THE ELUCIDATION OF THE MECHANISM OF FOLATE CATABOLISM IN THE RAT

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Summary

It has been demonstrated that after a sufficient equilibration period the principle catabolite of a tracer dose of [^{3}H]pteroylglutamate is acetamidobenzoylglutamate. This catabolite was formed prior to excretion from p-aminobenzoylglutamate, a small amount of which also appears in the urine and indicates that the principle mechanism of cellular folate catabolism proceeds via cleavage of the C9-N10 bond. The inability of others to demonstrate this simple catabolic mechanism resides in that they used too short a study period which shows a complex equilibration pattern in the urine or that they may have wrongly identified the other cleavage product, a pteridine, as an intact folate derivative. In addition the further metabolism of p-aminobenzoylglutamate has not been appreciated until now. Apart from the two catabolites mentioned three others have been recognized and preliminary evidence suggests that they may be pteridines which have become labelled during metabolic cleavage.

Introduction

In man since at least ten times as much folate is absorbed as is excreted, it is clear that a considerable amount of catabolism of some or all of the folate cofactors takes place. It has also been speculated that an increase in this catabolism may cause the folate deficiency which frequently occurs in pregnancy (1) anticonvulsant therapy (2) various forms of malignancy (3) Crohn's disease (4) and homocystinuria (5). Since the manner in which folates are catabolised or what the resultant products are, has never been established experimentally, support for these important clinical claims does not exist. As suggested by ourselves (6) and others (7) the most obvious mechanism by which folates might be catabolised would be cleavage of the C9-N10 bond, with subsequent excretion of aminobenzoylglutamate (p-ABGlu) and one or more pteridines. However, the apparent absence of pABGlu in the urine of rats fed labelled pteroylglutamate (PteGlu) has led to the belief that

biologically inactive forms of the vitamin, in which the C9-N10 linkage remains, are the excreted catabolites (8-10). In this communication we demonstrate that the majority of folate catabolism does in fact result from the cleavage of the C9-N10 bond after an initial period when a complex excretion pattern pertains. In addition most (but not all) of the pABGlu is further metabolised within the rat to form p-acetamidobenzoylglutamate.

Thus, after a period when the incorporated label would have all been converted to reduced polyglutamyl forms (11), a large amount of the excretion is due to the cleaved product acetamidobenzoylglutamate, and so it appears that the following catabolic process is the major one; one or more of the folate polyglutamate cofactors cleave between the C9-N10 bond; the resulting p-aminobenzoylpolyglutamate is enzymatically deconjugated to pABGlu; most of the pABGlu becomes acetylated; this acetylated product is excreted in the urine together with a small amount of unaltered pABGlu. In addition to these two catabolites three others are seen in these equilibrated urines. Preliminary evidence suggest that they are pteridines which have derived their radioactivity from the tritium on C9 during cleavage.

Materials and Methods

Chemicals: All chemicals were of AnalaR grade.

Folates: PteGlu and 5-formyltetrahydropteroylglutamate (5-CHO-H_4PteGlu) were a gift of Lederle, Pearl River, New York. Tetrahydropteroylglutamate (H_4PteGlu), dihydropteroylglutamate (H_2PteGlu), pteroic acid (Pte), 5-methyletrahydropteroylglutamate (5-CH_3-H_4PteGlu), 10-formyltetrahydropteroylglutamate (10-CHO-H_4PteGlu), 5, 10-methylidynetetrahydropteroylglutamate (5, 10-CH=H_4PteGlu) and 5, 10-methylenetetrahydropteroylglutamate (5, 10-CH_2-H_4PteGlu) were prepared as previously described (12-13). 5-Methyldihydropteroylglutamate (5-CH_3-H_2PteGlu) and its oxidation product, which is referred to in the literature as 4a-hydroxy-5-methyltetrahydropteroylglutamate (4a-OH-5-CH_3-H_4PteGlu) were prepared as originally described (14). 10-Formylpteroylglutamate (10-CHO-PteGlu) was prepared by the air oxidation of 10-CHO-H_4PteGlu. p-Aminobenzoate (pAB) and p-aminobenzoylglutamate (pABGlu) were purchased from Sigma Ltd. London. Acetamidobenzoylglutamate was prepared from pABGlu by the method of Baker et al. (15).

