammonium bicarbonate. Peak fractions of binding activity (Fig. 3) were pooled, lyophilized to dryness, and dissolved in 0.05 M potassium phosphate buffer, pH 7.6. The results of this purification are shown in Table 2.

Analyses of binding specificity and affinity were done according to methods described for the binding factor from hog kidney (4). "Bound" and "free" folate were separated by adsorption of the free folate onto charcoal coated with albumin (4), by zonal centrifugation through sucrose gradients, or by filtration through Sephadex G-25 or G-100 (Fig.

Abbreviations: PteGlu, pteroylglutamic acid; $PteGlu_7$, pteroylheptaglutamic acid.

Table 1. Folate levels in maternal and neonatal serum

Source no.	Free	Bound	Total	Unsaturated binding* (ng of PteGlu per ml)
	(ng of folate per ml of serum, ± SD)			
Matched samples†				
Neonatal sera (15)	9 ± 2.4	15 ± 6.0	24 ± 9.1	0
Maternal sera (15)	6 ± 3.0	4 ± 5.0	10 ± 3.0	0
Individual samples‡				
Neonatal sera (65)	8 ± 3.2	17 ± 7.2	25 ± 12.4	0
Pooled samples				
Neonatal sera				
Untreated	4	12	16	0
Extracted§	0	0	0	1.2

* Determined at several concentrations of extracted or unextracted serum and [^{3}H]PteGlu. This represents the maximal binding of [^{3}H]PteGlu by the sample.

† Maternal samples were obtained upon admission for childbirth; neonatal samples were obtained from umbilical vein immediately after delivery of placenta.

‡ Randomly collected as for matched maternal and neonatal samples.

§ Pooled serum was extracted with acid, treated with charcoal, neutralized, and purified by affinity chromatography and filtration through Sephadex G-100.

3). Binding capacity was determined by subsaturating concentrations of binder as detailed elsewhere (4).

Reduced folate derivatives used in this study were synthetically prepared and were all *d,l* isomers.

RESULTS

Analysis of 15 samples of umbilical cord serum and matched maternal serum showed no significant differences with respect to free folate content. In contrast, the bound fraction of folate was four to five times greater in the cord serum than in the matched sample of maternal serum (Table 1). For 65 individual cord serum samples the mean value of bound folate (1) was 17 ± 7.2 mg/ml of serum. While more than 80% of the cord serum samples contained greater than 8 ng of bound folate per ml, none bound exogenously added [^{3}H]PteGlu at any concentration of serum or [^{3}H]PteGlu tested. This means that none of the cord serum samples contained endogenous "unsaturated" binder.

After acid extraction and charcoal treatment, the pooled cord serum contained no detectable folate and showed a binding capacity of 1.2 ng of PteGlu per ml (Table 1). This compared with a pre-treatment value of bound folate of 12 ng/ml. Assuming bound folate and binding capacity to be equivalent, this would represent a recovery of 10%. The results of the treatment and purification procedure are summarized in Table 2.

Table 2. Purification of folate binder in umbilical cord serum

Stage of purification	Total volume (ml)	Total protein* (mg)	Total binding (ng of PteGlu)	Specific activity†
Pooled Serum	250	17,500	0	0
Crude acid extract	800	15,700	480.54	0.03
Post affinity chromatography	10	27	411.19	15.23
Post Sephadex G-100 and lyophilization‡	3	1.2	385.50	321.25

Increase in specific activity = 1×10^4

Recovery of activity of initial acid extract = 80%

* Determined by the methods of Lowry *et al.* (16) and Böhlen *et al.* (17).

† Refers only to the capacity of the preparation to bind exogenously added [^{3}H]PteGlu and is expressed as ng of PteGlu bound per mg of protein.

‡ Assuming a molecular weight of 40,000 for the binder and that it is univalent, this preparation was 3–4% pure.

