HIGH MOLECULAR WEIGHT COMPLEXES OF FOLIC ACID IN MAMMALIAN TISSUES

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SUMMARY: Twenty-four hours after injection of tritiated folic acid into normal rats, a large amount of labeled folates were found to be associated with the high molecular weight fraction of liver, kidney and intestine. The bound folate was associated with three fractions in the liver cell supernatant having approximate molecular weights of ≥ 350,000, 150,000 and 25,000 daltons, respectively. A fourth fraction which had an approximate molecular weight of 90,000 daltons was isolated from the liver nuclear fractions. The bound folates associated with these fractions were almost exclusively polyglutamate forms.

In the early 1960's Iwai, Luttner and Toennies (1) found that folic acid in human erythrocytes was bound to a macromolecular substance of at least 50,000 daltons. This material, called folate precursor substance, bound primarily polyglutamates of 5-CH_3-H_4-Folate. More recently, binding proteins for folates have been identified in human plasma (2,3) human leukemic cells (4), leukocytes (5) and cow's milk (6) but it was not until this year that folate-binding in other tissues was reported (7,8,9).

We would now like to report the identification and partial characterization of several macromolecular-complexes of folate derived from crude rat liver cell supernatant and nuclear fractions. The folate appears to be bound to proteins in a non-covalent manner, is relatively stable, and can be demonstrated by *in vivo* or *in vitro* experiments.

MATERIALS AND METHODS: Normal male Sprague-Dawley rats, fed on Wayne Lab Blox (Allied Mills, Inc., Chicago) were injected i.p. with 25 uCi of ^{3}H-folic acid (Amersham-Searle, generally labeled, 1.2 or 5 Ci/mmole) and sacrificed 24 hours later. The tissues were removed and homogenized in either a mini-Waring blendor in 4 volumes of 0.05 M potassium phosphate buffer, pH 7.0 containing 0.5% ascorbate or, when the isolation of cellular organelles was desired,

in a Potter-Elvejhem homogenizer with a Teflon pestle using 5 volumes of isotonic sucrose with 0.01 M 2-mercaptoethanol (10). This latter procedure provided a crude separation of liver into nuclear, mitochondrial, microsomal and supernatant (58,000 x g for 60 min) fractions.

The distribution of bound and free folate was determined using small columns of Bio-gel P-6. Fractions were collected and counted. Large scale chromatography on Sephadex G-150 was carried out by applying 20 ml of cytoplasm or 10 ml of sonicated nuclei supernatant plus 10 ml of buffer to the column. Molecular weights of the folate complexes were estimated from this column after calibration with known protein standards (11). Microbiological assays of the folate content of various fractions were carried out on boiled samples using Lactobacillus casei and Streptococcus faecalis (12) both before and after treatment with hog kidney conjugase (13).

A mixture of biosynthesized folate derivatives for use in in vitro labeling experiments was obtained by i.p. injection of ^{3}H-folic acid into rats and sacrifice after 24 hours. The livers were removed, homogenized in isotonic sucrose, and fractionated by sedimentation (10). The cell supernatant and nuclear fractions were made 0.1 M with 2-mercaptoethanol and then boiled 10 min to denature protein. The precipitates were removed by centrifugation, and the supernatants of these fractions were used as sources of labeled folate coenzymes (14).

RESULTS AND DISCUSSION: Fig. 1 shows that a significant portion of the label obtained from the liver and kidney but not heart was eluted in the void volume of Bio-gel P-6 columns and corresponds to bound folate. Similar experiments showed that the intestine also contained a significant amount of high molecular weight labeled material but that skeletal muscle and spleen did not.

About 25% of the injected folate was found in the liver after 24 hours. After the liver was homogenized and fractionated, 32.5% of the label was in the nuclei, 20.3% in the mitochondria, 4.5% in the microsomes, and 43% in