Radiochemicals: (3'5'9[^{3}H])PteGlu with a specific activity 56 Ci/mmole was supplied by the Radiochemical Centre, Amersham, England. The same company carried out a tritium exchange on p-amino-3,5-dibromobenzoylglutamate supplied by us, to prepare (3,5[^{3}H])pABGlu with a calculated specific activity of 5 Ci/mmole. Both of these radioactive materials were purified by Column Method A described below.

Column Method A: Salt gradient: Glass columns 0.8 x 40 cms were packed under gravity to a height of 30 cm with deaerated A25 QAE Sephadex which had previously been equilibrated with 5 mM Tris HCl pH 7.5. After washing with several column volumes of the same buffer, rat urines containing the appropriate markers, were adjusted to pH 7.5, diluted with distilled water to a conductivity of less than 3 mohms^{-1}, and applied to the column. Elution was effected by a linear gradient of 300 ml of the starting buffer and 300 ml of the same buffer which was 0.6 M with respect to NaCl. A flow rate of 20 ml/hr was maintained and usually 5 ml fractions were collected.

Column Method B: pH gradient: Here columns of similar dimensions were packed with deaerated A25 QAE Sephadex which had previously been equilibrated with 0.01 M disodium citrate pH 5.0. After washing with several column volumes of this buffer, urine samples, whose pH had been adjusted to 5, were diluted to a conductivity of less than 1 mohms^{-1} with distilled water and applied to the columns. Elution was effected by a pH gradient of reducing pH, composed of 500 ml of 0.01 N HCl and 500 ml of 0.03 N HCl. A similar flow rate and sample volume as Column Method A were maintained.

The wash and column fractions were analysed for radioactivity (16) or spectrophotometrically at the appropriate wavelength for the standard folate.

Thin layer chromatography (TLC) was on cellulose plates (MN300, UV_{254}) as previously described (17), using butanol: acetic acid: water (4:1:5) upper phase, as solvent. When column peaks were being analysed they were concentrated by lyophilisation before application.

Standards of acetamidobenzoylglutamate were de-acetylated to pABGlu by heating for 1 hr at 100°C in 0.2 N HCl and estimated as previously described (18). Male and female albino Wistar rats (150-450 g) were injected intramuscularly with 50 µCi of [^{3}H]PteGlu on two consecutive days and their urines collected for the duration of the study.

Results and Discussion

Daily urine collections of rats given a tracer dose of [^{3}H]PteGlu were analysed using Column Method A. They showed at first a very complex mixture of intact PteGlu and many other known folate derivatives as reported by others (8-10, 19-21). This pattern presumably represents equilibration of the tracer with the various folate pools and does not represent true catabolism. The pattern becomes progressively more simple until after about thirteen days almost all of the tritium in the urine elutes as a single peak on Column Method A (Fig. 1). The peak did not contain a polyglutamyl form since its position was unaltered by conjugase treatment. The non-identity of this main peak with all but three possible folate derivatives, including 4a-OH-5-CH_3-H_4 PteGlu which has been suggested to be the folate catabolic product by others (9, 10), was established by cochromatography. One of the three derivatives, 5-CH_3-H_2PteGlu, that coeluted with the main catabolite was ruled out by treatment of the standard and urine with 2-mercaptoethanol, which caused the

