THE FATE OF FOLIC ACID AND RELATED COMPOUNDS IN THE RAT

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DECLARATION

This work was carried out between 1968 and 1971 at the University of Aston in Birmingham.

The work was done independently and has not been submitted for any other degree.

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NOMENCLATURE

In general the conventions adopted here follow those of the Tentative Rules and Proposals of the IUPAC-IUB Commission on Biochemical Nomenclature [see Biochem.J.(1966) 101,1] and those symbols and abbreviations acceptable to the Biochemical Journal (see Policy of the Journal and Instructions to Authors (1970); The Biochemical Society, London).

Abbreviations and nomenclature for folates and pteridines are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [see Biochem.J. (1967) 102,15]

The conventions for isotopically labelled compounds are those adopted by the Editorial Board of The Biochemical Society and the Editors of The Chemical Society (see Handbook for Chemical Society Authors, p186).

SUMMARY

The physiological disposition and metabolism of folates was studied by purification, characterisation and specific radioactivity determinations of compounds derived from oral doses of labelled folates.

At doses of 22 and 320µg of pteroyl-L-glutamic acid/kg, 50 and 25% respectively was absorbed. With doses of 320µg of [2^{14}-C]pteroyl-L-glutamic acid/kg, radioactivity was detected in the peripheral circulation 10 minutes after administration. Pteroyl-L-glutamic acid was absorbed without metabolism and was present in systemic blood for up to 4 hours after administration.

With doses of pteroyl-L-glutamic acid from 3.1 to 56µg/kg approximately 6% of the dose was excreted in 24 hours; whilst at 320µg/kg 30% was excreted mainly in the 2 to 4 hour period.

Three urinary folates were isolated. One of the major metabolites was 5-methyltetrahydro-pteroylglutamic acid. The others were unidentified but were not pteroylglutamic acid, 7,8-dihydro-, 5,6,7,8-tetrahydro-, 5- or 10-formyl-tetrahydro-, 5,10-methylidyne-tetrahydro-, 5,formimidoyl-tetrahydro-, 5,10-methylene-tetrahydro-, 5-methyltetrahydro-pteroylglutamic acid, nor any decomposition products of these compounds formed during isolation. Labelled unconjugated pteridines were absent.

Six hours after administration of pteroyl-L-glutamic acid, more than seven labelled compounds were isolated from liver homogenates. Extractions performed up to 10 days after administration or after prior starvation only slightly increased the incorporation of label into

conjugates. 5-methyltetrahydro-pteroylglutamate was the major monoglutamate and represented 10% of the total hepatic folates.

The physiological disposition of the natural diastereoisomer of 5-methyltetrahydro-pteroylglutamate was similar to that of pteroylglutamate. At a dose of 84µg/kg, 80% of the urinary folates was 5-methyltetrahydro-pteroylglutamate, but at lower doses a greater proportion was present as metabolites.

The major unidentified metabolite excreted after pteroylglutamic acid administration showed a similar absorption and excretion pattern to 5-methyltetrahydro-pteroylglutamic acid and was metabolised to 5-methyltetrahydro-pteroylglutamate.

The results are discussed with reference to the nutritional, metabolic and clinical importance of folates in previous work.

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In 1931 Wills[1] reported an extract of autolysed liver which was effective in the treatment of tropical macrocytic anaemia in humans. Later observations, apparently unrelated, showed new factors (vitamins M,G,B_c and Factor U) which prevented nutritional cytopenia in the monkey[2] and in chicks[3,4]. There then followed a series of important findings showing that these factors were essential nutrients for lactic acid bacteria[5] and a factor, active for Streptococcus faecalis R, was purified from four tons of spinach. This factor was named folic acid[6] and, although criticised at the time, the name has remained. In 1946 a large group of chemists showed that these nutritional factors were identical and were N[4{[(2amino-4hydroxy-6pteridyl)methyl]amino}benzoyl]-glutamic acid, for which the name pteroylglutamic acid was proposed[7]. The structure and numbering of PteGlu* is shown in Fig.1.

PteGlu was synthesised in 1948 and a vast amount of literature has since been published on folates and other pteridines. Many reviews have appeared and books by Blakley (1969)[8] and Chanarin (1969)[9] are excellent and comprehensive reviews. However a good deal of confusion, concerning folates in particular, has arisen partly from the different techniques employed by different workers and there follows a brief description of the methods, and then a more detailed account of folate and pteridine metabolism

*Abbreviations: PteGlu, pteroyl-L-glutamic acid; H_2PteGlu and H_4PteGlu, 7,8-dihydro- and 5,6,7,8-tetrahydropteroyl-L-glutamic acids; 5(and 10)-HCO-, 5(and 10)-formyl-; 5,10-CH=, 5,10-methylidyne-; 5,10-CH_2-, 5,10-methylene-; 5-CH_3-, 5-methyl-; 5-HCNH-, 5-formimidoyl-derivatives; $PteGlu_2$, pteroyl-L-glutamyl-γ-L-glutamic acid; $PteGlu_3$, pteroyl-L-glutamyl-γ-L-glutamyl-γ-L-glutamic acid; and their derivatives corresponding to those for PteGlu.

in normal and diseased metabolism.