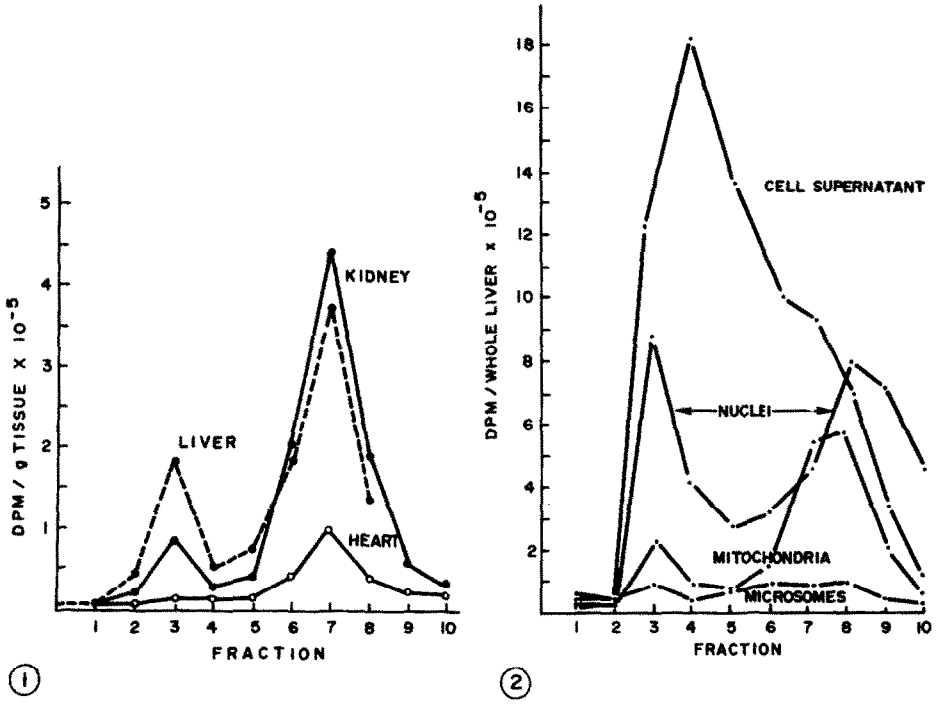

Figure 1. The tissues obtained from a rat which had been injected with 25 μCi of ^{3}H-folic acid (1.2 C/mmole) were homogenized in a mini-Waring blendor and centrifuged at 28,000 x g. The supernatant (0.1 ml) was chromatographed on a 3 ml Bio-gel P-6 column (0.7 x 6.0 cm) using 0.05 M phosphate buffer pH 7 containing 0.5% ascorbate. The fractions (10-drops each) were collected and counted. The columns were calibrated with blue dextran and folic acid.

Figure 2. Samples (0.1 ml) of the cell supernatant (cytoplasm) and the supernatants obtained after centrifugation of sonicated microsomes, mitochondria, and nuclei were chromatographed on 3 ml Bio-gel P-6 columns as in Fig. 1.

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the cell supernatant. The distribution of bound and free folate in these liver fractions, determined by using Bio-gel P-6 columns, is shown in Fig. 2.

Because the liver nuclei and cell supernatant contained most of the label and had the largest percentages of bound folate, further studies of these fractions were undertaken. Portions of each fraction were applied to a calibrated Sephadex G-150 column (Fig. 3). Chromatography of the cell supernatant resulted in the elution of four peaks containing radioactive folate from the column; one in the void volume, two others associated with smaller

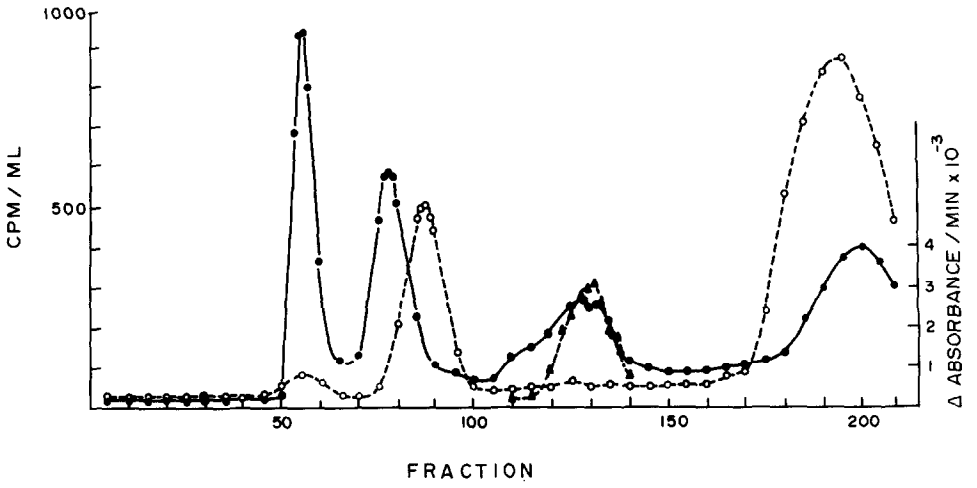

Figure 3. Samples of liver cell supernatant or nuclei (20 ml) were chromatographed on a large Sephadex G-150 column (87 x 5 cm). The column was equilibrated with 0.01 M phosphate buffer, pH 7, containing 0.01 M mercaptoethanol and was run at 0°C using upward flow at a rate of one ml/min. About 220 fractions of 10 ml each were collected, and 1 ml samples were counted. Dihydrofolate reductase was measured using the method described by Osborn and Huennekens and expressed as the change in absorbance/min at 340 nm per 0.1 ml sample. ●——● , radioactivity of cell supernatant; ○——○ , radioactivity of nuclear fraction; ▲——▲ , dihydrofolate reductase activity.

molecular weight species, and a fourth corresponding to unbound folate derivatives. The bound folate peaks will subsequently be referred to as the supernatant I, II, and III peaks and had molecular weights of greater than 350,000, about 150,000, and 25,000 daltons. When the nuclear fraction was applied to the column, two major radioactive peaks were eluted. The first major peak corresponded to bound folate with a molecular weight of about 90,000 daltons and the second corresponded to free folate derivatives.

