The Distribution and Chemical Nature of Radioactive Folates in Rat Liver Cells and Rat Liver Mitochondria

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Subcellular fractionation of rat liver cells revealed that a mixture of ^{14}C - and ^3H -labelled folic acid was distributed approximately equally between the mitochondria and cytosol 2, 24, 48 and 72 h after oral administration. Subfractionation of liver mitochondria 48 h after oral administration showed that the radioactivity was mainly associated with the inner membrane (27.7%) and matrix (51.5%). Hot-ascorbate extraction of the cell cytosol, mitochondrial inner membrane and matrix showed the majority of folates were present as polyglutamates. Acid treatment of isolated folates from cytosol, inner membrane and matrix produced breakdown products consistent with scission of tetrahydrofolates. The folates isolated in the mitochondrial matrix were bound to protein that had an estimated mol. wt. of 90000.

The subcellular distribution of folates and folate-linked enzymes in liver has previously been investigated in the mouse (Siva-Sankar *et al.*, 1969), guinea pig (Corrocher & Hoffbrand, 1972), sheep (Gawthorne & Smith, 1973) and rat (Brown *et al.*, 1965; Wang *et al.*, 1967; Shin *et al.*, 1976; Zamierowski & Wagner, 1974, 1977*a,b*). In all cases the majority of the folates were found in mitochondria and the cytosol. Time-course studies have shown that initially there are significant concentrations of folate associated with the microsomal fraction that decrease markedly with time (Corrocher & Hoffbrand, 1972; Shin *et al.*, 1976; Zamierowski & Wagner, 1977*a*).

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The folates in rat, guinea-pig and sheep livers were reported to be predominantly reduced polyglutamate forms.

The present paper describes an investigation into the distribution of a mixture of $[2\text{-}^{14}\text{C}]$ folic acid and $[3',5',7,9\text{-}^3\text{H}]$ folic acid (Fig. 1) in rat liver subcellular fractions and mitochondria after oral administration. The use of a mixture of radioactively labelled folic acids should reveal any scission of folate molecules by showing a segregation of radioactive label recovered in cellular fractions and mitochondrial subfractions. Barford *et al.* (1977*a,b*), Connor *et al.* (1977) and Murphy *et al.* (1976) have shown that in both short- and long-term experiments there are folate-scission products in both rat urine and faeces. Chemical investigations of the folates in the mitochondrial matrix

Fig. 1. Structures of (a) $[2\text{-}^{14}\text{C}]$ folic acid and (b) $[3',5',7,9(n)\text{-}^3\text{H}]$ folic acid to show the positions of radioactive labels
 * represents ^{14}C in (a) and ^3H in (b).

and inner membrane indicate that the majority are protein-bound tetraglutamate derivatives of 10-formyltetrahydrofolate. Similar investigations suggest that the major portion of the cytosol folate is protein-bound 10-formyltetrahydrofolate tetraglutamate. The subcellular distribution of folic acid in rat liver was also investigated by using a centrifugation procedure that gives purer fractions than in previous reports.

Materials and Methods

Animals

Male Wistar rats were orally given a mixture of [2-¹⁴C]folic acid and [3',5',7,9-³H]folic acid (Fig. 1) in the ratio 1 μ Ci of ¹⁴C : 2.5 μ Ci of ³H/100 g body wt. Animals were then housed in cages designed to prevent coprophagy. Animals were allowed food and water *ad libitum* during the experiment except for 16 h before killing, when they were starved. Starvation decreased the concentration of liver glycogen, which facilitated easier cellular and mitochondrial fractionation.

Subcellular fractionation of livers

(a) *Fractionation.* Livers were fractionated by a modified method of Fleischer & Kervina (1974) to give the following fractions: nuclei, mitochondrial fraction 1, mitochondrial fraction 2 + lysosomes, microsomal fraction and cell supernatant. Homogenates were prepared in 5 vol. of 0.25 M-sucrose/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5, as described for fresh liver by Fleischer & Kervina (1974) except that the homogenate was filtered through two layers of muslin.