Bioassay techniques using the larvae of Aedes aegypti L.[10], the haematopoetic response or parallel growth in monkeys[11,12], swine[13,14], chick[16,17] and rat[18], have now been superseded by more specific and sensitive microbiological assays using Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae for folates and Crithidia fasciculata for pteridines[20](review ref.21). The response is routinely measured turbidimetrically, but the size of growth on a thin-layer agar plate has been used, ref.20 page 642. The differential growth response of L. casei, S.faecalis and P.cerevisiae to synthetic folates is shown in Table I. Even with this data the growth response to other folates and pteridines cannot be predicted and must await chemical identification of synthetic or natural folates. Microbiological assays have also been criticised for their lack of specificity[8,23] and that the growth response is altered by mutation[24], the presence of antibiotics[24], folate antagonists[25] and contamination with other bacteria (although strictly aseptic conditions are not essential[26]). Large concentrations of ascorbate may increase[27] or decrease[27,28] S.faecalis growth; compounds other than folate stimulated the growth of L.casei and S.faecalis[29]; autoclaving of food decreased the L.casei relative to the S.faecalis response[28] and these organisms do not respond directly to folate derivatives which occur in largest amounts. Despite these criticisms, which are particularly important when impurities are present, microbiological assay is still the only folate assay technique used routinely and is the most sensitive (down to 10^{-10}M) assay

technique developed for folates. The microbiological response is shown in Table 1.

Radiolabelled PteGlu, commercially available since 1960, offers advantages in a reliable and sensitive radiochemical assay method, and has greatly aided metabolic studies both *in vitro* and *in vivo*. Tritiated-PteGlu is non-specifically labelled[205], tritium being present in the pteridine ring, the C_9-methylene group and in the glutamic acid moiety. Tritiated-PteGlu is of high specific radioactivity and may be used for studies in humans, but possible tritium-exchange reactions, isotopic effects and biochemical or chemical conversions to pterins (with the loss of labelled glu or p-aminobenzoyl-glu) make it unsuited for detailed analysis. Specifically labelled [2-^{14}C]PteGlu is therefore advantageous in folate and pterin studies and was used almost entirely throughout this study.

Enzymatic assays (see Jaenicke[20] page 605), involving reactions (a) to (e) followed by the spectrophotometric assay of 5,10-CH=H_4PteGlu, the nicotinamide coenzyme or radiochemical assay, have only recently been developed.

(a) dihydrofolate reductase:

$$H_2\text{PteGlu} \xrightarrow[NADPHH^+ \quad NADP^+]{} H_4\text{PteGlu} \xrightarrow[H^+]{HCOOH} 5,10\text{-CH=}H_4\text{PteGlu}$$

(b) tetrahydrofolate formylase:

$$H_4\text{PteGlu} \xrightarrow[HCOOH \quad ATP \quad ADP+Pi]{} 10\text{-HCO-}H_4\text{PteGlu} \xrightarrow{H^+} 5,10\text{-CH=}H_4\text{PteGlu}$$

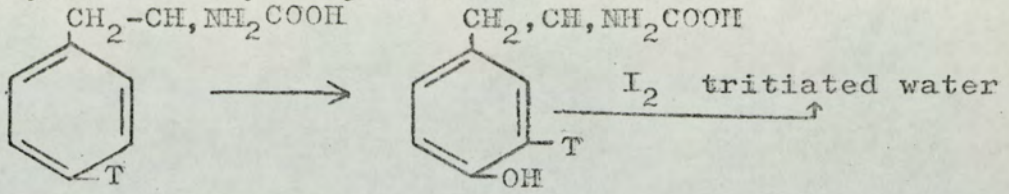
(c) methylenetetrahydrofolate dehydrogenase:

$$5,10\text{-CH}_2\text{-}H_4\text{PteGlu} \xrightarrow[NADP^+ \quad NADPH \; H^+]{} 5,10\text{-CH=}H_4\text{PteGlu} \xrightarrow{H^+} 10\text{-HCO=}H_4\text{-PteGlu}$$

(d) methionine synthetase:

$$5\text{-CH}_3\text{-}H_4\text{PteGlu} \xrightarrow[met]{homocys} H_4\text{PteGlu} \xrightarrow[H^+]{HCOOH} 5,10\text{-CH=}H_4\text{PteGlu}$$

(e) phenylalanine hydroxylase: -



Although these methods are highly specific and folates down to 10^{-7}M can be assayed, the enzymes are not yet available commercially and the methods developed are only for monoglutamate assays, but the future potential of enzymatic assays seems indisputable.

Fluorimetry[31,32,33], although sensitive down to 10^{-8}M, has a limited use for folates, of which only H_2PteGlu, 10-HCO-PteGlu and 5,10-CH=H_4PteGlu are fluorescent, and is relatively non-specific for pterins.

The structural similarity of many natural folates and pterins causes separation and identification problems, but have been separated by a choice of solvents used with paper or thin-layer chromatography. Separation of pteridines[15] and folates[39] on paper are reviewed by Schoen[34], Slavik[35] and Blakley[8]. T.l.c. has further advantages over paper in speed, greater resolution and choice of absorbent. Folate and pterins have been separated on cellulose[19], silica gel[36,37,38,40] and on mixtures of silica gel and alumina with plaster of Paris[39]. The sensitivity (down to 100ng per spot) is greatly increased (down to 10pg per spot) with microorganisms (bioautography[41,42,43]) but diffusion of folates and dye through the culture medium causes enlargement of spots and thus reduces the separation. Compounds are also difficult to recover from the bioautograms.