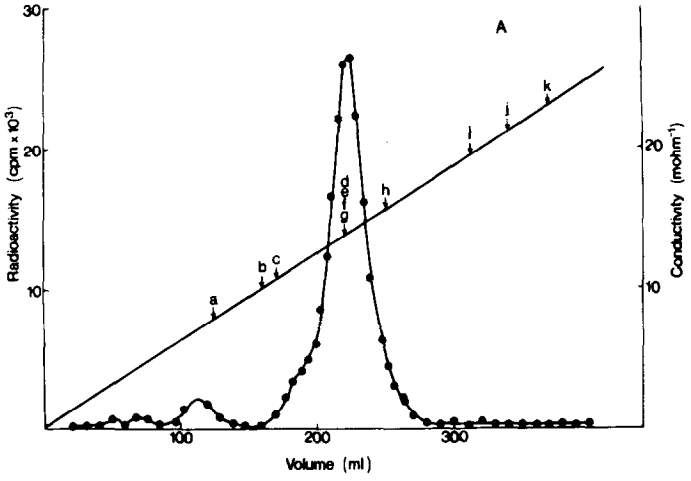

Fig. 1. Fractionation of a day 13 rat urine by Column Method A on QAE Sephadex using a NaCl gradient. The elution position of authentic chemical folate standards and their possible degradation products are: a, pAB; b, an unidentified degradation product of standard 5-CH_3-H_4PteGlu; c, 4a-OH-5-CH_3-H_4PteGlu; d, acetamidobenzoylglutamate; e, 5-CH_3-H_2PteGlu; f, 10-CHO-PteGlu; g, pABGlu; h, 5-CHO-H_4PteGlu; i, 5-CH_3-H_4PteGlu; j, Pte; k, PteGlu. Under these conditions 10-CHO-H_4PteGlu and 5, 10-CH=H_4 PteGlu would elute with 10-CHO-PteGlu and 5, 10-CH_2-H_4PteGlu, H_4PteGlu and H_2PteGlu would degrade.

standard to move to a new elution position at (i) but left the radioactive distribution unaltered. To determine how much, if any, of the excreted material was pABGlu or 10CHOPteGlu, a new chromatographic procedure (Column Method B) based on a pH rather than a salt gradient was devised. This new procedure separates the two standards and also divides the urine into four peaks, two major and two minor, which we have designated I, II, III and IV in order of their elution. The minor peaks I and IV do not coelute with the two standards while peak II elutes close to pABGlu and peak III exactly with 10CHOPteGlu. While difference in elution by cochromatography can exclude possible identity, coelution of itself does not establish that two compounds are the same. Consequently it was decided to compare the major peaks with their coeluting standards on TLC. Such analysis showed in fact that peak II contains only a small amount of pABGlu (Fig. 3A). On TLC peak III in early day excretions was found to contain some 10CHOPteGlu but by day 13 virtually

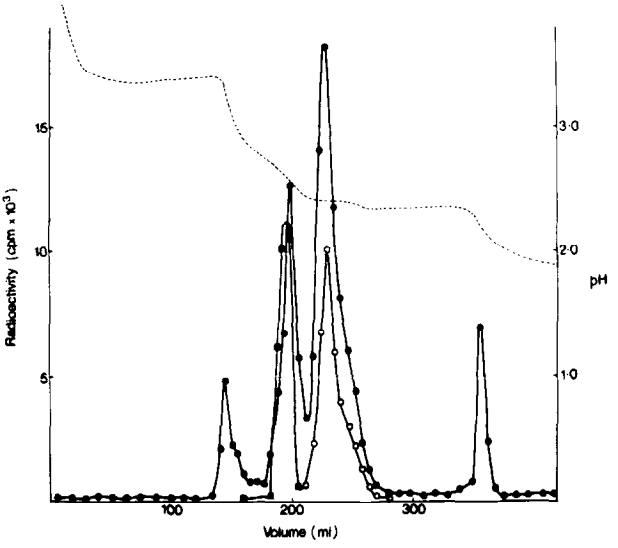

Fig. 2. Fractionation of a day 13 rat urine by Column Method B on QAE Sephadex using a pH gradient (·····). The elution position of standard pABGlu (x——x), and acetamidobenzoylglutamate (o——o) were determined by the Bratton Marshall method (18) either directly or after acid treatment.

