BBA 26247

THE SUBCELLULAR LOCALIZATION OF PTEROYL POLYGLUTAMATE HYDROLASE AND FOLATE IN GUINEA PIG INTESTINAL MUCOSA

A. V. HOFFBRAND AND T. J. PETERS

Department of Haematology and M.R.C. Intestinal Malabsorption Research Group, Royal Postgraduate Medical School, London, W.12, (Great Britain)

(Received June 30th, 1969)

SUMMARY

1. Guinea pig intestinal mucosa was fractionated by two methods and the subcellular localization of pteroyl polyglutamate hydrolase (formerly known as folate conjugase) was determined.

2. Pteroyl polyglutamate hydrolase appeared to be localized predominantly in the lysosomal fraction: no significant brush-border localization of the enzyme was noted.

3. Folate was concentrated in the mitochondrial fraction.

4. Possible mechanisms of pteroyl polyglutamate absorption are discussed in the light of the findings.

INTRODUCTION

Folate occurs in the diet mainly as pteroyl polyglutamates[1]. Recently HERBERT[2] has suggested that the free or unconjugated forms of folate (pteroyl monoglutamates) may be absent from uncooked foodstuffs. Pteroyl polyglutamates containing more than three glutamic acid residues are inactive in the *Lactobacillus casei* microbiological assay, and in this paper the term pteroyl polyglutamates is used to describe these *L. casei* inactive folate compounds.

There is a rise in the plasma *L. casei* activity after ingestion of pteroyl polyglutamates suggesting that these compounds have been hydrolysed to pteroyl, tri-, di- or monoglutamates during absorption. It has therefore been proposed that intestinal pteroyl polyglutamate hydrolase (formerly known as folate conjugase) is responsible for this hydrolysis[3,5]. This enzyme is not listed by the Enzyme Commission. It is a γ-glutamyl carboxypeptidase which removes the glutamate residues from pteroyl polyglutamates[6,7] and there is some evidence that it is distinct from other intestinal peptidases[7].

Some workers have detected low levels of pteroyl polyglutamate hydrolase in the lumen of the gastro-intestinal tract of both man and guinea pig (unpublished results; ref. 8). On the other hand, others [9,10] have failed to detect any such activity in lumenal contents. The enzyme has a sharply defined pH optimum of 4.6 (unpublished result), and thus in either case is unlikely to play a significant role in polyglutamate digestion within the small intestinal lumen whose pH is between 5.5 and

6.5 (ref. 11). Furthermore the concentration of pteroyl polyglutamate hydrolase in the mucosal cells is at least 100 times that found in the intestinal lumen (unpublished result) suggesting that the intestinal mucosa may play a significant role in polyglutamate digestion. It is therefore important to study the subcellular localization of this enzyme.

Recent work by CRANE[12] and his colleagues has stressed the role of the brush border region of the cell in the final stages of carbohydrate and protein digestion. Pteroyl polyglutamates might also be hydrolysed at the brush border before their absorption and therefore particular care was paid to the isolation of brush borders and the study of their pteroyl polyglutamate hydrolase content. In addition the subcellular localization of folate in the small intestinal mucosa of the fasted guinea pig was studied.

METHODS

Male guinea pigs (Hartley strain), weighing 350–450 g, were fasted for 24–48 h and killed by a blow on the head. The small gut was rapidly removed and washed with ice-cold 0.15 M NaCl, everted over a metal rod and washed in four changes of the saline. The superficial mucosa was scraped off using glass slides; histological examination confirmed that only villi were removed. The mucosa was homogenized and differentially centrifuged either by the method of HÜBSCHER *et al.*[13] or by the method of EICHHOLZ AND CRANE[14]. The purity of the various fractions was assessed by phase-contrast and electron microscopy and by studying the distribution of several marker enzymes. It was found that the brush borders prepared by the technique of HÜBSCHER *et al.* were less pure than those prepared by the technique of EICHHOLZ AND CRANE, as has been previously noted by these workers.

The combined mitochondrial/lysosomal fraction ($335000 \times g \cdot \text{min}$) prepared by the technique of HÜBSCHER *et al.* was suspended in 0.3 M sucrose. It was subfractionated into partially purified lysosomes and partially purified mitochondria by isopyknic centrifugation on 1.5 M sucrose for $4300000 \times g \cdot \text{min}$ (P. SHAKESPEARE, personal communication). In order to assess the degree of separation of mitochondria and lysosomes, the distribution of cytochrome oxidase (EC 1.9.3.1.)[15], β-glucuronidase (EC 3.2.1.3)[16] and aryl sulphatase A (EC 3.1.6.1)[17] was studied in the two subfractions.