The amphoteric nature of natural folates and pterins

and their differing basicities and acidities can, in part, account for their separation with ion-exchangers on Dowex[20,44,45], phosphocelluloses[20] DEAE- and TEAE-cellulose columns[46] and on ion-exchange thin-layer plates[48], but stereochemical configurations must play a part in the separation of diastereoisomers of 5,10-CH_2-H_4PteGlu[49,50]. Despite its low capacity, advances in the production of DEAE-cellulose has now established its use in the separation of natural folates although the separation of certain folate coenzymes is insufficient and an improved separation has recently been claimed using DEAE-Sephadex; see ref.20 page 661.

Other reports for separating and identifying natural folates and pterins from biological extracts have sporadically appeared and include high voltage electrophoresis[52,53], u.v.[8] and i.r.[20,54,55] spectroscopy, polarography[56,57], n.m.r.[58,59,60,61,62] and mass spectrometry[63,64].

The development of reliable and reproducible assay methods together with the growing recognition of the lability and similarity of folate and of pterin coenzymes has, since 1960, reduced the previous confusion in the literature about folates and pterins. Folates and pterins have been found in many species of animals, plants and micro-organisms and probably occur universally throughout nature but pterins, unlike folates, have received little attention in mammals, even though biopterin was first isolated from human urine[81].

The biosynthesis of pterins has been extensively studied in insects, amphibia and micro-organisms and the currently held pathway is shown in Fig.2. Guanosine tri-

phosphate is converted to a substituted diaminopyrimidine by loss of the C8 carbon atom; cyclization then forms the 6-substituted pterin from which is derived the naturally occuring pteridines with and without a carbon side chain. The biosynthesis of lumazines and riboflavin from pterins is shown in Fig.3(b). The deamination of pterin to lumazine has recently been demonstrated in rat liver[22] and riboflavin biosynthesis in micro-organisms and plants is reviewed by Winestock and Plaut (1965)[108]. The origin of biopterin found in mammalian urine and liver is speculative. Possible sources of biopterin are food, by synthesis by intestinal flora, or by biosynthesis from pteridines (reversal of lumazine and riboflavin biosynthesis) or folates. These sources have been tested only on three occasions when it was shown that biopterin excretion was not altered by antibiotics, which suppressed the growth of intestinal flora[65,80], nor by folate or riboflavin deficiency[65,66] and was not diminished by a biopterin-free diet, even in the F_2 generation and therefore cannot be derived from stored biopterin. Orally administered biopterin was well absorbed but not utilised[80], and Pabst and Rembold (1966)[65] concluded that the excreted biopterin was synthesised in the animal. Coenzyme forms of pterins found in mammalian systems are an unknown form of neopterin and tetrahydrobiopterin which may be derived by reduction of sepiapterin, biopterin (?) and dihydrobiopterin, see Fig.3(a). Sepiapterin reductase[67] and dihydropterin reductase[68] have been isolated from mammalian and avian liver. Tetrahydrobiopterin (and other pterins?) functions as a coenzyme for oxidation/reduction reactions and the conver-

sion of phenylalanine to tyrosine [see page 4 reaction(e)] has been used to estimate the level of tetrahydrobiopterin in rat liver (13μg/g), kidney (1.5μg/g) and brain (0.75μg/g)[69]. Estimates of serum biopterin in man using Crithidia fasciculata were higher (27ng/ml)[70] than folate (10ng/ml)[20].

Other reactions requiring pterin coenzymes are, lipid synthesis: the sparing action in the biosynthesis of dicarboxylic acids by biopterin or neopterin in Crithidia fasciculata[71]; lipid catabolism:

$R.CH_2.O.CH_2R^1 \xrightarrow{\text{tetrahydropterin}} R.CH_2OH + R^1CHO \longrightarrow R^1COOH$

as in the production of stearic acid and glycerol from batyl alcohol in rat liver homogenates[72]; and a possible role in steroid hydroxylation:

progesterone ⟶ 17α-OH-progesterone, which is stimulated by relatively large doses of 6methylpterin or H_4PteGlu in the rat testis[73]. A mechanism suggested for the hydroxylation of phenylalanine is the production of a quinonoid.dihydropteridine intermediate[74], analogous to the flavin semiquinone intermediate[75], however the quinonoid dihydropteridine has recently been criticised and a peroxide intermediate proposed[76]. Although these oxidation systems are as yet unknown in composition and mechanism they are of considerable physiological and pharmacological importance.

Folates can also be synthesised from purines (Fig.2). The proposed 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine intermediate is coupled to p-aminobenzoylglutamic acid or p-aminobenzoic acid giving 7,8H_2-PteGlu and 7,8 dihydropteroate respectively. Glutamic acid may also be coupled to 7,8 dihydropteroate giving 7,8H_2-PteGlu. However

in a healthy adult, the now classic study by Herbert[77] showed that folate synthesis, if any, was not sufficient to prevent serum folate deficiency and anaemia when fed a folate deficient diet (5µg folate/day). No correlation was observed in patients between the types and frequency of intestinal microflora and serum folate[78] indicating that there was no net folate synthesis useful to man and thus dietary folate is the sole source of folate available to man. Folate coenzymes are biosynthesised by reduction to H_4PteGlu and then a 1-Carbon unit (formate, formaldehyde or methyl oxidation states) is attached at N5 or N10, see Fig.1. PteGlu, which has no known coenzyme function and probably does not exist naturally, is reduced to H_2PteGlu by dihydrofolate reductase. Enzymes catalysing all these folate interconversions have been isolated from mammalian liver.