All centrifugation steps (Fig. 2a) were performed on an MSE Superspeed 50TC centrifuge at 0–4°C. The filtered homogenate was centrifuged at 3000 rev./min in an 8 × 50 ml angle rotor for 10 min (1000g at r_{av} , 7.62 cm). The supernatant (S_1) was decanted through four layers of muslin to remove congealed lipid and was used for the isolation of mitochondria, lysosomes, microsomal fraction and cell supernatant. The pellet (R_1) was used for the isolation of nuclei and mitochondria. R_1 was resuspended to a total volume of 30 ml in 2.4 M-sucrose/10 mM-Hepes/1 mM-MgCl₂, pH 7.5. This procedure was performed in the centrifuge tube (50 ml) in which the pellet was obtained by using a hand-operated loose-fitting Teflon pestle. The resulting suspension was carefully overlaid with about 15 ml of 0.25 M-sucrose/10 mM-Hepes/1 mM-MgCl₂, pH 7.5, to fill the tube. The tubes were centrifuged at 30000 rev./min in an 8 × 5 ml angle rotor for 70 min (76000g at r_{av} , 7.62 cm). The nuclei formed a small white pellet, whereas the mitochondria formed a 'button' on the side of the tube at the 2.4 M/

0.25 M interface. The 0.25 M-sucrose was removed by aspiration and the mitochondrial 'button' was freed from the side of the tube with a spatula and poured with a minimum volume of 2.4 M-sucrose into a homogenizer tube. Mitochondria were resuspended in 30–40 ml of 0.25 M-sucrose/10 mM-Hepes/1 mM-EDTA, pH 7.5, and centrifuged at 17000 rev./min in an 8 × 50 ml angle rotor for 10 min (25000g at r_{av} , 7.62 cm). The resulting pellet was resuspended in about 5 ml of 0.25 M-sucrose, pH 7.0, and is referred to as mitochondrial fraction 1. The white pellet containing nuclei was washed and resuspended in 3 ml of 2.2 M-sucrose/1 mM-MgCl₂, pH 7.0.

Fraction S_1 was centrifuged at 17000 rev./min in an 8 × 50 ml angle rotor for 10 min (25000g at r_{av} , 7.62 cm). The supernatant (S_2) was decanted and used in the isolation of microsomal fraction and cell supernatant. The pellet (R_2) was resuspended in 0.25 M-sucrose/10 mM-Hepes/1 mM-EDTA, pH 7.5, and re-centrifuged at 17000 rev./min for 10 min (25000g at r_{av} , 7.62 cm). The supernatant was discarded and the pellet resuspended in about 5 ml of 0.25 M-sucrose, pH 7.0. This fraction is referred to as mitochondrial fraction 2.

Fraction S_2 was centrifuged at 50000 rev./min in a 10 × 10 ml angle rotor for 60 min (157000g at r_{av} , 5.61 cm). The supernatant was decanted and is referred to as cell supernatant. The pellet was washed with 0.25 M-sucrose, pH 7.0, and resuspended in about 5 ml of 0.25 M-sucrose, pH 7.0. This is termed the microsomal fraction.

The above procedure gives pure subcellular fractions with the exception that mitochondrial fraction 2 contains the majority of cell lysosomes. All fractions and a sample of the homogenate were stored at –20°C before determination of radioactivity, protein content and enzyme-marker activities.

(b) *Enzyme assays.* Fractions were assayed for protein by using the biuret method (Layne, 1957) with bovine serum albumin (fraction V) as standard. Succinate-cytochrome *c* reductase was the mitochondrial marker enzyme and was assayed by the method of Tisdale (1967). The microsomal marker enzyme was rotenone-insensitive NADH-cytochrome *c* reductase. The assay was performed as for succinate-cytochrome *c* reductase, but with 0.1 M-Tris/HCl buffer, pH 8.5; NADH (0.2%) was the substrate and 4 μ g of rotenone in 4 μ l of ethanol was added as the inhibitor. The lysosome marker enzyme was acid phosphatase, which was measured by the method of Trouet (1974). Lactate dehydrogenase was used as the cytosol marker and was assayed as described by Stolzenbach (1966).

