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CELLULAR FOLATE BINDING PROTEINS; FUNCTION AND SIGNIFICANCE

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INTRODUCTION

In recent years a number of proteins from a variety of tissues have been characterized as folate binding proteins. Studies have been made possible by the availability of radioactive folic acid and by the development of simple and rapid methods of separating the free labeled folic acid from that which is protein bound. In practice, the material is incubated in vitro with a trace amount of radioactive folic acid for a short period; the unbound tracer is removed by adsorption with coated charcoal, or by some other method of separating the small folate molecules from the large proteins (gel filtration, dialysis, etc). The protein-bound radioactivity is then counted. Folic acid, however, is not a natural, physiological form of the vitamin. Natural folates are reduced and may carry one-carbon substituents. Moreover, within cells, these folates are predominantly found as the polyglutamate derivatives (Figure 1). Therefore, studies on the binding of the natural forms of folate have been carried out by measuring the ability of the natural, reduced forms to compete with radioactive folic acid for binding to the protein.

This method of in vitro binding has been used successfully to identify a variety of proteins that bind folate rapidly and specifically with high affinity. Alternatively in a few cases, radioactive folic acid has been administered either orally or parenterally to experimental animals for various periods before removal of the tissue. The folate binding proteins are thus labeled in vivo and can be identified by procedures that separate molecules on the basis of size.

The binding proteins identified thus far may be classified as being either extracellular—i.e. in fluids such as milk, serum, cerebrospinal fluid, etc—

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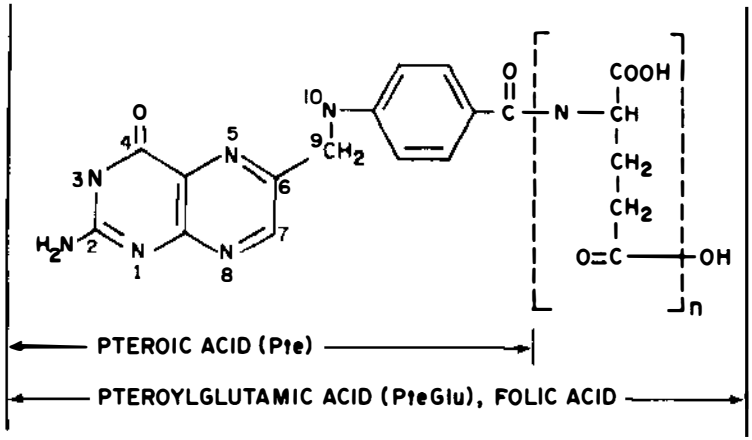

$PTEGLU_5$	PTEROYLPENTAGLUTAMIC ACID (N=5)
$H_4PTEGLU$	5,6,7,8-TETRAHYDROPTEROYLGLUTAMIC ACID TETRAHYDROFOLIC ACID
$H_4PTEGLU_5$	5,6,7,8-TETRAHYDROPTEROYLPENTAGLUTAMIC ACID
$5\text{-}CH_3\text{-}H_4PTEGLU$	5-METHYLTETRAHYDROFOLIC ACID
$5\text{-}HCO\text{-}H_4PTEGLU$	5-FORMYLTETRAHYDROFOLIC ACID
$5,10\text{-}CH_2\text{-}H_4PTEGLU$	5,10-METHYLENETETRAHYDROFOLIC ACID
$5,10\text{-}CH{=}H_4PTEGLU$	5,10-METHENYLTETRAHYDROFOLIC ACID

Figure 1 Structures and nomenclature of the folate derivatives.

or associated with cellular material. It is convenient to subdivide the cellular folate binding proteins further into those that are membrane bound and those that are intracellular and soluble. The membrane-bound folate binding proteins are found associated with membranes of kidney, intestinal epithelium, choroid plexus, and liver as well as the plasma membrane of bacteria. These membrane-associated folate binding proteins can be solubilized by detergents and are believed to participate in the transport of folate derivatives. Two sources of intracellular folate binding proteins have been studied most extensively—those derived from chronic granulocytic leu-

kemia cells and those derived from mammalian liver. The former represent a specialized type of circulating lymphocyte, and the folate binding proteins derived from these cells resemble the folate binding proteins present in serum more closely than those present in liver.

The granulocyte folate binding proteins are, therefore, usually discussed along with the extracellular folate binding proteins. They have been reviewed thoroughly in recent articles (6, 38, 39). Not much more can be added at this time; therefore, I do not consider these binding proteins here except where such discussion is pertinent to those of primary interest. Instead, I confine this review to the membrane-derived and the intracellular folate binding proteins.

MEMBRANE-DERIVED FOLATE BINDING PROTEINS

Mammalian Sources

In 1972, Leslie & Rowe (20) demonstrated that although rat intestinal epithelial cells could not accumulate radioactive folic acid, they could bind it rapidly in a process that was saturable and showed structural specificity for the components of the folate molecule. Reduced folate compounds competed slightly less well for binding of labeled folic acid than oxidized derivatives. The binding of [^{3}H] folic acid to the cell membrane was very tight, and the brush-border fraction of cells labeled by incubation with [^{3}H] folic acid could be isolated with the label still associated. Solubilization of the brush-border membrane fraction with sodium dodecyl sulfate resulted in radioactivity associated with a molecular species greater than 100,000 M_r as well as with smaller molecules having M_r of 24,000 to 16,000.