The function of the tetrahydrofolate coenzyme lies in its ability to accept a carbon source, producing the other folate coenzymes and, in turn, their ability to transfer the carbon unit, and possibly further hydrogens, producing either tetrahydro- or dihydro-folate. Reduction of dihydrofolate to tetrahydrofolate then completes the folate cycle of 1-Carbon transfer. The immediate carbon sources are formate; the amino acids glycine, serine, histidine and methionine (and S-adenosylmethionine); trimethyl-sulphonyl chloride and carbon dioxide. The ultimate carbon sources may be proteins, carbohydrates, lipids or carbon dioxide (photosynthesis). Folate interconversions convert the carbon source into a form to be utilized in the synthesis of amino acids, proteins and nucleic acids (see Table 2)

although folate involvement in the synthesis of choline, methane, lipid and as a source of energy have been suggested. The involvement of different folate coenzymes in intermediary metabolism is summarised in Table 2.

The mediation and interconversions of folate coenzymes has been studied in detail and found to be associated with vitamins B_{12}, C, riboflavin and iron.

Folate- and vitamin B_{12}- deficiencies produce conditions which are haematologically indistinguishable, giving rise to megaloblastic anaemias: both B_{12}, isolated from liver in 1948[84], and folate are antimegaloblastic factors. Small doses of PteGlu produce a haematological response only in megaloblastic anaemia due to folate deficiency as does cyanocobalamin only in B_{12} deficiency, but large (pharmacological) doses of either compound produce haematological responses in both types of anaemia. However the response to PteGlu in cobalamin deficiency was incomplete due to the progressive development of neurological symptoms (or PteGlu caused the neurological disorders) or the occurrence of neurological relapse. The many experiments performed have failed to clarify any relationship between folate and cobalamin and the levels of folate in red cells, the clearance of folate from plasma, the urinary excretion of folate after intravenous folate administration and of formimidoylglutamic acid after histidine were similar in both cobalamin- and folate- deficient patients. The biochemical role of folate has been centred around its greater requirement in synthesis of thymidylate (and hence DNA) than uracil (and hence RNA), see Fig.4, and thus the development of the megaloblastic state. The 'methyl-trap'

hypothesis[85,86] proposed that B_{12} was active only in the transmethylation of homocystine to methionine requiring 5-CH_3-H_4PteGlu, and thus in B_{12}- deficiency it was proposed that 5-CH_3-H_4PteGlu was increased at the expense of the other folate coenzymes causing a reduced synthesis of nucleic acids. However there is little evidence that this methyl transfer reaction is the sole one and recent evidence[82] shows that on a diet deficient in B_{12} and limiting in methionine, the metabolism of L-histidine ([2-^{14}C] imidazole) to $^{14}CO_2$ was reduced compared with a diet containing either B_{12} and/or methionine. The production of $^{14}CO_2$ from 5[^{14}C]-CH_3-H_4PteGlu was not altered in B_{12}-deficiency nor by the methionine level. These results show that B_{12} independent transmethylation reactions are operating and thus the 'methyl-trap' hypothesis must be at least modified. Other proposals for the role of B_{12} are in the conversion of folate to polyglutamate[88,89] in neurological disorders[90,91,92], the reduction of ribonucleotides to deoxyribonucleotides[93] (consistent with the observed increase in plasma lactic dehydrogenase in megaloblastic anaemias[94]), the reduction of 5,10-CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu[95] and the role of arsenic[96] and cyanide[91] in megaloblastic anaemias. Evidence is lacking and indirect, and these hypotheses highly conjectural. A method for the estimation of 5-CH_3-H_4PteGlu in rat liver and urine was used during this work and should be of value in diseased conditions involving 5-CH_3-H_4PteGlu coenzyme.

Biochemical relationships between folate and ascorbate remain obscure but a large proportion of patients with an-

aemia and clinical scurvy do show evidence of megaloblastic haemopoesis and patients responded to ascorbate and also to folate enhanced by ascorbate[98] but not to iron, folate or cobalamin[97]. Folate and ascorbate were related in irradiated rats[99] and folate was required for ascorbate synthesis in rats[100] but no relationship could be found in guinea pigs[101]. May et al.[102] concluded that the requirement for folate was increased in scurvy and preliminary results presented here suggest a role for ascorbate in normal folate metabolism.

Iron deficiency, per se, may lead to folate deficiency. A relationship between iron and folate metabolism has been shown in experimental animals and in man. Patients with iron deficiency had an increased formimidoylglutamate excretion[103]. A similar secondary folate deficiency has been shown in rats when a decreased activity of formimidoyltransferase was reported[104].

Biotin treated rats had increased folate levels[105] and high doses of PteGlu increased the urinary excretion of riboflavin[106] although folate and B_{12} failed to prevent decreases in blood haemoglobin, erythrocytes and serum proteins in riboflavin deficiency[107] and thus a folate relationship with riboflavin has yet to be confirmed.