Submitochondrial fractionation

(a) *Subfractionation.* Liver mitochondria from rats that were given a mixture of ³H- and ¹⁴C-labelled folic

Fig. 2. Fractionation schemes for (a) rat liver and (b) rat liver mitochondria

acid 48 h before being killed were subfractionated to give: outer membranes, soluble intermembrane proteins, inner membranes and soluble matrix proteins by the method of Greenwalt (1974) (Fig. 2b). The method involves the removal of the outer membrane by digitonin followed by the disruption of the inner

membrane with Lubrol WX. The isolation medium used in the procedure contained 70 mM-sucrose, 220 mM-DL-mannitol, 2 mM-Hepes and 0.5 mg of bovine serum albumin (fraction V, defatted)/ml. The medium was adjusted to pH 7.4 with KOH before use. All centrifugation steps and incubation steps were

performed at 0–4°C. Centrifugation was performed on an MSE Superspeed 50TC centrifuge with an 8 × 50ml angle rotor for low-speed (up to 11 000 rev./min) spins and a 10 × 10ml angle rotor for the high-speed (42 500 rev./min) spins.

(b) *Enzyme markers.* Kynurenine hydroxylase (EC 1.14.13.9) was the marker enzyme for the outer membrane and was assayed by the method of Okamoto (1970). The soluble intermembrane proteins were characterized by adenylate kinase measured by the method of Sottocasa *et al.* (1976). The inner-membrane marker was succinate-cytochrome *c* reductase, which was assayed by the method of Tisdale (1967). Glutamate dehydrogenase was used to characterize the matrix proteins and was measured by the method of Sottocasa *et al.* (1976). The degree of lysosome contamination of mitochondria and sub-fractions was estimated by assaying acid phosphatase in the fractions (Trouet, 1974). In all cases the activity of acid phosphatase in the starting mitochondrial suspension was below 10% of the total liver homogenate. Acid phosphatase activity was only detected in the soluble intermembrane-protein fraction.

Measurement of radioactivity in homogenates, sub-cellular and submitochondrial fractions

Samples (200 µl) were oxidized in a Beckman biological-material oxidizer. The oxidation products were passed through a solid CO₂/methanol-cooled water trap, which removed ³H₂O, and then into 15 ml of Fisons Absorber 'P' (Fisons, Loughborough, Leics., U.K.), a scintillation 'cocktail' designed for collection of ¹⁴CO₂. The condensed ³H₂O in the cold trap was melted and mixed with 10 ml of Fisons Absorber 'H'. Samples were oxidized in duplicate together with appropriate controls. Samples were counted for radioactivity in a Nuclear Enterprises liquid-scintillation counter (type NE8310). Appropriate corrections were made for quenching and background.

Column chromatography

(a) *Sephadex G-15 chromatography.* This procedure was performed as described by Barford *et al.* (1977b). The columns were calibrated with authentic folate-monomonoglutamates, pterin and *p*-aminobenzoyl-glutamate, which were detected either by an LKB Uvicord II and a chart recorder (LKB Instruments, Croydon, Surrey, U.K.) or by spectrophotometric analysis with a Pye-Unicam SP.1800 u.v. spectrophotometer (Pye Instruments, Cambridge, U.K.).

(b) *Sephadex G-75 chromatography.* Samples were applied to a column (0.8 cm² × 30 cm) of Sephadex G-75 in 0.05 M-phosphate buffer, pH 7.0, containing 5 mg of dithiothreitol/100 ml of buffer. Columns were eluted with the same buffer. Fractions (30 × 2 ml) were collected from each column.

(c) *Sephadex G-150 chromatography.* Samples were applied to a column (4 cm² × 60 cm) of Sephadex G-150 in 0.05 M-phosphate buffer, pH 7.0, containing 5 mg of dithiothreitol/100 ml of buffer. Columns were eluted with the same buffer and fractions (100 × 3 ml) were collected from each column. The molecular weights of folate-protein complexes were estimated by calibrating this column with the following protein standards: cytochrome *c*, α-chymotrypsinogen A, bovine serum albumin (fraction V), bovine γ-globulins, equine apoferritin and Dextran Blue 2000 as described by Andrews (1966). [All the proteins used for column calibration were supplied by Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.]