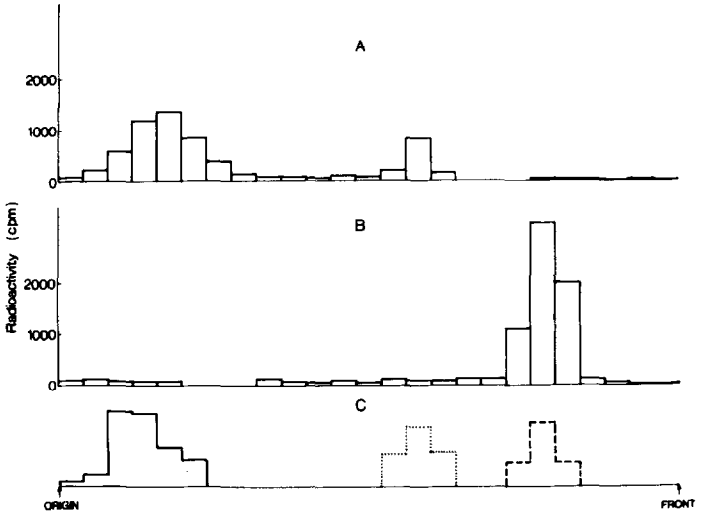

Fig. 3. Fraction on TLC of peak II (A) and of peak III (B) from Fig. 2; the positions of standard 10-CHO-PteGlu (——), pABGlu (·····) and acetamidobenzoylglutamate (-----) shown in C were determined by the Bratton Marshall method (18) either directly or after conversion to pABGlu. The solvent used was the upper phase of butanol: acetic acid: water (4:1:5).

all of the catabolite present ran as a single fast moving spot which was also a hitherto unknown catabolite and remained to be identified (Fig. 3B). This however was resolved when it was realized that in vivo pABGlu would be likely to be further metabolised by acetylation. That this catabolite was in fact acetamidobenzoylglutamate was confirmed by cochromatography on TLC (Fig. 3) and subsequently on Column Methods A and B (Fig. 1 and 2) and by the demonstration that mild acid treatment converts both the standard acetamidobenzoylglutamate and this catabolite to pABGlu. Furthermore it was found that after intravenous administration of [^{3}H]pABGlu to a rat, substantial conversion to the acetamido form took place. That cleavage of the C9-N10 bond actually takes place intracellularly was clear since acetylation could not have occured extracellularly i.e. in the urine. As acetamidobenzoylglutamate is the most abundant catabolite in these fully equilibrated urines it follows that the majority of folate catabolism proceeds via cleavage of the C9-N10 bond.

The three other catabolites while they are quantitatively smaller occur in all equilibrated urines. They are apparently unaltered by permanganate oxidation which would cleave them had they an intact C9-N10 bond. This, coupled with the fact that they do not correspond to any known standard, would suggest that they are not intact folates. Other work in this laboratory has shown that a possible cleavage product of 10-CHO-H_4PteGlu is a formyl derivative of pABGlu but none of these three catabolites correspond to this compound. Neither are they pABGlu or acetamidobenzoylglutamate. Studies in hand indicate that they may be pteridines which have derived some of the tritium atoms attached to C9 during cleavage.

Finally there is clearly a marked discrepancy between our finding and those of Blair and his coworkers (8-10). While they did not examine urines sufficiently removed from initial labelling to ensure that equilibration had taken place, they find that with time the major emerging component is 4a-OH-5-CH_3-H_4PteGlu. The major methodological difference between the two studies has been that they have used (2[^{14}C]PteGlu. This would explain why they do

not detect pABGlu or acetamidobenzoylglutamate since they would not be labelled after cleavage. However, if 4a-OH-5-CH_3-H_4PteGlu were excreted, we would also observe it with our label and cochromatography with authentic standard showed no radioactivity on our columns in this area. A possible explanation is that the compound to which they are ascribing the 4a-OH-5-CH_3-H_4PteGlu structure is in fact a pteridine which is not labelled by the C9 tritium on cleavage and consequently not seen in our studies. They emphasise that they found less [^{14}C] than [^{3}H] in the urine of rats fed doubly labelled PteGlu. They conclude from this that 'exchange' of the [^{3}H] label must occur. However, after cleavage of the C9-N10 bond the pteridine portions might either be excreted more slowly or a greater proportion might pass through the bile to be excreted in the faeces, thus causing a lower amount of [^{14}C] than [^{3}H] to emerge in the urine.

Acknowledgment

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