Thus deficiencies of B_{12}, ascorbate or iron may lead to secondary folate deficiency symptoms which may be difficult to distinguish from primary folate deficiency. Folate deficiency, however, is probably the most common hypovitaminosis of man and is often associated with low income groups[79] or a disinterest with food[109]. On the other hand pterin deficiency is unlikely to occur since it is

synthesised in the body; deficiency diseases have not been reported and phenylketonuriacs have normal tetrahydropterin and dihydropterin reductase. The daily requirement of PteGlu has been estimated (at 50-100µg for adults and 20-50µg for children aged 1-4 years) by restoration or maintenance of blood folate or normal haematology. These studies, however, do not relate to the effect of dietary folates (which are largely unknown) nor do they indicate if there was selective replenishment of the several forms of body folate. 5-CH_3-H_4PteGlu, a naturally occurring dietary folate, was compared in metabolic profile with PteGlu studies in this work.

Folate deficiency may thus arise due to malabsorption, an increased folate demand, in association with other nutritional factors or an increased excretion and these in turn may be enhanced by drugs, alcohol, states of stress or in pregnancy.

Malabsorption of PteGlu has been demonstrated in tropical sprue, idiopathic steatorrhea, intestinal disease and infection with intestinal worms and megaloblastic anaemia is frequently associated. It is important to note that, although the mean absorption is lowered, estimates vary widely and the use of PteGlu may have obscured important biochemical and physiological variations in these conditions.

The importance of folates in cell growth and division has led to the development of folate antimetabolites for use in cancer chemotherapy and a treatment for leukaemia using aminopterin was described as long ago as 1948[110]. Since then aminopterin, and to a greater extent metho-

trexate, have produced impressive results with Burkitts lymphoma, psoriasis, leukaemia and suppression of immune response in homograft rejection although only a limited success has been achieved with solid tumours, These folate antimetabolites are thought to act by inhibiting dihydrofolate reductase and such an inhibition in vitro is well documented[8]. This inhibition causes a depletion in tetrahydrofolate (and hence other tetrahydrofolate coenzymes) producing similar effects to those in folate deficiency. A crucial effect is in depleting the level of 5,10-CH_2-H_4-PteGlu for thymidylate synthesis. The incorporation of formate into DNA- purines was inhibited to a greater extent than RNA- purines in rat tissues[111] and Ehrlich ascite cells[112]. Because of the non-specific action in depleting all tetrahydrofolates these antimetabolites are extremely toxic even though leukaemic lines P-815 and 70429 in mouse contained folate patterns different from mouse liver[113]. The toxicity is greatest in rapidly proliferating cells such as the intestinal mucosa and antimetabolite therapy can lead to abdominal pains, nausea, vomiting and diarrhoea. Coe and Bull[114] warned that "there is need for caution in the use of methotrexate in benign disease. A prospective study should be carried out to determine the best method of monitoring hepatotoxicity of this drug during long term administration". Only limited success has been achieved in reducing the toxicity of these antimetabolites by using 5-HCO-H_4PteGlu or 5-CH_3-H_4PteGlu and with 5-HCO-H_4PteGlu the antitumour effects were decreased[115], presumably because the 5-HCO-H_4PteGlu afforded a greater protection to leukaemic cells(L1210 in mice) than the host.

The uptake, metabolism and physiological disposition of folate analogues has been extensively reviewed[8].

The many studies performed to establish the occurrence, the physiology and biochemistry of folates have produced confusion by conflicting reports, some of which have been clarified by work presented in this thesis.

Results on food folates vary not only with the type and preparation of the food but with the extraction and assay techniques. The protective effect of ascorbate and a comparison of eight extraction procedures has been compiled[28]. The levels of four American daily diets were 157µg before and 689µg after conjugase treatment using L.casei[116], although some estimates varied by an order of magnitude. High cost diets contained only slightly higher folate (193µg) than low cost diets (157µg)[117]. Butterworth et al.[118] later attempted separation of food folate by chromatography and found 55% as 10-HCO-PteGlu, 34% as 5-HCO-H_4PteGlu and 10% as PteGlu but they did not rule out decomposition, no reference was made to 5-CH_3-H_4PteGlu and conjugates were not detected. In a more recent study[119] of 111, 24 hour diets collected in neutral ascorbate, of the total folate, 10% was active for S.faecalis and 25% for L.casei and that 25% of the total folate was microbiologically active before conjugase; they concluded "the increase in L.casei over S.faecalis after conjugase represented a persistence of methylated and/or triglutamate forms" and continued "that the assay of S.faecalis before conjugase represented pteroates other than methylated forms and may have none, one or two glutamate residues". Derivatives in milk were 20ng/ml without conjugase treat-