The positions of peaks of radioactivity on columns were determined by counting the radioactivity in 0.5 ml or 1 ml portions of each fraction in a liquid-scintillation counter by using 10 ml of a 'cocktail' of toluene (1000 ml), Fisons Emulsifier Mix no. 1 (500 ml), 2,5-diphenyloxazole (5.0 g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g). Appropriate corrections for background and quenching were made. Unless otherwise stated the recovery of radioactivity from columns was complete.

Extraction of folates from binding proteins

Samples of protein-bound folates were extracted by one of two methods. (1) Samples of partially purified proteins or mitochondrial matrix proteins were added to cold (4°C) trichloroacetic acid (8%, w/v). Precipitates were removed by centrifugation and the supernatant was adjusted to pH 7 with conc. NaOH. (2) Samples were added to boiling 0.05 M-phosphate buffer, pH 7.0, containing 2% (w/v) sodium ascorbate and 5 mg of dithiothreitol/100 ml, and maintained at 100°C for 10 min. Precipitated protein was removed by centrifugation after cooling the extract to room temperature.

Chemicals and reagents

[2-¹⁴C]Folic acid (sp. radioactivity 58.2 µCi/µmol) and [3',5',7,9(n)-³H]folic acid (sp. radioactivity 500 µCi/µmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

All chemicals used for buffers and isolation media were AnalaR grade. All biochemicals used for enzyme assays and isolation procedures were obtained from Sigma, Poole, Dorset, U.K. The exceptions were: digitonin (AnalaR) was obtained from BDH Chemicals, Poole, Dorset, U.K.; 5-formyltetrahydrofolate (calcium leucovorin) was obtained from Lederle Laboratories, London, U.K.; *p*-aminobenzoyl-L-glutamate was supplied by Koch-Light, Colnbrook, Bucks., U.K.; 5-methyltetrahydrofolate was supplied by Eprova Research Laboratories, Schaffhausen,

Switzerland; 10-formylfolic acid was prepared by the method of Blakley (1959).

Results

Subcellular distribution of ^{14}C - and ^3H -labelled folic acid in rat liver

Groups of three male rats were orally given a mixture of ^{14}C - and ^3H -labelled folic acid and killed

after 2, 24, 48 or 72 h. The livers were rapidly excised and fractionated as described in the Materials and Methods section. The results of cell fractionations are shown in Table 1. The lysosomes were mainly associated with the mitochondrial fraction 2, as shown by the distribution of acid phosphatase. Radioactivity in the various fractions was determined as described in the Materials and Methods section and the results are shown in Table 2. The majority of the radioactivity recovered from 2 to 72 h was found in the

Table 1. *Distribution of marker enzymes in liver fractions*

Each percentage is the mean for results from three animals.

		Activity (% of the total activity recovered in the fractions)					
	Fraction ...		Mitochondrial fraction 1	Mitochondrial fraction 2	Nuclei	Microsomal fraction	Cytosol
	Time (h)	Activity recovered from homogenate (%)					
Protein	2	66.3	33.1	12.1	4.6	14.9	35.3
	24	61.4	28.1	11.8	4.5	15.5	40.1
	48	64.0	22.1	9.5	5.6	17.1	45.7
	72	62.0	34.1	14.5	2.4	14.4	34.6
Succinate-cytochrome c reductase	2	42.3	69.2	28.7	2.1	0.0	0.0
	24	18.7	72.1	25.1	2.8	0.0	0.0
	48	67.3	70.9	26.9	2.2	0.0	0.0
	72	42.5	84.3	15.7	0.0	0.0	0.0
Rotenone-insensitive NADH-cytochrome c reductase	2	74.1	10.8	14.0	1.5	62.1	11.6
	24	48.0	11.3	10.6	2.2	68.2	7.7
	48	63.7	5.5	7.9	2.7	75.0	8.9
	72	59.8	9.6	22.7	0.3	57.3	10.1
Acid phosphatase	2	41.0	14.3	40.6	1.2	27.5	16.4
	24	57.2	13.9	57.0	1.5	17.4	10.2
	48	13.1	13.1	49.3	2.9	17.0	17.7
	72	15.7	15.7	51.0	0.6	14.9	17.8
Lactate dehydrogenase	2	82.0	0.5	1.3	0.5	4.6	93.1
	24	58.0	1.2	1.6	0.3	8.8	88.1
	48	78.0	1.2	0.7	0.5	6.8	90.8
	72	66.0	0.0	1.1	0.3	11.5	87.1

Table 2. *Distribution of ^3H and ^{14}C radioactivity in liver fractions*

Radioactivity is expressed as a percentage of the total radioactivity recovered in the fractions. Each percentage is the mean for results from three animals.