Selhub & Rosenberg (29) demonstrated the presence of folate binding macromolecules in a membrane preparation from rat kidney that was highly enriched for the brush border. Binding was not measured in a solubilized membrane fraction; however, solubilization of the membrane preparation subsequent to binding of radioactive folic acid demonstrated that the label was associated with the particulate material, rather than the material trapped within vesicles formed by these membrane preparations. The binding of folic acid was saturable with a binding constant, K_b, of 4.2×10^{-11}M and showed specificity for oxidized rather than reduced derivatives. The binding was optimal at pH 6.4–7.7 but was reversible in that endogenous folate could be removed by acid treatment. At pH 9.0, 5-CH_3-H_4PteGlu was as effective as PteGlu in competing for the binding of labeled folic acid. Selhub & Rosenberg speculated that these folate binding proteins might be involved in the tubular reabsorption of folic acid by the proximal tubule cells of the kidney. There is no doubt that folate conservation by the

kidney is extremely important. It has been shown that soon after administration of radioactive folic acid to mice (26) most of the radioactivity is found in the kidney. Only later does the majority of the radioactivity appear in the liver.

The properties of the material studied by Selhub & Rosenberg are similar to the folate binding protein identified some years earlier by Kamen & Caston (19). The latter authors isolated a soluble binder from a commercial preparation of hog-kidney acetone powder. The acetone powder was extracted at acid pH (3.5) at 4°C overnight in the presence of charcoal. This material bound radioactive folic acid tightly between pH 6.0 and 9.0 but was dissociated at acid pH. Binding activity was destroyed by trypsin. The material showed high specificity for H_4PteGlu and 5-CH_3-H_4PteGlu as well as PteGlu at neutral pH. It had a molecular weight of 35,000 to 40,000 as determined by gel filtration. The solubility of the material prepared by Kamen & Caston, as opposed to the particulate nature of the preparations obtained by Selhub & Rosenberg, may be attributed to the use of acetone powder as the starting material by the former group. Delipidation brought about by acetone extraction and the extensive treatment at pH 3.5 may have resulted in the liberation (and possible modification) of the protein from the membranes of the kidney tubules.

Indirect evidence that membrane-associated folate binding proteins are probably concerned with transport was first obtained by Zamierowski & Wagner (44) in studies performed using the in vivo binding technique. The distribution of radioactivity in various subcellular fractions of the liver was measured at time intervals following an intra-peritoneal injection of [^{3}H] folic acid. At the earliest time (0.5 hr following injection) most of the radioactivity was present bound to components of the crude nuclear and microsomal cell fractions. Subsequent purification of these fractions on sucrose density gradients showed that the radioactivity was bound to the plasma membrane components of these cell fractions. The radioactive material bound to the plasma membrane fraction after 0.5 hr was dissociated and shown to be unchanged folic acid. After longer periods the majority of the folate was bound to proteins in the cytosol and mitochondria (see below). The folates bound to these latter proteins are primarily reduced and polyglutamate forms. These kinetic studies suggest that the injected folic acid is first bound to a transport protein in the plasma membrane, and that upon entering the cell the folic acid is reduced and converted to polyglutamates.

Evidence that a folate-binding protein is located in the choroid plexus was first obtained from studies designed to investigate transport. The choroid plexus is a vascular structure in the brain believed to transport substances selectively between the cerebrospinal fluid and the blood. Chen & Wagner (5) and Spector & Lorenzo (33) using the choroid plexus from hog and

rabbit, respectively, showed that labeled 5-methyltetrahydrofolate taken up during in vitro incubations was bound to macromolecules. In both these studies, following uptake, the choroid plexus was dispersed by sonication (5) or homogenization (33) and centrifuged at low speed. The radioactivity in the supernatant was bound to a macromolecule. Because of the low speed of centrifugation it was possible that the radioactivity in the supernatant was associated with a membrane component rather than a soluble macromolecule. Spector has extended these studies on the folate-binding protein of the choroid plexus in a series of papers (30–32). Rabbit choroid plexus, previously incubated with radioactive PteGlu or 5-CH_3-H_4PteGlu and homogenized, contained tightly bound radioactivity (31). Treatment of the homogenates with Triton X-100 produced a soluble preparation in which most of the radioactivity was bound to a macromolecule. The binder was insoluble at concentrations of Triton X-100 below 0.02%. The solubilized binder has a M_r of 340,000–400,000 as estimated by chromatography on Sephadex G-200 and Sepharose 4B. The bound folate derivatives were dissociated at acid pH in much the same manner as the folate-binder complex of hog kidney (19). Using an in vitro binding assay in which albumin-coated charcoal was used to separate the free labeled folate from that which was bound, it was shown that the binder had a higher affinity for PteGlu than for 5-CH_3-H_4PteGlu. The protein nature of the macromolecule was indicated by the loss of binding activity after treatment with papain. This folate-binding protein has been presumed to be located in the plasma membrane (30, 32) of the choroid plexus, though data supporting this contention rest largely on the fact that the preparation is particulate and that uptake of folate derivatives by the intact choroid plexus is a carrier-mediated process. Treatment of intact choroid plexus with Pronase, a crude extract of *Streptomyces griseus* having proteolytic activity, decreased the uptake of radioactive folate (30), prompting speculation that the binding protein is on the exterior surface of the structure. The possibility exists, however, that uptake was impaired by nonspecific alteration in membrane structure, though the preparation appeared unaltered under light microscopy and lactic dehydrogenase, a cytosolic enzyme, was not lost by Pronase treatment.