Pteroyl polyglutamate hydrolase was assayed by incubating the various subcellular fractions with partially purified yeast pteroyl polyglutamates for 90 min at pH 4.6 and assaying the micobiologically active folate released with *L. casei*[5,18,19].

The pteroyl polyglutamate substrate was prepared as follows . 50 g of Difco yeast was fractionated on a DEAE-cellulose column using gradient elution with phosphate buffer containing 0.2 % 2-mercaptoethanol as described by SCHERTEL *et al.*[18]. The eluant was assayed for folate using *L. casei* with and without prior incubation with chick pancreas pteroyl polyglutamate hydrolase (Folate Conjugase, Difco). The fractions containing pteroyl polyglutamates and no free folate were combined and the phosphate partially precipitated by the addition of 2.5 vol. methanol. Remaining phosphate buffer was removed using a column of Sephadex G-10, the polyglutamates eluting in the void volume.

The pteroyl polyglutamates consisted of approx. 1 % of the dry weight of the final product and is stated to consist of reduced, formylated and methylated poly-

glutamates containing 6, 7, 11 or possibly more glutamate residues[20]. No detectable *L. casei* active folate was present in the substrate prior to treatment with pteroyl polyglutamate hydrolase.

Pteroyl polyglutamate hydrolase was assayed as follows. Doubling dilutions of the intestinal homogenates or subcellular fractions were prepared and 0.1 ml of each solution, 0.1 ml of polyglutamate substrate (0.5 μg/ml) and 1.8 ml of citrate–phosphate buffer (pH 4.6) containing 1% freshly added ascorbic acid and 10 mM $CaCl_2$ were incubated for 90 min at 37°, together with suitable controls. The ascorbic acid was added to the buffer to inhibit oxidative destruction of the folate derivatives during the incubation, autoclaving and assay of the cell fractions. The reaction was terminated by autoclaving at 115° for 5 min and the microbiologically active folate released assayed using *L. casei* as described by HERBERT[20]. The release of folate was linear for at least 90 min. Using results obtained from the sensitive part of the standard growth curve for *L. casei*, there was satisfactory correspondence between the amount of enzyme and bacterial growth.

Total folate content of the fractions was assayed with *L. casei* after incubation for 90 min at 37° at pH 4.6 without the addition of polyglutamate substrate. No attempt was made to distinguish microbiologically active folate and pteroyl polyglutamates in the various fractions. During the incubation period endogenous pteroyl polyglutamate hydrolase was shown to release all the microbiologically active folate. Neither more prolonged incubation nor further incubation in the presence of added pteroyl polyglutamate hydrolase released additional *L. casei* active material. Protein estimations were by the technique of LOWRY *et al.*[21] using bovine serum albumin (Armour) as a standard.

RESULTS

Subcellular localization of pteroyl polyglutamate hydrolase

Table I shows the distribution of the enzyme in the highly purified brush borders prepared by the technique of EICHHOLZ AND CRANE[14]. Approx. 0.5% of the recovered enzyme was located in the brush borders, the rest of the enzyme being present in the combined supernatant fractions. Although there was some loss of enzyme

TABLE I

DISTRIBUTION OF PTEROYL POLYGLUTAMATE HYDROLASE IN BRUSH BORDERS PREPARED BY THE TECHNIQUE OF EICHHOLZ AND CRANE

All results ± S.E. 1 unit of enzyme activity corresponds to the release of 1 ng *L. casei* active folate after incubation for 90 min at 37°. All fractions frozen and thawed before assay.

Fraction	*Recovered enzyme* (%)	*Specific activity (units per mg protein)*
Original homogenate	—	107 ± 20
Brush borders	0.58 ± 0.21	3.9 ± 2.1
Combined supernatants	99.4 ± 0.2	57.5 ± 18
Recovery		74.2 ± 2.0*

* Number of experiments: 4.

activity during the procedure, the specific activity confirmed that the enzyme was not concentrated in the brush border.