		Radioactivity (% of total radioactivity recovered in the fractions)				
	Fraction ...		Mitochondrial fraction 1	Mitochondrial fraction 2	Nuclei	Cytosol
	Time (h)					
^3H	2	27.3	12.1	0.8	7.0	52.8
	24	42.2	9.4	0.8	1.3	46.3
	48	33.0	12.0	1.2	1.8	52.0
	72	42.0	10.1	0.7	1.8	45.4
^{14}C	2	27.1	12.5	0.7	7.9	51.8
	24	43.9	9.0	0.9	1.0	45.2
	48	33.7	11.5	1.1	1.7	52.0
	72	37.8	10.1	0.7	2.6	48.8

mitochondrial and cytosol fractions with about equal amounts in each. A significant amount of radioactivity was associated with the microsomal fraction after 2h; this radioactivity decreased after 24h. Only a trace of radioactivity was found in the nuclei at all times.

The percentage recoveries of ^{14}C and ^3H in each fraction indicated that there was no apparent scission of the folate molecule in the various subcellular fractions up to 72h after oral administration.

The distribution of radioactivity shows that 2h after oral administration a substantial amount of folate had been accumulated into the mitochondria of the liver via the gut and suggests the presence of a specific transport system for folates into liver cells and into mitochondria.

Column chromatography of cytosol fractions

Chromatography of cytosol fractions on Sephadex G-15 showed three major peaks of radioactivity 2h after oral administration: (1) a mixed-label ^{14}C and ^3H peak close to the void volume; (2) a ^3H peak that was eluted close to *p*-aminobenzoylglutamate; (3) a ^{14}C peak that was eluted in the same region as pterin. Sephadex G-15 chromatography of the 24h, 48h (Fig. 3a) and 72h cytosol fractions revealed one peak close to the void volume, which contained equal percentages of ^{14}C and ^3H , indicating an intact folate.

Sephadex G-15 chromatography of acid-treated (see the Materials and Methods section) ^{14}C - and ^3H -labelled peak isolated from the void volume Sephadex G-15 column fractions from 24h and 48h (Fig. 3b) and 72h cytosol fractions showed two singly labelled peaks: (1) a ^3H peak that was eluted in the same region as *p*-aminobenzoylglutamate and (2) a ^{14}C peak that was eluted in the same region as pterin. In all the elution profiles there was a small mixed-label peak that was eluted in a similar region to 10-formylfolate. Extraction of the same ^{14}C and ^3H peaks with boiling phosphate buffer (0.05M) plus ascorbate (2%) as described in the Materials and Methods section followed by Sephadex G-15 chromatography showed similar results to those of acid treatments except that the mixed-label peak was absent (Fig. 3c). Sephadex G-15 chromatography of a boiling-ascorbate extract of whole cytosol showed two mixed-label peaks, one close to the void volume and another that was eluted in the same region as 5-methyltetrahydrofolate (Fig. 3d). Small amounts of singly labelled scission products, similar to those described above, were also observed.

These results show that the cytosol folates at 24, 48 and 72h are polyglutamates that are predominantly bound to proteins. The scission products obtained by acid treatment and boiling indicate that the folates isolated by Sephadex G-15 chromatography were tetrahydrofolate derivatives.