ment. Liver contained some 0.6-20μg/g mainly as conjugates[121,122]. Thus natural folates in the diet foods are mainly conjugates which are tetrahydro- derivatives and may be methylated or formylated, but these may be affected by cooking. Ghitis[123] found that boiling for 5mins reduced the folate content of pasteurised and fresh milk by up to 90% and 60% respectively, whilst Hurdle[124] found no loss with pasteurised milk and no loss on boiling or frying liver or white chicken meat. Hurdle reported losses of up to 90% on cooking broccoli and eggs. Little is known about the chemistry of cooking nor storage of food folate although it is generally accepted that folates (or at least the growth of folate-assay organisms) are reduced by cooking; many more studies are needed. These estimates of food folate show that the mean total daily monoglutamates (and up to triglutamates) is similar to the requirements of PteGlu(50-200μg daily) and is discussed later. Some of the required folate must then be derived from conjugates, either by autolysis accompanied by a conversion into microbiologically-active folates, and/or by utilization of conjugates. Folates containing up to three glutamic acid groups are well absorbed[125-8] but the absorption of $PteGlu_7$ varied from significant[129,130] and slight[128,131,132] to undetectable[133]. 75% of dietary folates were conjugates[116,119] which were still evident after cooking[134] and thus cooking alone produces little, if any, conversion of conjugates to monoglutamates. It is therefore likely that conjugates are nutritional folates even though earlier evidence did not rule out the absorption of impurities[135]. A crucial point is whether or not synthetic polyglutamates are sufficiently

similar to dietary folates for the results to be significant. Yeast[30] and liver conjugates were reduced and methylated or formylated derivatives, and the small amounts of unreduced forms may have been derived from reduced forms during isolation. The nature of the "conjugates" has not been studied sufficiently. Conjugase is an enzyme which has been isolated from many sources and is a carboxypeptidase converting $PteGlu_7$ to PteGlu[137]. Bacterial conjugase is relatively non-specific, hydrolysing a variety of mono- di- and tripeptides[138] and the chicken-pancreas enzyme attacks not only $PteGlu_3$ but also p-aminobenzoyl-γ-γ-triglutamate[139,140]. Although pteroyl-hepta-glutamic acid was isolated from yeast and characterised by chemical and biological properties[141-2] only an L.casei-active factor containing three glutamic acid molecules was characterised from liver[142]. The characterisation of other hepatic folates relies on ion-exchange properties compared with synthetic polyglutamates, but elution patterns are profoundly altered by reduction or substitution of polyglutamates. Attempts were made during this work to further characterise hepatic polyglutamates using t.l.c., ion-exchange and Sephadex chromatography.

Since little is known about food folates, most of the metabolic studies have been done with PteGlu as a model compound.

Shortly after its chemical synthesis, metabolic studies with PteGlu showed that it was efficiently absorbed and a transient rise in urinary folate followed[131,143,144]. Later studies, also on humans, showed a peak in the serum (systemic blood) between 1-6h and most frequently at about

2h after the oral dose and that higher doses produced elevated serum folate peaks. This rise in systemic folate was dependent upon the absorption, tissue uptake, metabolism (chiefly hepatic) and excretion of the oral dose, the study therefore gave little direct evidence of PteGlu absorption. When assaying systemic blood, it was suggested that a preloading saturation dose (15mg) gave a more reliable index of PteGlu absorption[159]. However, in view of the findings presented here, the argument and validity of such a preloading dose is questionable. Despite the disadvantages in that relatively large doses (1-5mg PteGlu for humans) must be used to obtain a significant increase in serum folate and that systemic folate may differ from portal folate, systemic folate assays have aided the diagnosis of folate malabsorption. Assays of the faecal excretion of folate might conceivably give more reliable estimates of absorption. Unfortunately microbiological assay of faeces[145] were higher than the oral folate due to bacterial synthesis[146] and/or biliary excretion of folate[147]. Faecal excretion showed extensive variation in humans, after oral administration of labelled PteGlu. Urinary excretion has also been used to study folate absorption but, since the metabolic pathway(s) were not defined, the results are inconclusive.

Detailed studies on PteGlu absorption have been performed in rats. Although Cohen *et al.*[148] found no variation in absorption along the small intestine, everted-sac[149, 150, 151] and *in situ*[152] techniques have shown a greater absorption in the proximal part of the small intestine. Impaired absorption of folate has been found in cases with diseases and resection of the small intestine[23]. PteGlu

absorption may be active or passive and in a detailed study of factors affecting transport in the everted sac, Smith et al.[151] concluded that PteGlu may be transported to some extent by passive diffusion in addition to transport by solvent drag. Little work has been done on the factors affecting the absorption of other folates.

It has been suggested that metabolism of PteGlu takes place during absorption and that, on the serosal side, 5-CH_3-H_4PteGlu appeared in man[125] and 5-CH_3-H_4PteGlu and 5-HCO-H_4PteGlu appeared in the hamster[154]. The suggestion that PteGlu is absorbed without metabolism[151,153,155,156] was confirmed during this work.

Little work has been published on the absorption of reduced folates.

Both direct[25,157,158] and indirect[157] studies have established that PteGlu is rapidly taken up by the tissues. Chanarin et al.[158] concluded that about 60% of an intravenous load (15µg/kg) was cleared into the tissues and extracellular fluid in 3min. In rats[157], 24h. after a dose of PteGlu 29% of the total remained in the kidney, liver, spleen, testes and duodenum, 10% in the plasma but less than 1% remained in the erythrocytes. The observed increase in systemic blood folate (at 2h.) has clearly had sufficient time to be taken up by the tissues.