Further evidence that pteroyl polyglutamate hydrolase is not localized in the brush borders was obtained by comparing the specific activity of the enzyme in the intestinal villi and crypts. Villi were prepared as previously described, and the remaining crypt regions were homogenized in 0.15 M NaCl and assayed for pteroyl polyglutamate hydrolase. Since the brush border is largely absent from the crypt region of the mucosa, enzymes which are localized to the brush border are absent in slices prepared from this region. This has been demonstrated for several known brush-border enzymes[22-24]. Two experiments were performed. The mean specific activity of the pteroyl polyglutamate hydrolase in the whole mucosal homogenate was 120 units per mg protein, in the villus homogenate 110 units per mg protein and the crypt homogenate 118 units per mg protein. The equal distribution of the enzyme between villus and crypt is inconsistent with a brush border localization of pteroyl polyglutamate hydrolase.

Table II shows the distribution of the enzyme in the various subcellular fractions prepared by the technique of HÜBSCHER *et al.*[13]. Nearly 50% of the recovered

TABLE II

DISTRIBUTION OF PTEROYL POLYGLUTAMATE HYDROLASE IN BRUSH BORDERS AND OTHER SUBCELLULAR FRACTIONS PREPARED BY THE TECHNIQUE OF HÜBSCHER *et al.*

Fraction	*Recovered enzyme (%)*	*Specific activity (units/mg protein)*
Original homogenate	—	111 ± 24
Brush borders and nuclei	20.5 ± 3.5	35.7 ± 12
Mitochondria and lysosomes	47.6 ± 0.8	349 ± 43
Microsomes	7.3 ± 2.9	27.9 ± 6.7
Soluble fraction	26.9 ± 5.6	92.8 ± 2.6
Recovery		117 ± 9.1*

* Number of experiments: 9.

TABLE III

ENZYMIC CHARACTERIZATION OF LYSOSOMES AND MITOCHONDRIA AND LOCALIZATION OF PTEROYL POLYGLUTAMATE HYDROLASE

Fraction	*Cytochrome oxidase (units/mg protein)**	*β-Glucuronidase (units/mg protein)***	*Aryl sulphatase (units/mg protein)***	*Pteroyl polyglutamate hydrolase (units/mg protein)*
Mitochondria and lysosomes	19.3 ± 1.6	0.128 ± 0.02	0.422 ± 0.09	349 ± 43
Purified lysosomes	9.99 ± 1.2	0.241 ± 0.01	0.628 ± 0.10	751 ± 130
Purified mitochondria	39.2 ± 3.5	0.088 ± 0.01	0.142 ± 0.05	327 ± 79
Recovery	74.6 ± 2.2****	89.6 ± 4.9****	89.8 ± 7.1§	90.0 ± 4.9****

* 1 unit of enzyme defined as in ref. 16.
** 1 unit of enzyme corresponds to the hydrolysis of 1 μmole substrate per h at 37°.
*** Number of experiments: 8.
§ Number of experiments: 3.

enzyme was present in the combined mitochondrial/lysosomal fraction. The rest of the enzyme appeared to be equally distributed between the soluble fraction and the relatively impure brush borders prepared by the technique of HÜBSCHER *et al.* The specific activity showed a 3–4-fold concentration of the enzyme in the mitochondrial/lysosomal fraction, and no concentration of the enzyme in any other subcellular fraction was noted.

Table III shows the specific activity of three marker enzymes in the combined mitochondrial/lysosomal fraction and in the mitochondrial and lysosomal subfractions. An approx. 3-fold increase in specific activity of the appropriate enzyme in the corresponding subfraction was observed. The specific activity of pteroyl polyglutamate hydrolase was also 2–3 times higher in the lysosomal than in the mitochondrial subfraction (Table III). Although the enzyme is concentrated in the lysosomal subfraction to approximately the same degree as the two lysosomal marker enzymes, it could still be that the enzyme associated with the mitochondria may represent partial localization of the pteroyl polyglutamate hydrolase in this organelle.

Subcellular localization of folate

The distribution of the folate in the various fractions obtained by the technique of HÜBSCHER *et al.* is shown in Table IV. The vitamin appeared to be concentrated in the combined mitochondrial/lysosomal fraction. The concentration and percentage distribution of folate in the two subfractions is shown in Table V and indicates that the mitochondria are the principle organelle containing the vitamin. However, since only

TABLE IV

DISTRIBUTION OF FOLATE IN SUBCELLULAR FRACTIONS PREPARED BY THE TECHNIQUE OF HÜBSCHER *et al.*

Fraction	*Recovered vitamin* (%)	*Concn.* (*ng folate per mg protein*)
Original homogenate	—	4.5 ± 0.4
Brush borders and nuclei	15 ± 2.4	1.7 ± 0.3
Mitochondria and lysosomes	67 ± 5.4	15 ± 2.0
Microsomes	5.4 ± 1.2	1.5 ± 0.3
Soluble fraction	13 ± 5.2	1.0 ± 0.3
Recovery		70 ± 5.2*

* Number of experiments: 4.