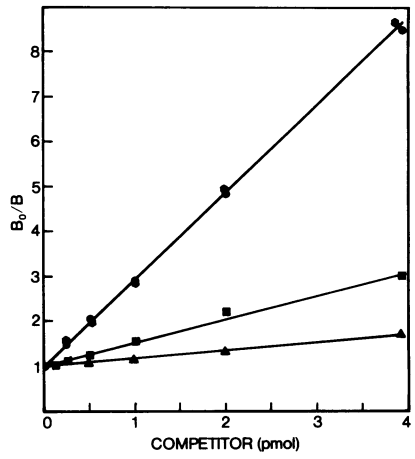

FIG. 1. Specificity of umbilical cord serum binder for several folate derivatives. The reaction mixture contained in 1.0 ml: 0.5 pmol of [^{3}H]PteGlu, unlabeled derivative as indicated, enough binder to bind about 0.4 pmol of PteGlu, 25 μmol of sodium ascorbate, and 50 μmol of potassium phosphate, final pH 7.6. The reaction was started by adding binder and proceeded for 30 min at 21° in the dark and was stopped by placing tubes on ice and adding 1.0 ml of a suspension of charcoal coated with albumin (4). Charcoal was pelleted by centrifugation at 3000 × *g* and the supernatant fluid containing the bound folate was counted (4) with an efficiency of 30%. Controls made by substituting buffer for binder determined counts not removed by charcoal, usually 0.5–1.0% of total counts incubated. The results have been plotted according to Burger *et al.* (18). B_0, net counts bound in absence of unlabeled folate; *B*, net counts bound in presence of any given concentration of folate or competitor. ●, PteGlu; ⬢, $PteGlu_7$ (the heptaglutamate was a gift of Dr. Charles Butterworth of the University of Alabama); ■, 5-methyltetrahydrofolic acid; ▲, methotrexate.

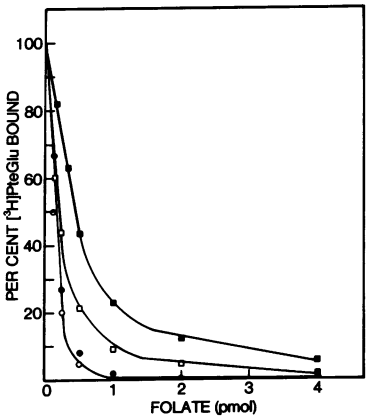

FIG. 2. Sequential addition of [³H]PteGlu and unlabeled folate. Reaction conditions were as described in Fig. 1 except that binder was preincubated for 20 min with unlabeled folate as indicated and then [³H]PteGlu was added. After an additional 30 min incubation period either at room temperature or at 4°, the reaction was stopped and the samples were processed for counting. Other experiments in which a constant amount of unlabeled folate (2.2 pmol) was first bound, 0.5 pmol of [³H]PteGlu then added, and samples taken at different time intervals showed that 5-methyltetrahydrofolic acid was displaced linearly at about 0.3%/min for at least 3 hr; no displacement of PteGlu occurred under identical conditions. ○, PteGlu at 4°; ●, PteGlu at 21°; □, 5-methyltetrahydrofolic acid at 4°; ■, 5-methyltetrahydrofolic acid at 21°.

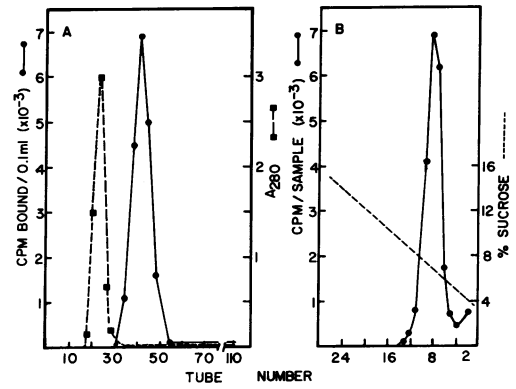

FIG. 3. (A) Sephadex G-100 chromatogram of umbilical cord serum binder of folate after initial purification by affinity chromatography. Five milliliters of the partially purified binder preparation were applied to a 2.5 × 90 cm column of Sephadex G-100 equiliberated in 0.1 M ammonium bicarbonate. Fractions (5 ml) were collected and 0.1 ml aliquots of each were tested for binding of [³H]PteGlu. The column had been calibrated with known molecular weight markers: γ-globulin, ovalbumin, myoglobin, and folic acid. The peak of binding activity fractionated with a molecular weight of about 40,000. (B) Sucrose gradient centrifugation of folate–binder complex. Reaction mixture (0.5 ml) containing 33,000 cpm of [³H]PteGlu and enough binder to bind 30,000 cpm was layered over a 10 ml linear 3–15% sucrose gradient (prepared with 0.05 M potassium phosphate buffer, pH 7.6) and centrifuged at 40,000 rpm for 24 hr in an SW 41 rotor in a Beckman L-75 ultracentrifuge at 4°. 10-Drop fractions were collected from the top of the gradient and counted as in Fig. 1. Unbound [³H]PteGlu was found only in the first two fractions.

As shown by competition experiments, the separated binding factor preferred oxidized folates to reduced derivatives (Fig. 1). The binding factor did, however, show strong binding affinity for 5-methyltetrahydrofolic acid. Note that only 3.5–4 pmol of 5-methyltetrahydrofolic acid produced the same degree of competition for the binding of [³H]PteGlu as 1 pmol of PteGlu. In contrast, the affinity of the binder for 5-formyltetrahydrofolic acid and methotrexate was slight, about 30 and 20 pmol, respectively, being required to equal the competition of 1 pmol of PteGlu. In addition, high concentrations of folate analogs such as *p*-aminobenzoylglutamic acid and 6-carboxypterin did not inhibit the binding of [³H]PteGlu. Finally, changes in the number of glutamic acid residues did not appreciably alter the binding affinity.