Fig. 3. Sephadex G-15 chromatography of radioactivity from rat liver cytosol

(a) Chromatography of the 48h cytosol fraction. (b) The high-molecular-weight peak from (a) treated with acid and rechromatographed. (c) Hot-ascorbate extraction of the high-molecular-weight peak from (a) rechromatographed. (d) Hot-ascorbate extraction of 48h cytosol fraction. Elution positions of authentic compounds are: 1, *p*-aminobenzoylglutamate; 2, 10-formylfolate; 3, 5-formyltetrahydrofolate; 4, 5-methyltetrahydrofolate; 5, pterin (which was eluted in a broad band). Symbols; ●, ^3H radioactivity; ○, ^{14}C radioactivity.

Distribution of ^{14}C - and ^3H -labelled folates in liver mitochondria

Male rats were orally given a mixture of ^3H - and ^{14}C -labelled folic acid, as described in the Materials and Methods section, and killed after 48h. Mitochondria were prepared from the liver and sub-fractionated as previously described (see the Materials and Methods section). The results of subfractionation

(Table 3) show a good separation of mitochondrial components. The contamination of the inner-membrane fraction with the matrix enzyme marker, glutamate dehydrogenase, may have resulted from non-specific binding to membrane fragments. Suspension of membrane fragments in isolation medium followed by resedimentation showed a 97% decrease in glutamate dehydrogenase activity in the inner-membrane fraction. The amount of ^{14}C and ^3H associated with the inner membrane was only decreased by 20% during the washing procedure.

The distribution of radioactivity in mitochondrial subfractions (Table 3) shows very little radioactivity (2.5%) associated with the outer membrane. Approx. 18% of the radioactivity was associated with the soluble intermembrane proteins. The majority of the recovered radioactivity was associated with the mitoplast fraction: 27.7% with the inner membrane and approx. 52% in the matrix. In all fractions the percentage recoveries of ^{14}C and ^3H were almost identical, indicating intact folate molecules.

Column chromatography of the mitochondrial matrix

Sephadex G-15 chromatography of the mitochondrial matrix showed a single mixed-label peak of radioactivity close to the void volume in which the percentage recoveries of ^{14}C and ^3H were exactly matched. Chromatography of a matrix sample on Sephadex G-75 resulted in a mixed-label peak of radioactivity close to the void volume that accounted for 35% of the recovered radioactivity. The remainder of the radioactivity was recovered in two singly labelled overlapping peaks that were eluted in the same region as folate monoglutamates. A similar result was obtained if a sample of the mixed-label peak from a Sephadex G-15 column was run on Sephadex G-75. Chromatography of the pooled singly labelled peaks from the Sephadex G-75 column on Sephadex

G-15 showed a ^3H peak that was eluted in a similar position to *p*-aminobenzoylglutamate and a ^{14}C peak that was eluted in a similar position to pterin.

Treatment of the high-molecular-weight mixed-label peak from Sephadex G-15 chromatography with trichloroacetic acid (8%, w/v) (as described in the Materials and Methods section) produced the same results as acid treatment of the mixed-label peak from the cytosol. Sephadex G-15 chromatography of the neutralized acid extract showed two peaks, one ^3H -labelled and the other ^{14}C -labelled, which were eluted in the same region as *p*-aminobenzoylglutamate and pterin respectively. Also present was a minor mixed-label peak that was eluted close to 10-formylfolate.

Extraction of the mitochondrial matrix with boiling phosphate buffer (0.05M; pH 7.0) plus ascorbate (2%, w/v) as described in the Materials and Methods section, followed by Sephadex G-15 chromatography, showed the majority of the radioactivity eluted as a mixed-label peak close to the void volume. Two minor singly labelled peaks were also eluted in similar positions to those noted above. Chromatography of the mixed-label high-molecular-weight peak on Sephadex G-75 produced two overlapping singly labelled peaks similar to those noted above for Sephadex G-75 chromatography of the matrix, which suggests scission of the folate molecule. Treatment of the polyglutamate fraction obtained by hot-ascorbate extraction of matrix and Sephadex G-15 chromatography with trichloroacetic acid followed by Sephadex G-15 chromatography showed a mixed-label peak of radioactivity close to the void volume, which contained approximately twice the recovered percentage of ^3H (44.2%) compared with ^{14}C (23.2%). The remainder of the recovered radioactivity was present in a ^3H peak and a ^{14}C peak that were eluted in similar positions to *p*-aminobenzoylglutamate and pterin respectively. A minor mixed-label peak that were eluted close to 10-formylfolate was also recovered.