TABLE V

CONCENTRATION OF FOLATE IN PARTIALLY PURIFIED LYSOSOMES AND MITOCHONDRIA

Fraction	*Recovered vitamin* (%)	*Concn.* (*ng folate per mg protein*)
Mitochondria and lysosomes	—	15 ± 2.4
Purified lysosomes	6 ± 2.1	1.9 ± 1.0
Purified mitochondria	94 ± 6.7	8.9 ± 3.0
Recovery		69.3 ± 11*

* Number of experiments: 6.

low recoveries of folate were obtained (Tables IV and V) a firm conclusion on this point cannot be reached.

DISCUSSION

The results indicate that pteroyl polyglutamate hydrolase is concentrated in the lysosomes of guinea pig intestinal mucosa. These findings are at variance with a preliminary abstract of ROSENBERG AND STREIFF[25], who claimed that the highest concentration of the enzyme was in the brush-border region of the intestinal cell. The reason for this discrepancy is not clear. It may represent a species difference, since the animal studied by ROSENBERG AND STREIFF was the rat, an animal with which we as well as other workers[26,27] have had difficulty in obtaining pure subcellular fractions. It is also noteworthy that the enzyme was assayed at pH 6.0–7.0 which is not the pH optimum for mammalian pteroyl polyglutamate hydrolase[5,28,29].

The pH optimum of 4.6 is consistent with a lysosomal location of the enzyme. This suggests that hydrolysis of pteroyl polyglutamate occurs within the intestinal cell rather than at the brush border. The mechanism of entry of the polyglutamate into the cell is unknown. It has been suggested that pteroyl monoglutamate may be absorbed by an active transport mechanism[30], but it is not known if pteroyl polyglutamates are absorbed by a similar process.

It has been previously suggested that the renal tubular and intestinal lysosomes play a role in the absorption of various substances[31–33]. In the proximal renal tubule endocytosis has been convincingly demonstrated. Invagination of the cell membrane in the crypts of the microvilli form small vesicles which subsequently acquire the histochemical characteristics of lysosomes[34–36]. Although endocytosis has been well described in the gut of the newborn[34–37], it is not clear whether this process can occur in adult intestinal mucosa. The similarity between renal and intestinal lysosomes has been noted by HSU AND TAPPEL[32] who suggested that they may play a role in intestinal absorption, a conclusion which is supported by their localization in the apical rather than the basal part of the cell [38,39]. The role of lysosomes in intestinal disease is uncertain but in coeliac disease, a condition in which folate malabsorption invariably occurs, lysosomal damage has been demonstrated[39].

The significance of the mitochondrial localization of folate is uncertain. SWENDSEID[40] and her colleagues were unable to demonstrate precise localization of folate in liver cells but examination of their data shows that the highest concentration was in the mitochondrial fraction. In a study of the subcellular localization of ten folate requiring enzymes in rat liver only two were localized to the mitochondria[41]. In addition, however, the enzyme complex which plays a key role in folate metabolism by converting pteroylglutamic acid to 5-formyltetrahydropteroylglutamic acid (folinic acid) is localized to the mitochondrion[42].

ACKNOWLEDGEMENTS

We are grateful to Professors C. C. Booth, R. K. Crane and G. Hübscher for helpful discussion during this work. We wish to thank Mrs. C. Griffin and Miss A. Quinlan for valuable technical assistance.

Part of this work was performed while A.V.H. was an M.R.C. Travelling

Scholar at the New England Medical Center, Boston, Mass., U.S.A.. T.J.P. is an M.R.C. Clinical Research Fellow.