Binding studies carried out with the Triton X-100 solubilized protein from rabbit choroid plexus indicated a tight association between the protein and radioactive folic acid (30). These studies were carried out using equilibrium dialysis and were probably complicated by the micellar nature of the Triton solubulized preparation, which resulted in an extremely long time for equilibrium to be reached ($\sim$ 7 days). The apparent dissociation constant (K_a) of radioactive PteGlu was dependent on the concentration of PteGlu used, being 0.4×10^{-12}M when folate was present at a concentration

of $.36 \times 10^{-9}$M, and 46×10^{-12}M when folate was present at a concentration of 3.3×10^{-9}M.

Sulieman & Spector have recently reported the purification of a folate-binding protein from hog choroid plexus (35). Triton X-100 was used to solubilize the homogenate of choroid plexus. After removal of endogenous folate by acidification, the preparation was placed over an affinity column consisting of folic acid bound to bovine serum albumin that was in turn bound to Sepharose 4B. The binding protein was eluted with an acidic buffer, and subsequent purification steps included chromatography on Sephadex G-200, DEAE-Sephadex, and finally, slab gel electrophoresis. Binding activity was followed by an in vitro charcoal binding assay. The final preparation was purified 6000-fold. The undenatured, but solubilized, binding protein had a M_r of about 200,000 by gel chromatography on Sephadex G-200 and a M_r of about 51,000 upon sodium dodecyl sulfate gel electrophoresis after denaturing in the presence of 2-mercaptoethanol. Binding activity was optimal at pH 7–8 and between 23° and 37°C. Significant binding did take place at 1° and 50°C. Relative binding affinities were measured by the inhibition of the binding of radioactive PteGlu. Both 5-CH_3-H_4PteGlu and H_4PteGlu competed equally for binding of PteGlu while pteroic acid and H_2PteGlu competed less well. Binding activity was also identified in the choroid plexus from a number of mammalian species, including humans. Assuming a one-to-one binding ratio and a molecular weight of 51,000, the highest concentration of the binding protein was found in the choroid plexus of the rat.

A binding protein involved in transport has been identified in the plasma membrane of L-1210 cells (10). These are mouse leukemia cells that can be propagated in an ascites form in mice or in tissue culture. They have been used extensively to study folate transport [see (17) for review]. Such studies have led to the conclusion that L-1210 cells contain two separate systems for the transport of folate compounds: one specific for folic acid and a second specific for 5-CH_3-H_4PteGlu, other reduced folate compounds, and the folate analog, methotrexate. Transport studies that show substrate specificity imply the existence of a protein as part of the "carrier" system.

The binding protein on the surface of L-1210 cells was detected by measuring the binding of radioactive 5-CH_3-H_4PteGlu and methotrexate to intact L-1210 cells at low temperatures (4°C). Under these conditions, little internalization of the bound ligand occurs, while at 37°C transport and metabolism of the substrates may take place. Thus experiments at the two different temperatures enable a comparison of the parameters that affect both binding and transport. Using this method, Henderson et al showed that the inhibition constants (K_i) for binding or transport of labeled 5-CH_3-H_4PteGlu by unlabeled 5-CH_3-H_4PteGlu, aminopterin,

5-HCO-H_4PteGlu, methotrexate, and PteGlu were in the same relative order of effectiveness. The absolute values of the K_i's for transport were about 3 times higher than the K_i's for binding, which may be due to the fact that they were performed at different temperatures. Additionally, it was noted that the dissociation constants, K_d, measured for binding of labeled 5-CH_3-H_4PteGlu and methotrexate were 0.11 and 0.35 μM, respectively, which agreed well with the values for the inhibition constants shown by unlabeled 5-CH_3-H_4PteGlu (0.11 μM) and methotrexate (0.44 μM) for the binding of radioactive 5-(CH_3H_4PteGlu. The binding was almost completely reversible at 4°C, and inhibition of binding by various anions was in the same relative order of effectiveness as for inhibitors of transport. About 1 pmol of ligand was bound per mg of cell protein, which corresponded to about 8×10^4 receptors per cell if there is one ligand per receptor. Although the binding protein of L-1210 cells has not yet been isolated, nor even extracted and measured in an in vitro system, the correlation of properties between binding and transport strongly suggests that this is a component of the transport system. Indeed, the authors are careful to refer to this material as a binding component rather than a binding protein. Further studies by this laboratory on the nature of this component are eagerly awaited.

A folate binder has been extensively characterized in cells of human origin grown in tissue culture. McHugh & Cheng used human KB cells grown in either folate-deficient (D) or folate-replete (R) medium (22). A crude membrane (particulate) fraction bound radioactive PteGlu in vitro at pH 7.5. Prior treatment of the fraction at acid pH followed by incubation at pH 7.5 resulted in greater amounts of bound radioactivity, which led to the conclusion that the latter treatment measured total binding capacity while only free (uncovered) binder was measured unless the covered binding sites were first stripped by acid treatment. Another possible conclusion is that the binding is not reversible at pH 7.5. When grown in deficient medium the amount of folate bound by the crude membrane fraction increased from a total of 1,731 to 8,307 pmole/10^8 cells. In replete medium about 5% of the binding sites were saturated while about half were saturated in deficient medium. This increase in the number of binding sites required growth in deficient medium for at least 2 months.