Table 3. Distribution of enzyme markers and radioactivity in liver mitochondrial subfractions

Rats were orally given a mixture of ^3H - and ^{14}C -labelled folic acid and were killed 48 h later. The livers were rapidly excised and mitochondria were subfractionated as described in the Materials and Methods section. Enzymes and protein were assayed, and radioactivity was determined, as described in the Materials and Methods section. The results are expressed as a percentage of the total activity/radioactivity recovered in the fractions and are the means for five experiments.

Fraction	Activity/radioactivity (% of total recovered in the fractions)			
	Outer membrane	Intermembrane proteins	Inner membrane	Matrix
Protein	19.3	14.5	31.5	34.7
Kynurenine hydroxylase	84.4	9.1	5.4	1.1
Adenylate kinase	3.8	89.0	1.0	6.2
Succinate-cytochrome <i>c</i> reductase	9.9	0.1	89.5	0.5
Glutamate dehydrogenase	1.2	15.4	29.9	53.5
^3H	2.2	17.9	27.7	52.2
^{14}C	2.5	18.8	27.7	50.9

These results show that the majority of matrix folates are polyglutamates and are bound to protein(s) that has a molecular weight in excess of 70000. The unstable nature of the isolated folatepolyglutamates suggests that they may be tetrahydrofolate derivatives and that they are stabilized *in vivo* by a binding protein(s).

Estimation of the molecular weight of the mitochondrial matrix-binding protein

Zamierowski & Wagner (1974, 1977a) showed that, in a sonicated extract of mitochondria, folate was bound to a protein with a mol.wt. of 90000. The folate present in the mitochondrial matrix was shown to be present in a high-molecular-weight peak when chromatographed on Sephadex G-15. The molecular weight of this folate species was determined by chromatography on Sephadex G-150 as described in the Materials and Methods section. Approx. 25% of the recovered ^{14}C and ^3H radioactivity was eluted at a position consistent with a mol.wt. of 90000. The remainder of the radioactivity was eluted as two separate ^3H and ^{14}C peaks in the same region as folate monoglutamates. Chromatography of the pooled ^3H and ^{14}C peaks of radioactivity on Sephadex G-15 showed two separate ^3H and ^{14}C peaks that were eluted in similar positions to *p*-aminobenzoyl-glutamate and pterin respectively.

Investigation of the nature of the folates associated with the mitochondrial inner membrane

The recovery of radioactivity with the inner-membrane fraction indicated that intact folate molecules were present. Washing and resedimentation of the membrane fragments released only 20% of the radioactivity (see above). Acid extraction of the inner membrane and subsequent Sephadex G-15 chromatography of the neutralized extract showed that the majority of ^3H radioactivity was eluted in two peaks, one of high molecular weight (20%) and the other (36%) in a similar position to *p*-aminobenzoyl-glutamate. The majority of the ^{14}C radioactivity (55%) was eluted as a single peak in a position similar to pterin. There was also a minor mixed-label peak that was eluted near 10-formylfolate.

Sephadex G-15 chromatography of a boiling-ascorbate extract of the inner membrane showed three peaks: (1) a mixed-label peak that was eluted close to the void volume and that accounted for approx. 40% of the recovered ^3H and ^{14}C radioactivity; (2) a ^3H peak that was eluted in a similar position to *p*-aminobenzoylglutamate (33% of recovered ^3H); (3) a ^{14}C peak that was eluted in a similar region to pterin (30% recovered ^{14}C).

These results are very similar to those obtained for the mitochondrial matrix and suggest that the folates

as isolated are present as protein-bound tetrahydro-folatepolyglutamates.

The recovery of radioactivity from the inner-membrane fragments was approx. 40% for acid extraction and 25% for boiling-ascorbate extraction, which suggests that the folates are firmly attached to the inner membrane. Acid and boiling-ascorbate extraction procedures performed on the cytosol and mitochondrial matrix always gave at least 90% recovery of radioactivity from the starting material.