REFERENCES

1 C. E. BUTTERWORTH, R. SANTINI AND W. B. FROMMEYER, *J. Clin. Invest.*, 42 (1963) 1929.
2 V. HERBERT, *Gastroenterology*, 54 (1968) 110.
3 R. R. STREIFF AND I. H. ROSENBERG, *J. Clin. Invest.*, 46 (1967) 1121.
4 H. BAKER, O. FRANK, S. FEINGOLD, H. ZIFFER, R. A. GELLENE, C. M. LEEVY AND H. SOBOKTA, *Am. J. Clin. Nutr.*, 17 (1965) 88.
5 A. V. HOFFBRAND AND T. F. NECHELES, *Lancet*, II (1968) 528.
6 A. KAZENKO AND M. LASKOWSKI, *J. Biol. Chem.*, 173 (1948) 217.
7 N. G. L. HARDING, D.Phil. Thesis, Oxford University, 1966.
8 F. A. KLIPSTEIN, *Am. J. Clin. Nutr.*, 20 (1967) 1004.
9 A. D. WELCH, R. Q. HEINLE, E. M. NELSON AND H. V. NELSON, *J. Biol. Chem.*, 164 (1946) 787.
10 H. G. BUYZE AND C. ENGEL, *Biochim. Biophys. Acta*, 2 (1948) 217.
11 B. BORGSTRÖM, A. DAHLQVIST, G. LUNDH AND J. SJÖVALL, *J. Clin. Invest.*, 36 (1957) 1521.
12 R. K. CRANE, *Ann. Rev. Med.*, 19 (1968) 57.
13 G. HÜBSCHER, G. R. WEST AND D. N. BRINDLEY, *Biochem. J.*, 97 (1965) 629.
14 A. EICHHOLZ AND R. K. CRANE, *J. Cell Biol.*, 26 (1965) 687.
15 S. J. COOPERSTEIN AND A. LAZAROW, *J. Biol. Chem.*, 189 (1951) 665.
16 W. H. FISHMAN, *Methods Biochem. Analy.*, 15 (1967) 77.
17 A. B. ROY, *Biochem. J.*, (1953) 12.
18 M. E. SCHERTEL, J. W. BOEHNE AND D. A. LIBBY, *J. Biol. Chem.*, 240 (1965) 3154.
19 A. V. HOFFBRAND, T. F. NECHELES, N. MALDONADO, E. ORTA AND R. SANTINI, *Brit. Med. J.*, II (1969) 543.
20 V. HERBERT, *J. Clin. Pathol.*, 19 (1966) 12.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
22 H. VAN GENDEREN AND C. ENGEL, *Ezymologia*, 5 (1938) 71.
23 F. MOOG AND R. GREY, *J. Cell Biol.*, 32 (1967) C1.
24 C. NORDSTRÖM, A. DAHLQVIST AND L. JOSEFSSON, *J. Histochem. Cytochem.*, 15 (1968) 713.
25 I. H. ROSENBERG AND R. R. STREIFF, *Gastroenterology*, 52 (1967) 117.
26 G. B. ROBINSON, *Biochem. J.*, 88 (1963) 162.
27 L. M. SRIVASTAVA, P. SHAKESPEARE AND G. HÜBSCHER, *Biochem. J.*, 109 (1968) 35.
28 O. D. BIRD, M. ROBBINS, J. M. VANDERBELT AND J. J. PFIFNER, *J. Biol. Chem.*, 163 (1946) 649.
29 I. H. ROSENBERG, H. A. GODWIN, R. R. STREIFF AND W. B. CASTLE, *Lancet*, II (1968) 530.
30 A. S. V. BURGEN AND N. J. GOLDBERG, *Brit. J. Pharmacol.*, 19 (1962) 313.
31 C. DE DUVE AND R. WATTIAUX, *Ann. Rev. Physiol.*, 28 (1966) 435.
32 L. HSU AND A. L. TAPPEL, *J. Cell Biol.*, 23 (1964) 233.
33 S. L. PALAY AND L. J. KARLIN, *J. Biophys. Biochem. Cytol.*, 5 (1959) 363.
34 S. L. CLARK, *J. Biophys. Biochem. Cytol.*, 4 (1959) 41.
35 H. HOLTER AND H. HOLTZER, *Exptl. Cell Res.*, 18 (1959) 421.
36 W. STRAUS, *J. Cell Biol.*, 20 (1964) 497.
37 J. G. LECCE, *J. Physiol. London*, 184 (1966) 594.
38 J. S. TRIER AND C. E. RUBIN, *Gastroenterology*, 49 (1965) 574.
39 E. O. RIECKEN, J. S. STEWART, C. C. BOOTH AND A. G. E. PEARSE, *Gut*, 7 (1966) 317.
40 M. E. SWENSEID, F. H. BETHELL AND W. W. ACKERMANN, *J. Biol. Chem.*, 190 (1951) 791.
41 F. K. WANG, J. KOCH AND E. L. R. STOKSTAD, *Biochem. Z.*, 346 (1967) 458.
42 J. M. NORONHA AND A. C. SREENIVASAN, *Biochim. Biophys. Acta*, 44 (1960) 64.