When intact KB cells from deficient medium were incubated with radioactive PteGlu for various periods of time and the cells then disrupted and separated into a crude membrane fraction and soluble fraction, the amount of PteGlu bound to the membranes was much greater than that present in the soluble fraction and did not increase much with time, whereas the amount of soluble folate did increase. These authors also showed that a considerable fraction (55%) of the soluble radioactivity was associated with

a folate polyglutamate fraction (as measured by elution position on a Sephadex G-25 column) after 15 hr incubation of the intact cells with labeled PteGlu. Almost none of the radioactivity bound to membranes appeared to be polyglutamate derivatives at this time. It was not determined how much of the radioactive folate in the soluble fraction was protein bound.

When KB cells grown in deficient medium were incubated with radioactive PteGlu for 16 hr and the crude membrane fractions further separated into plasma membrane, post-mitochondrial (presumably endoplasmic reticulum) membrane, nuclear membrane, and mitochondrial membrane fractions, it was found that most of the radioactivity was associated with the mitochondrial membrane when expressed per mg of protein. On a cellular basis, most of the radioactivity was associated with the plasma membrane. It was not established what form of folate was bound to each of these membrane fractions although it is presumed that it was no longer PteGlu.

McHugh & Cheng (22) also attempted to investigate the specificity of binding by incubating intact KB cells with radioactive PteGlu for 1.5 hr either alone or in the presence of unlabeled analogs. The crude membrane fraction was isolated and the radioactivity was counted. Only those compounds with an intact pteroylglutamate structure inhibited to any significant extent. The experiment was also carried out using a preparation of crude membranes from KB cells solubilized with the non-ionic detergent NP-40. Binding was measured by incubation with labeled PteGlu at 37°C for 15 min. Unbound PteGlu was removed with charcoal and albumin. It was found that 10-CH_3-PteGlu, H_2PteGlu, and H_4PteGlu were the most potent inhibitors. Since these experiments were carried out in the absence of reducing agents to protect reduced folate compounds that were added, the results obtained with these compounds should be viewed with caution. The fact that the entire crude membrane fraction was used as a source of the binder makes interpretation even more difficult since the different types of membranes may well have binding proteins with different specificities.

The purifications of a folate binding protein from human placenta was recently reported by Antony et al (1). This material is referred to as a folate receptor by the authors because it was isolated from a particulate, presumably membrane, preparation of placenta and is therefore believed to be involved in the transport of folate. The particulate preparation was solubilized by the use of Triton X-100, and binding activity was measured in vitro using [^{3}H] PteGlu. Unbound radioactivity was separated by adsorption onto dextran-coated charcoal. Purification was accomplished largely through adsorption onto a folic acid affinity column. A purified glycoprotein was obtained after a 61,000-fold purification. This had a molecular weight of about 38,500 as determined by SDS-polyacrylamide gel electro-

phoresis. Carbohydrate analysis showed that the purified material contained about 12% carbohydrate which was covalently bound. One mole of PteGlu was bound per mole of protein. The binding of PteGlu to the protein was tight, as evidenced by the fact that radioactive PteGlu bound in vitro to the protein did not dissociate during polyacrylamide gel electrophoresis in 0.1% Triton X-100. This tight binding was pH-dependent since endogenous folate was stripped of the crude binder by incubation at pH 3.0.

The purified binding protein had an association constant (K_a) of 3.5 mM^{-1} for PteGlu. The K_a values for 5-HCO-H_4PteGlu and methotrexate were 0.1% and 0.3%, respectively, of the value obtained for PteGlu. Antony et al noted that other reduced folate analoges, H_2PteGlu, H_4PteGlu, and 5-CH_3-H_4PteGlu were less than 90% pure and were unstable during measurement of the K_a. The latter three compounds had K_a values in the range of 5–20% less than that of PteGlu. Such studies indicate a greater affinity of the binding protein for PteGlu than for the natural, reduced folate monoglutamates.

Antiserum raised to the purified binding protein permitted an investigation of the distribution of immunologically similar proteins in various tissues. Immunological cross-reacting material was demonstrated on the membrane of human erythrocytes by an immunofluorescence technique. This antiserum also cross-reacted with both the high- and low-M_r folate binding proteins of human milk (38).

Bacterial Sources

Some of the best evidence that a membrane-associated folate binding protein is involved in transport comes from studies carried out with *Lactobacillus casei.* This microorganism is the one generally used for assay of folates. Much of this has been reviewed recently in detail (17). Briefly, Henderson, Zevely & Huennekens (11, 16) showed that intact cells of *L. casei* rapidly bound radioactive PteGlu at 4°C in a saturable manner. Transport of PteGlu took place at 37°C and required an energy source (16). Compounds such as iodoacetate that block transport had no effect on binding at 4°C. A crude preparation of the folate binding protein was made by Triton extraction of *L. casei* protoplasts. [^{3}H] PteGlu was added to the suspension of protoplasts before Triton extraction, and the radioactivity remained bound to the protein following chromatography on Sephadex G-25. These authors showed that when *L. casei* was grown in media containing various levels of folates, the transport ability was inversely proportional to the folate levels; maximal transport was seen at 2 nM folate, and transport was completely repressed at 1 μM. The binding activity of whole cells and the levels of Triton X-100 solubilized binding protein exactly paralleled the transport activity of the cells grown at different levels of folate. Purification

of the folate binding protein from *L. casei* was achieved simply by adsorption to and elution from microgranular silica (Quso G-32) and chromatography on Sephadex G-25 (12). At this point the protein–folic acid complex was pure but was contained in a Triton micelle with an apparent M_r of 220,000. This micelle could be disrupted by chaotropic agents (SCN^- and ClO_4^-) and by ethanol, resulting in loss of the bound radioactive folic acid. The protein was pure as judged by polyacrylamide electrophoresis in sodium dodecyl sulfate and had an M_r of 25,000. The amino acid composition was notable for the absence of cysteine and the high levels of methionine, tryptophan, and other nonpolar amino acids. There was no detectable carbohydrate. The ratio of PteGlu to protein in the purified preparation was 1:1.