Exactly what happens after PteGlu is taken up is uncertain. Johns et al.[25] found that PteGlu could be flushed, up to 3 days later and concluded that PteGlu was slowly metabolised. Indirect arguments produced in support of this conclusion are based on the low activity of dihydrofolate reductase at physiological pH and, although the re-

duction of H_2PteGlu is continually required, the reduction of PteGlu is not required since most of the food folates are in reduced forms. On the other hand, PteGlu was reduced on absorption [125,154] and rapidly reduced after intramuscular injection[125] but the intestine contains much lower levels of dihydrofolate reductase than the liver and none could be detected in muscle[160]. In view of the proposed slow reduction of PteGlu, the observation of reduced folates appearing in blood and urine was explained by displacement[161] rather than by metabolism. Chanarin and McLean, using tritiated-PteGlu, showed unlabelled metabolites in the urine[161] in ten out of fourteen cases; however the characterisation of metabolites was ambiguous and specific activities were not reported[162]. Butterworth *et al.*[156], using [2-^{14}C]-PteGlu, found a greater dilution than expected and concluded "While this may indicate some dilution by displacement of non-radioactive folate from tissue, it does not approach the nearly complete displacement as described by Chanarin and McLean ------ further investigation will be needed to determine if this discrepancy might be due to differences in labelled folate, patient material, dosage or route of administration." Such further observations, carried out on rats, are presented in this thesis.

The haemolytic response and the effectiveness afforded in folate therapy show that PteGlu is metabolised, ultimately at least, into naturally occurring folates. Microbiological assays have shown that the majority of body folate is in the liver. As with food folates, estimates of

hepatic folates vary with the extraction and assay conditions and present-day investigations are based on extraction, separation on DEAE-cellulose and microbiological assay of fractions before and after conjugase treatment[121,122]. Bird et al.[121] observed that alternate freezing and thawing or autolysis of rat liver decreased the conjugates and altered other folates, compared to an extraction obtained by heating fresh liver for a short period. They concluded that endogenous conjugase was released by either freezing and thawing or autolysis but was inactivated by the short heating period. It was also pointed out that the temperature and heating period were critical as folates were altered by changing either of these parameters. Using this technique 75% of the hepatic folates were conjugates (more than five types), 67% were 5-CH_3-H_4PteGlu or its derivatives and the total folate was 20µg/g wet weight. Other forms of folate were 5-(and 10)-HCO-H_4PteGlu and their conjugates with little unsubstituted H_4PteGlu, although decomposition of H_4PteGlu was not ruled out and materials with chromatographic behaviour of pteridines (the likely decomposition products) were found. Other workers have found folate levels ranging from 0.1 to 20µg/g for rat liver[121,163-5] and 0.6 to 17µg/g for human liver[120,166]. Rat kidney, spleen and intestine contain 1.6, 0.6 and 0.6µg of folate/g respectively[163,165] and bone marrow 95 to 726ng/ml[167], but no indication is given on the degree of hydrolysis which occurred before microbiological assays and the types of folate are unknown. Serum folate contains only 5-CH_3-H_4-PteGlu and accounts for the much greater growth of L.casei than either S.faecalis or P.cerevisiae[168]. The resting

level is about 10ng/ml in man. Rat serum folate[121,169] was significantly higher than that of cattle, sheep, horse, rabbit and chicken which were similar to man[70]. Erythrocytes and leucocytes contain various folate conjugates.

Folate excretion has been used to determine the absorption, renal handling and tissue uptake in an attempt to diagnose folate deficiency. Folate excretion in sweat has been studied only on one occasion when it was shown that, under profuse sweating, S.faecalis and L.casei response was five to six times higher than that in urine and that the growth was not increased after incubation with conjugase.

A few recent studies on biliary folates showed that oral PteGlu and 5-HCO-H_4PteGlu were rapidly converted into biliary folates[147,170,171]. Purification of biliary folates tentatively identified the presence of 10-HCO-PteGlu, 10-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu (20-55%) and possibly a triglutamate form[147]. An interesting finding was that the concentration of biliary folates exceeded that in the plasma[147,170,171], but a concentration or secretion mechanism has not been suggested. The presence of conjugase[172] enzyme has been disputed[147] and folate conjugates are absent. The extent of the cycling of folates from intestine via plasma to the bile and back to the intestine has not been studied quantitatively and the nutritional effects of biliary excretion are unknown. PteGlu may, in certain circumstances, pass into the intestinal lumen directly through the mucosa of the stomach, duodenum and jejunum[173].

The major excretion of folate is in the urine where, in humans, 1-18μg of folate are excreted per day[130,132],

[143,144,174,175,185] but the type(s) of folate excreted have not been studied at this level. Folate excretion is increased following oral, intravenous and intramuscular injection of PteGlu and the pattern of excretion is well documented. After oral administration of PteGlu there is a transient urinary rise at, or shortly following, the peak of serum folate[143,176] and at physiological doses (up to 500µg in humans) up to 12% of the dose is excreted[144]. The amount excreted increased with the dose[178,186] and with 15mg PteGlu 77% was excreted[144]. However after pteroic acid, which is ineffective as a replacement for PteGlu[7,177], much less (<3%) was excreted and it was concluded that pteroic acid was both poorly absorbed and poorly converted to folates[177].

Folate excretion following PteGlu has been used as a diagnostic aid in diseased conditions. Following PteGlu load, folate excretion was decreased in patients with steatorrhea[24,183,184,185], pregnancy[159], sprue[179,180], pernicious anaemia[181,185,190], leukaemia[181,182], Hodgkin's disease[182] and in folate and B_{12} deficiencies[161]. The decreased excretion has been shown by several routes of administration of PteGlu to be caused by malabsorption and/or folate deficiency, possibly involving "deranged folate metabolism[9]". However the urinary folates were not identified and therefore "folate deficiency and deranged metabolism" may reflect an increase in tissue uptake or conversion into folate material which is not excreted. Deranged folate metabolism has also been suggested in viral hepatitis, cirrhosis, cardiac failure with congestive hepatomegaly[187], in riboflavin deficiency, high dietary methion-

ine levels[188], and hyperthyroidism[189] where folate excretion is increased.