Discussion

The recoveries of ^3H and ^{14}C radioactivity after oral administration in all subcellular and submitochondrial fractions strongly suggests that in the liver there was little or no breakdown of folates. The majority of the radioactivity from Sephadex G-15 column chromatography of cytosol and mitochondrial matrix was recovered as mixed-label high-molecular-weight peaks, suggesting either polyglutamate forms, protein-bound polyglutamates or protein-bound monoglutamates or a mixture of all three. Boiling-ascorbate extracts of the matrix showed the majority of folates to be present as polyglutamates, whereas the same extraction procedure revealed both polyglutamates and 5-methyltetrahydrofolate when applied to samples of cytosol. The radioactivity recovered from the cytosol 2 h after oral administration contained only a small proportion of high-molecular-weight folate and a large amount of singly labelled scission products, suggesting that reduced folates were present and that polyglutamate biosynthesis follows the reduction of accumulated folates at a low rate (Blair, 1976).

The extent of binding of folates to mitochondrial matrix protein remains unresolved. Sephadex G-15 chromatography showed the majority as high-molecular-weight derivatives, but did not distinguish between polyglutamates or protein binding. Sephadex G-75 and G-150 chromatography showed that approx. 40% and 25% respectively of matrix radioactivity was associated with protein. The result of Sephadex G-150 chromatography showed that the folate was bound to a single protein that had a mol.wt. of 90000. This result agrees with that of Zamierowski & Wagner (1974, 1977a). The reason for the differences between the amounts of high-molecular-weight folate-protein complex may be that it is loosely bound to the protein and that passage through the gel results in dissociation of the folate-protein complex. The dissociation was then followed by scission of the folate molecule.

Extraction of mitochondrial matrix with boiling ascorbate and subsequent Sephadex G-15 chromatography showed that the majority of folate was present as polyglutamate. The data obtained from Sephadex G-75 chromatography and acid extraction of matrix

polyglutamates show them to be highly unstable, yielding scission products consistent with the breakdown of tetrahydrofolate polyglutamates.

Acid treatment of the high-molecular-weight mixed-label peak of radioactivity isolated by Sephadex G-15 chromatography from cytosol or mitochondrial matrix resulted in complete scission of the folate molecule to give ^3H -labelled fragments that were eluted close to *p*-aminobenzoylglutamate and ^{14}C -labelled fragments that were eluted in the same region as pterin. These results indicate that the folate present was a tetrahydrofolate derivative. Pearson (1974) and Blair & Pearson (1974a) have shown that tetrahydrofolate undergoes rapid oxidation and subsequent scission under acid conditions to yield pterin and an unidentified fragment.

Extraction of the mitochondrial inner membrane with boiling ascorbate showed the majority of the folates to be polyglutamates, and acid extraction produced a pattern of scission products similar to the cytosol and matrix, suggesting tetrahydrofolate derivatives.

Connor *et al.* (1977) have shown that the carbon framework of the major liver folate of the rat is 10-formylfolate tetraglutamate. The results reported in the present paper indicate that the major folate present in the cytosol, mitochondrial matrix and mitochondrial inner-membrane fractions, as isolated by these procedures, is tetrahydrofolate tetraglutamate. These folate derivatives are probably protected against oxidation and subsequent scission by binding to a particular protein. The oxidation of tetrahydrofolates and tetrahydropterins by O_2 is greatly retarded by steric hindrance to the approach of the O_2 molecule and by a decrease in the polarity of the reaction medium (Pearson, 1974; Blair *et al.*, 1975; Blair & Pearson, 1974b; Pearson & Blair, 1976). The minor mixed-label peak present in all acid extractions may be 10-formylfolate.

The total cell folate isolated by Sephadex G-15 chromatography from a hot-ascorbate extraction showed no evidence of scission on further chromatography (Connor *et al.*, 1977) and was identified as 10-formylfolate tetraglutamate. A possible reconciliation of these observations with those reported in the present paper is that the original cell folate was 10-formylfolate tetraglutamate (minor component) and its tetrahydro derivative (major component) and that the latter was enzymically deformylated to its tetrahydrofolate derivative during isolation (Scrutton & Beis, 1979).

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