Further studies were carried out using mutants of *L. casei* that were resistant to amethopterin (methotrexate) (13). These cells have a defective transport system for uptake of folic acid and also have a comparable defect in the ability to bind folate. Such results are powerful evidence that the folate binding protein of *L. casei* is involved with the transport of folate into the cell.

INTRACELLULAR FOLATE BINDING PROTEINS

Presence in Erythrocytes

The earliest evidence for the existence of an intracellular folate binding protein is probably the discovery by Iwai, Luttner & Toennies in 1964 (18) that folic acid in human erythrocytes was bound to a macromolecular substance having a M_r of at least 50,000. This was called folate precursor substance and it bound primarily polyglutamates of 5-CH_3-H_4PteGlu, based upon differential microbiological assay with *L. casei, Streptococcus faecium,* and *Pediococcus cerevisiae.* The macromolecule was purified about 700-fold on DEAE cellulose and was clearly separated from hemoglobin. It could be precipitated with ammonium sulfate and was presumably a protein. The partially purified material contained about 30% of the folate originally present in the red-cell hemolysate. Unfortunately, this work was never followed up, and the nature of the protein in human erythrocytes to which the folate is bound remains unknown.

Evidence for Several Folate Binding Proteins in Liver

In 1974, two papers reported the presence of folate binding proteins in rat liver. The first, by Corrocher et al (8), studied the binding of radioactive folate both in vivo and in vitro. The in vitro experiments were carried out by incubating [^{3}H] folic acid with a crude liver homogenate at room temperature. The mixture was then chromatographed on a column of Sephadex

G-75. About 15% of the radioactivity was bound to two macromolecular species eluting at positions roughly corresponding to that shown by the "Y" and "Z" proteins of liver. These "Y" and "Z" proteins were believed to be involved in the binding of organic anions and were shown to have molecular weights of 44,000 and 10,000, respectively (2). In vivo labeling was carried out by injecting [^{3}H] PteGlu intraperitoneally. After 24 hr the animal was killed, its liver was homogenized and centrifuged, and the supernatant was chromatographed on Sephadex G-75. In this case two peaks of bound radioactivity were seen, one at the void volume of the column and the other corresponding to peak "X." They were not further characterized.

The second paper, by Zamierowski & Wagner (43), also utilized in vivo administration of [^{3}H] PteGlu to label the macromolecular species to which folate was bound. Twenty-four hours after injection of the labeled PteGlu into normal rats a large amount of radioactive folate was shown to be associated with a high molecular weight fraction of liver, kidney, and intestine. Most of the radioactivity was found in the liver. A crude fractionation of the liver into nuclei, mitochondria, microsomes, and cytosol by differential centrifugation showed that most of the bound radioactivity was in the nuclear and cytosol fractions. [The activity bound in the nuclear fraction was subsequently shown (45) to be associated with mitochondria that contaminated this fraction in the procedure originally used for subcellular separation.] Chromatography of the cytosol fraction on Sephadex G-150 columns showed three peaks of bound radioactivity: one that eluted close to the void volume of the column with a M_r of about 300,000, a second with a M_r of about 150,000, and a third with a M_r of about 25,000. When the material in the mitochondrial fraction was chromatographed in a similar way it had a M_r of about 90,000. These folate binding proteins are referred to as FBP-CI, FBP-CII, FBP-CIII, and MFBP. The fact that radioactivity could be dissociated from the macromolecular complex by boiling indicated it was not covalently bound, and microbiological assay with and without conjugase treatment showed that the bound material contained primarily polyglutamate forms. Unbound folate primarily consisted of monoglutamates (they supported the growth of *L. casei* without prior hydrolysis). Microbiological assay with *L. casei* and *S. faecium* also provided some information about the type of folate bound to each of the peaks. FBP-CII (M_r of 150,000) was associated with 5-CH_3-H_4PteGlu polyglutamates, while little if any 5-CH_3-H_4PteGlu polyglutamate was found in any of the other peaks. Further characterization of these binding proteins was provided in a subsequent paper (44). The two high molecular weight peaks in the cytosol that contained most of the bound radioactivity (FBP-CI and FBP-CII) and the peak from the mitochondria (MFBP) were shown to be sensitive to trypsin treatment. This together with the fact that binding

was destroyed by boiling indicated that they were probably protein. These proteins were able to bind folates in vitro as well as in vivo as long as the proper (i.e. polyglutamate) form of folate was used. A mixture of labeled, biosynthesized, folate polyglutamates was prepared from liver cytosol or mitochondria of animals previously injected with [^{3}H] PteGlu. The cytosol or mitochondria was boiled to denature the protein, centrifuged, and the respective supernatant incubated at 4°C with unlabeled cytosol or mitochondrial extract. Chromatography on Sephadex G-150 resulted in the same pattern of labeled folate binding proteins as was obtained by in vivo labeling.