Because the supernatant III peak had a molecular weight similar to dihydrofolate reductase, which is known to have a high affinity for certain forms of folate (15), assays for this enzyme were performed on the fractions eluted from the column in this region (16). It may be seen that the peak of dihydrofolate reductase activity corresponded closely with the peak of radioactive folate (Fig. 3).

Studies on the stability of the bound folate showed that all binding was

destroyed by boiling for 10 min as determined by chromatography on small Bio-gel P-6 columns. These results suggest that a non-covalent association is involved between the folates and a series of proteins in the cell.

The results of two representative sets of microbiological folate assays on the radioactive peaks eluted from the Sephadex G-150 column are shown in Table I. It can be seen that the bound forms of folate in the cell supernatant and nucleus are primarily folate polyglutamates whereas the unbound folates are mostly monoglutamates. Peak II consistently contained the highest concentration of bound folate in the cell supernatant and was the only peak which contained primarily 5-CH_3-H_4-Folate. In some experiments, the free fraction of the cell supernatant also contained appreciable amounts of 5-CH_3-H_4-Folate.

Studies of *in vitro* binding of labeled natural folate derivatives by cell supernatant and nuclear fractions were carried out. When the cell supernatant fraction from uninjected rats was mixed with equivalent amounts of the labeled biosynthesized folate derivatives, obtained as described under Materials and Methods, and incubated for 10 min at $4^{o}C$, 20 to 40% of the label was bound as determined by chromatography on Bio-gel P-6 columns. When the mixture was chromatographed on the Sephadex G-150 column, all three peaks from the cell supernatant were labeled, but the labeling of peak II was proportionately less than seen during the *in vivo* experiments. Similar *in vitro* binding using the supernatant of sonically disrupted nuclei resulted in binding of about 20 to 30% of the labeled material. Sephadex G-150 chromatography showed that the label was bound primarily to the same nuclear binding protein as in the *in vivo* binding studies. The rapid binding seen in these *in vitro* experiments may reflect the fact that the ligand used is a mixture of natural folate derivatives which are mainly polyglutamate forms (14) and not folic acid itself which is absent in most tissues.

Corrocher *et al.* (8) have also recently described several folate binding proteins in rat liver cytosol and suggest that these binding proteins may

TABLE I: Folate Composition of Radioactive Peaks[a]

Peak	Exp	Total Folate[b]	5-CH_3-H_4-Folate[c]	Other Folate[d]	% of Folate as Polyglutamates[e]
Cell Supernatant I	1	12	0	12	100
	2	10	0	10	94
Cell Supernatant II	1	42	34	8	96
	2	19	11	8	97
Cell Supernatant III	1	10	0	10	92
	2	8	0	8	94
Cell Supernatant Free	1	24	2	22	21
	2	41	25	16	2
Nuclear Bound	1	13	0	13	92
	2	11	0	11	95
Nuclear Free	1	37	0	37	19
	2	26	8	18	10

a. Material for assay was obtained from the central portion of the peaks illustrated in Fig. 3. Values are ng/ml.
b. Measured by microbiological assay using L. casei after conjugase treatment.
c. Measured by microbiological assay after conjugase treatment as the difference between values obtained with L. casei and S. faecalis.
d. Measured by microbiological assay using S. faecalis after conjugase treatment.
e. Measured as the difference in microbiological assay using L. casei before and after conjugase treatment divided by the total folate multiplied by 100.

function in the uptake of folate into the tissues. The predominant form of folate in the plasma is the monoglutamate form of 5-CH_3-H_4-Folate (13). If the folate binding proteins of the cytoplasm were simply serving to facilitate transport by binding a freely diffusable form of folate in the plasma, then one would expect the predominant form of folate bound to protein to be 5-CH_3-H_4-Folate monoglutamate. It is the polyglutamate forms of folate which are primarily bound, however, (Table I). These results suggest that the protein bound forms may have roles other than simply serving as a tissue binder. There has been speculation about the possibility that the polyglutamate forms of folate are the functional coenzyme forms (17,18). The observations (Fig. 3) that the supernatant peak III co-chromatographs with

dihydrofolate reductase and that the polyglutamate forms of folate are primarily bound to this peak is consistent with this idea.

The ultimate assignment of functional roles to these proteins which bind folic acid in its various forms must await their purification and study of their properties. Such studies are now being carried out in our laboratory.

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