Although the factor showed binding determinants for 5-methyltetrahydrofolic acid, at pH 7.6 the affinity for this derivative was somewhat lower than that for PteGlu. This was more clearly seen by sequential binding measurements (Fig. 2). Regardless of the temperature, [³H]PteGlu did not displace unlabeled PteGlu. However, at room temperature 5-methyltetrahydrofolic acid was displaced by [³H]PteGlu at the rate of 0.3%/min. At 4°, the rate of this displacement was less than 0.1%/min.

The binder, either saturated or unsaturated with PteGlu, has an apparent molecular weight of 40,000 as judged by Sephadex G-200 and G-100 chromatography in 0.1 M potassium phosphate buffer, pH 7.6. Zonal centrifugation through 3–15% sucrose gradients in the same buffer gave results consistent with the 40,000 molecular weight value (Fig. 3).

The binding of PteGlu showed strong pH dependency, as has been the case with binders of folate from other sources (2, 4, 10). Although the folate–binder complex disassociated at pH 3.0, it reformed when the solution was neutralized. At a starting concentration of 10^{-9} M PteGlu and an equivalent concentration of binding sites, binding of [³H]PteGlu occurred at an initial rate of 2%/sec at 4° and pH 7.6. No significant exchange between [³H]PteGlu–binder complex and unlabeled PteGlu could be measured after 1 hr at pH 7.6 and 37°, even in the presence of a 100-fold excess of unlabeled PteGlu.

DISCUSSION

Although binders of folate have been found in several tissues (2–4, 8, 9), their function is not known. Johns *et al.* suggested that specific carriers of folate must exist to account for the rapid plasma clearance and cellular uptake of this cofactor (10). Lichtenstein *et al.* described a carrier-mediated, saturable transport system for folate in culture L1210 cells (11). Also, Ford and Salter reported some of the properties of unsaturated binder in bovine and human milk (12). Markkanen *et al.* (13) demonstrated that serum folate, as judged by growth of *Lactobacillus casei* in a microbiological assay, fractionated on Sephadex G-200 into three bands of high molecular weight in addition to the major low-molecular-weight fraction that corresponded to "free" folate. When chromatographed on DEAE-Sephadex A-50, the "*L. casei* activity" appeared in the α_2-macroglobulin and transferrin regions (14). More recently several laboratories have described some properties of "serum folate binder" from a few patients with chronic granulocytic leukemia (2) or folate deficiency (3) and from some pregnant women and women taking oral contraceptives (15). Binding factor in these cases apparently existed in an "unsaturated" state in the serum. The level of endogenous, saturated binder was not assessed in those studies, however.

This report shows that endogenously bound serum folate can be dissociated from a specific binding factor and subsequently removed from the serum. The unsaturated binder can then react with [³H]PteGlu and other folate derivatives. This facilitates the study of its binding specificity, affinity, and other physical and chemical properties. The low recov-

ery of endogenous binder shown in Table 1 may be a reflection of several problems. No doubt conditions used to dissociate the folate–binder complex and to produce and concentrate unsaturated binder contributed to the low yield of separated binder.

The molecular weight of 40,000 for the separated binder from cord serum is similar to that of binder from hog kidney (4) and milk (12, *). Also, the binding preference at pH 7.6, mole PteGlu bound: mole 5-methyltetrahydrofolic acid bound, of 3.5–4:1 for cord serum is intermediate between the 6:1 for milk binder* and the 2:1 for hog kidney binder (4). Moreover, neither hog kidney nor cord serum binder has great affinity for 5-formyltetrahydrofolic acid or methotrexate. However, the cord serum binder differs appreciably from the hog kidney factor in the displacement of 5-methyltetrahydrofolic acid by PteGlu. Only insignificant displacement was noted with the hog kidney factor, whereas a displacement of about 0.3%/min occurred with the cord serum factor at 21°. The binding factor in cord serum also differs from the "unsaturated" binder reported in serum of some pregnant women (15). The latter failed to bind 5-methyltetrahydrofolic acid. Whether those sera also contained a "saturated" binder that could bind 5-methyltetrahydrofolic acid has not yet been ascertained.

The binding of 5-methyltetrahydrofolic acid by the factor in cord serum, and the high concentration of the saturated factor in neonatal relative to maternal circulation presents a possible molecular mechanism by which the fetus can sequester and concentrate folate across the placenta from the maternal circulation. More information is needed to decipher the biological role(s) of high-affinity binders of folate; future studies can be expected to yield valuable insights into the possible relationship between the binder in cord serum and the so-called folate deficiency of pregnancy.

* Kamen, B. A., Doctoral Dissertation, in preparation.

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