The possibility that these intracellular folate binding proteins are enzymes involved in folate metabolism was also considered (36, 43–45). Fractions of the Sephadex G-150 column were assayed to determine whether the folate binding proteins co-eluted with any enzymes known to metabolize folate derivatives. These were dihydrofolate reductase (EC 1.5.1.3), methionine synthase (EC 2.1.1.13), glutamate formiminotransferase (EC 2.1.2.5), 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68), serine hydroxymethyltransferase (EC 2.1.2.1), methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and formyltetrahydrofolate synthase (EC 6.3.4.3). In only one case was there any evidence for co-elution of any of these enzymes with any of the binding proteins. The elution position of the low molecular weight binding protein of liver cytosol (FBP-CIII) partially overlapped that of dihydrofolate reductase. This is a minor binding protein and suggests that at least a portion of the folate binding activity may be due to dihydrofolate reductase.

Evidence for a folate binding protein in bovine liver was also provided by Watabe (37). In the course of purifying the enzyme, dihydropteridine reductase (EC 1.6.99.7), it was found that a high molecular weight material contained a folate compound that was apparently $H_4PteGlu_2$. The $H_4PteGlu_2$ was not covalently bound. This material was called tetrahydrofolate ·protein complex and abbreviated as TFPC. It was detected because it catalyzed the transfer of electrons from NADH to reduce cytochrome c in the presence of purified dihydropteridine reductase. The series of reactions are presumed to take place as follows:

$$\text{Protein-}H_4\text{PteGlu}_2 + Fe^{3+}\ \text{Cyt c} \rightarrow \text{Protein-}H_2\text{PteGlu}_2 + Fe^{2+}\ \text{Cyt c}$$

$$\text{Protein-}H_2\text{PteGlu} + \text{NADH} \xrightarrow[\text{reductase})]{\text{(dihydropteridine}} \text{Protein-}H_4\text{PteGlu}_2 + \text{NAD}^+$$

The reduction of Fe^{3+} Cyt c can take place in the presence of dihydropteridine reductase if free (unbound) $H_4PteGlu_2$ is used, and this formed one

of the bases for its identification. Other evidence for the form of folate bound as $H_4PteGlu_2$ included growth-promoting behavior in several types of microbiological assays, stability of the compound, chromatographic behavior, and conversion to $H_4PteGlu$ after conjugase action. The TFPC was partially purified and shown to have an estimated M_r of about 70,000 by gel filtration. Although this protein was discovered by virtue of its ability to participate in the reduction of cytochrome c and be reduced in turn by dihydropteridine reducatase, Watabe speculated that it may not necessarily function this way in vivo. The subcellular location of TFPC was not determined, but its size was similar to that of the mitochondrial folate binding protein of rat liver (90,000) found by Zamierowski & Wagner (44). It should be noted (see below) that both $H_4PteGlu_5$ and $H_4PteGlu$ were subsequently found as the folate ligands bound to the mitochondrial protein of rat liver (41).

Characterization of a Folate Binding Protein of Liver Cytosol

The folate binding protein of rat liver cytosol that showed a specificity for $5\text{-}CH_3\text{-}H_4PteGlu$ polyglutamates (FBP-CII) was purified to homogeneity by Suzuki & Wagner (36). It was shown to have a M_r of about 150,000 by gel chromatography and sucrose density gradient centrifugation. Electrophoresis under denaturing conditions showed a single protein band of 32,000 daltons, while amino acid analysis indicated a minimum molecular weight of about 35,000. This suggests the protein is composed of four subunits. When isolated, the purified protein contained endogenous $5\text{-}CH_3\text{-}H_4PteGlu_5$ bound to it. In addition, it was shown that radioactive $5\text{-}CH_3\text{-}H_4PteGlu_5$ was the form of radioactive folate specifically bound in vitro by FBP-CII.

We may speculate on the possible role of FBP-CII in the liver. An enzymatic role for this protein appears to be excluded. FBP-CII in vivo contains bound $5\text{-}CH_3\text{-}PteGlu$ polyglutamates almost exclusively (36). The only two enzymes known to participate in the metabolism of $5\text{-}CH_3\text{-}H_4\text{-}PteGlu_n$ are methionine synthase and 5,10-methylenetetrahydrofolate reductase. Both have been shown to use polyglutamate as well as monoglutamate forms of folate, but both of these enzymes clearly separated from FBP-CII by gel chromatography. The folate substrates are also less tightly bound to these enzymes than $5\text{-}CH_3\text{-}H_4PteGlu_5$ is to FBP-CII.

It is possible to make a circumstantial case for FBP-CII serving a storage role in the liver. The major single form of folate in rat liver is $5\text{-}CH_3\text{-}H_4PteGlu_n$—a form metabolically active only for the synthesis of methionine. In vitamin B_{12} deficiency, folate is trapped in this form because of decreased methionine synthase activity. This is the basis of the methyl trap hypothesis to explain the functional folate deficiency that occurs in pernicious anemia (vitamin B_{12} deficiency) (15, 24). In addition, Scott & Weir have recently

speculated on the physiologic function of the "methyl trap" (28) and have suggested that it operates also in folic acid deficiency as well as vitamin B_{12} deficiency. Previous studies carried out by Zamierowski & Wagner measured bound and free folate levels in folate-deficient and pair-fed animals using differential microbiological assay (45). These results showed that during deficiency the free folate pool was most severely depleted (Table 1). The percent of folate in cytosol bound to FBP-CI and FBP-CIII did not change, but the amount bound to FBP-CII increased from 31 to 50%. Likewise, in mitochondria the majority of folate lost during deficiency comes at the expense of the unbound fraction. These data support the hypothesis that 5-CH_3-H_4PteGlu$_n$ accumulates and the "methyl trap" is in operation during folate deficiency. Thus 5-CH_3-H_4PteGlu$_n$ is trapped—bound to FBP-CII. Moreover, when radioactive PteGlu was injected into these folate deficient animals, almost all the radioactivity of the cytosol was associated with FBP-CII, in contrast to the situation in normal animals where most of the bound radioactivity of the cytosol is bound to FBP-CI or is not bound at all (45). 5-CH_3-H_4PteGlu$_5$ is the most abundant single species of all the folate derivatives in rat liver, making up about 31% of the total (27, 34). Liver contains most of the body stores of folates, amounting to about 50% of the total in man (14). Therefore, if 5-CH_3-H_4PteGlu$_n$ can be considered to be a storage form of folate, then the protein that binds it, FBP-CII, may be a storage protein!

Consistent with this hypothesis is the distribution of FBP-CII in various tissues. A radioimmunoassay was established by Cook & Wagner to measure FBP-CII (7). This permits measurement of the apoprotein regardless of the state of saturation with the ligand. These data showed that the liver

Table 1 Folate levels bound to proteins in rat liver in control and folate deficient animals[a]

Cellular constituent	Column fraction[b]	Control		Deficient	
		Folate[c]	Percent[d]	Folate	Percent
Cytosol	FBP-CI	13.0	18	5.2	18
	FBP-CII	21.8	31	14.1	50
	FBP-CIII	7.8	11	3.2	11
	Unbound	27.8	40	5.9	21
Mitochondria	MFBP	7.5	18	4.1	39
	Unbound	33.3	82	6.5	61

[a] Data taken from (45)
[b] The folate binding proteins were separated by Sephadex G–150 chromatography of the cytosol and the mitochondria.
[c] Values are expressed as ng/ml of the peak fraction isolated as above. Measured by microbiological assay with *L. casei* after conjugase treatment.
[d] Values of percent of total folate in either the cytosol or mitochondrial fractions.

contains much greater levels than the kidney, the next most abundant source. The levels of total folate are also much greater in the liver, though they tend to vary with diet (4). Using the value of FBP-CII as determined by radioimmunoassay, and assuming that four moles of 5-CH_3-H_4PteGlu$_5$ can be bound per mole of protein (there are four identical subunits per mole), one can calculate that the total binding capacity of FBP-CII in rat liver is about 12 $\times 10^{-3}$ μmole/g. This is about twice the amount of 5-CH_3-H_4PteGlu$_5$ normally present and provides significant excess to bind additional folate stores. In addition, although FBP-CII contains less bound folate during an period of folate deficiency, measurement of the FBP-CII by radioimmunoassay showed that the protein itself was not decreased. This indicates that it becomes progressively unsaturated during folate deficiency.

Of further interest is the observation that the level of FBP-CII in both liver and kidney is low in rat embryos (7). The levels increase about ten-fold immediately before birth and may reflect the absence of any need for storage in the fetal tissue since nutrients can be obtained from maternal circulation.

The Folate Binding Protein of Liver Mitochondria

The protein-bound folate of mitochondria, first identified by Zamierowski & Wagner (43) and shown to have a M_r of about 90,000 by chromatography on Sephadex G-150, has recently been found to consist of two closely related enzymes not previously recognized to be involved in folate metabolism. Wittwer & Wagner (41) showed that the chemical structure of the folate ligand bound to the partially purified mitochondrial folate binding protein was H_4PteGlu$_5$. H_4PteGlu was also bound to the protein, but this is probably a result of conjugase activity since rapid processing decreased the amount of H_4PteGlu found. The fact that H_4PteGlu was bound permitted the development of a rapid in vitro binding assay to follow purification. When the material eluted from the Sephadex G-150 column was chromatographed on DEAE cellulose, two binding proteins with similar molecular weights were separated. The first was shown to have dimethylglycine dehydrogenase activity (40, 41). It is also capable of oxidizing sarcosine and several other N-methyl amino acids. The second protein was shown to have only sarcosine dehydrogenase activity.

$$\text{Dimethylglycine} \xrightarrow[\text{(Dimethylglycine dehydrogenase)}]{} \text{Sarcosine} + \text{Formaldehyde}$$

$$\text{Sarcosine} \xrightarrow[\text{(Sarcosine dehydrogenase)}]{} \text{Glycine} + \text{Formaldehyde}$$

Final purification of dimethylglycine dehydrogenase (EC 1.5.99.2) and sarcosine dehydrogenase (EC 1.5.99.1) to homogeneity was achieved using

an affinity column of aminohexyl Sepharose to which 5-HCO-H_4PteGlu had been bound. The molecular weights were estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Dimethylglycine dehydrogenase has a M_r of about 89,000, and sarcosine dehydrogenase has a M_r of about 105,000. Both enzymes are flavoproteins in which the flavin is covalently bound (42). This was shown by the characteristic flavin absorbance spectra of the purified enzymes. Injection of [^{14}C]-labeled riboflavin into rats resulted in the incorporation of the label into both purified enzymes. The radioactive flavin, folate binding activity, as well as enzyme activity copurified with each of the two enzymes. The [14C]flavin bound to each of these enzymes was not dissociated by 6 M urea, sodium dodecyl sulfate, boiling or precipitation with 5% trichloroacetic acid.

The properties of dimethylglycine dehydrogenase have been studied in somewhat more detail than those of sarcosine dehydrogenase, though the reactions carried out by both these enzymes are very similar. The specificity of ligand binding to dimethylglycine dehydrogenase has been determined by competition of unlabeled folate analogs for the binding of [^{3}H]-labeled H_4PteGlu. The enzyme displayed the greatest affinity for H_4PteGlu$_5$ followed by H_4PteGlu, 5-HCO-H_4PteGlu, and 5-CH_3-H_4PteGlu. As a group the reduced folates were bound 100-fold tighter than folic acid or methotrexate. Since 5-CH_3-H_4PteGlu is more stable than H_4PteGlu, radioactive 5-CH_3-H_4PteGlu was used to determine the equilibrium constant for binding to dimethylglycine dehydrogenase. Two independent methods gave values of 2 μM for the dissociation constant of 5-CH_3-H_4PteGlu. Values of 0.2 μM and 0.4 μM were calculated for the dissociation constants of H_4PteGlu$_5$ and H_4PteGlu, respectively; 0.90 moles of 5-CH_3-H_4PteGlu were bound per mole of enzyme, indicating a one-to-one stoichiometry. Participation of the flavin moiety in the reaction mechanism was demonstrated by the fact that the flavin spectrum was reduced by the substrate of the enzyme.

The involvement of protein-bound H_4PteGlu$_5$ (or H_4PteGlu) in the mechanism of dimethylglycine dehydrogenase and sarcosine dehydrogenase is suggested in the scheme pictured in Figure 2. Because dimethylglycine dehydrogenase can use both dimethylglycine and sarcosine as substrates, while sarcosine dehydrogenase uses only sarcosine, the same scheme may be used for both enzyme reactions. Electron transfer is shown as occurring first with the production of a Schiff base–type adduct and reduction of the protein bound flavin. If no H_4PteGlu is available, the Schiff base may be hydrolyzed to give free formaldehyde. In the presence of bound H_4PteGlu, the formaldehyde produced by hydrolysis of the Schiff base may react directly with H_4PteGlu to produce 5,10-CH_2-H_4PteGlu. This reaction is well known to occur non-enzymatically (3). The fact that this does take

Figure 2 Scheme for the involvement of $H_4PteGlu$ in the mechanism of action of dimethylglycine and sarcosine dehydrogenase.

place was shown by an experiment in which purified dimethylglycine dehydrogenase was equilibrated with H_4PteGlu of high specific radioactivity (42). The radioactive ligand–protein complex was treated with dimethylglycine as substrate for the enzyme. This was followed immediately by the addition of sodium borohydride, which will reduce any 5,10-CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu. The latter compound is more stable than the former, which dissociates to formaldehyde and H_4PteGlu during chromatography. Radioactive 5-CH_3-H_4PteGlu was detected in this manner, indicating that formaldehyde produced during the reaction is converted to 5,10-CH_2-H_4 PteGlu on the enzyme as shown in Figure 2. The reaction of enzyme-bound H_4PteGlu with formaldehyde may prevent accumulation of the latter, which may be toxic. The 5,10-CH_2-H_4PteGlu thus formed may also be available for subsequent reactions in the mitochrondria (e.g. formation of serine).

The role of folate coenzymes in the oxidation of dimethylglycine and sarcosine has been a matter of speculation for over 25 years. Mackenzie (21) found that mitochondria (made permeable with phosphate) incorporated the N-methyl groups of dimethylglycine and sarcosine into carbon 3 of serine in high yield. Free formaldehyde, however, was not incorporated into carbon 3 of serine under these conditions. It was suggested that "active formaldehyde" was the product of dimethylglycine and sarcosine oxidation which could be directly incorporated into serine (21) and that a folate derivative might be involved in some way (21, 25). Dac & Wriston (9) found that folate deficiency did not impair sarcosine oxidation in isolated mitochondria. (Table 1 shows that the protein-bound folate is not readily lost during folate deficiency.) With partially purified sarcosine dehydrogenase, Mell & Huennekens (23) found that H_4PteGlu was not required for the reaction, and the hypothesis that folate was involved in the oxidative demethylation of dimethylglycine and sarcosine was no longer considered tenable. The fact that both of these enzymes were purified by virtue of their ability to bind H_4PteGlu in spite of the fact that the involvement of folate in these reactions was unknown is indeed a tribute to serendipity.

SUMMARY

It appears that specific functions may be assigned to some of the cellular folate binding proteins with some degree of certainty. Those that are membrane bound or derived from membranes probably have a role in transport of folate molecules into the cell. This is in spite of the fact that the localization of this protein to the plasma membrane has been carried out in only a limited number of cases. The role of the folate binding protein of *L. casei* in transport is much clearer. Bacteria provide the opportunity to obtain

mutants defective in both transport and binding, and such mutants are more difficult to obtain with mammalian cell lines.

The intracellular folate binding proteins have been discovered so far only in liver. The fact that the folate binding proteins in rat liver mitochondria are two enzymes, dimethylglycine dehydrogenase and sarcosine dehydrogenase, suggests that enzyme activities may eventually be discovered for the other intracellular folate binding proteins. This may not be possible, however, and a reasonably strong case has been made that the folate binding protein of cytosol, FBP-CII, serves in a storage role. Such a storage role is difficult to prove since it depends, in part, on the demonstration that the protein becomes progressively less saturated during deficiency—a situation true also for enzymes.

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