The renal handling of PteGlu has been studied in detail by Johns and co-workers[25,199] when it was found that PteGlu was reabsorbed mainly by storage in the renal tubules, unlike glucose which is rapidly transported from the tubular lumen to peritubular blood[200]. This reabsorption may be viewed as PteGlu binding to a protein (dihydrofolate reductase) which is blocked by methotrexate[199].

Attempts to characterise urinary folates in normal folate metabolism has led to confusion. Two folates which have been characterised were PteGlu and 5,10-CH=H_4PteGlu. PteGlu[125,184] is inconsistent with more recent microbiological data[162]. 5,10-CH=H_4PteGlu[191,192] is both inconsistent with microbiological assays[162] and with serum folate. Attempts were therefore made to purify and characterise urinary folates and then to extend this approach to the metabolism of natural folates and to the study of hepatic folates.

MATERIALS

Animals. The animals used were adult male Wistar rats, weighing 200-350g, purchased from Scientific Products Farm, Ash, Canterbury, Kent, U.K. Experiments on rats (which had not previously been used for experimentation) were conducted under Home Office Licence with certificate A.

Food. This was diet 41B, supplied by Pilsbury, Edgbaston, Birmingham, U.K. Dietary constituents are shown in Table 3. Pellets of diet 41B were broken into pieces small enough to prevent blocking of the urine/faeces separator. Food, replenished daily, and water were given ad libitum, unless otherwise stated.

U.v. spectra were recorded from 200 to 400nm on SP. 700 or SP.800 (Pye Unicam, Cambridge, U.K.), and at fixed wavelengths on a Uvispek (Hilger and Watts) and fluorescence spectra on an Aminco-Bowman spectrophotofluorimeter.

Pteridines and PteGlu were purchased from Koch-Light Laboratories Limited, Colnbrook, Bucks., U.K.

10-HCO-PteGlu was prepared by a modified method of Silverman, Law and Kaufman (1961)[113]. 5g PteGlu and 500ml of 98% formic acid were refluxed in the dark at 75° for 30min. The preparation was freeze-dried and gave a straw-coloured product (5.1g). U.v. and fluorescence spectra are shown in Table 4. The product showed a fluorescent impurity on t.l.c. which was removed by t.l.c., and by column (15x3.5cm) chromatography on De Acidite-G (chloride form, 150-200 mesh, 3-5% cross-linked). The product (2.5g) was applied to the column in 20ml of 1%(v/v)-NH_3 and eluted (1.5ml/min) with 500ml of 0.05M-HCl followed by 1500ml of

0.1M-HCl. The fluorescent impurity was eluted with 0.05M-HCl and 10-HCO-PteGlu with 0.1M-HCl (peak at 1200ml). This lengthy procedure gave an impurity of PteGlu (identified on t.l.c.) due to hydrolysis of 10-HCO-PteGlu. 10-HCO-PteGlu was routinely used without purification.

H_2-PteGlu was prepared by reduction of PteGlu with sodium dithionite[193]. The white product showed one light-blue fluorescent spot on t.l.c. The product was stored as a suspension in 0.005M-HCl at -15°C for up to four weeks without appreciable decomposition.

H_4-PteGlu was freshly prepared by catalytic hydrogenation of PteGlu over Pt in glacial acetic acid[194], followed by freeze-drying. T.l.c. showed small amounts of two fluorescent impurities. U.v. spectra indicated a purity of 60-80%.

5-HCO-H_4PteGlu, as Ca leucovorin, was a gift of Lederle Laboratories Ltd., Pearl River, N.Y., U.S.A. and was readily converted to 5,10-CH=H_4PteGlu in dil-HCl immediately before use[196]. U.v. and fluorescence spectral data are shown in Appendix II.

5,10-CH_2-H_4PteGlu was prepared by suspending PteGlu (3g) in 100ml tris buffer (pH 7.8), to which was added sodium borohydride in water (25ml). The reaction mixture was kept under nitrogen and shielded from the light throughout the preparation. After 15min stirring, excess borohydride was destroyed by adjusting the pH to 5.5 with 5M acetic acid. Formaldehyde (4ml, 38% w/v) was added and the pH adjusted to 5 with 1M acetic acid. After a further 15min the reaction was cooled in an ice bath, 1.5ml of 2-mercaptoethanol added and the pH adjusted to 7 with 0.5M-

NH_4OH. Purification was done on DEAE-cellulose (DE 52, Whatman, W.& R. Balston Ltd., Maidstone, Kent, U.K.) washed with 2 litres of 0.4M-ammonium acetate and then water until free from acetate, tested with $Pb(NO_3)_2$. A 3.5x45cm column was prepared and washed with 3 litres of 4.10^{-3}M ammonium acetate, pH 7.0. The product was applied to the column and was eluted with an ammonium acetate gradient produced by passing 0.4M ammonium acetate into a mixing flask containing 500ml of 4.10^{-3}M ammonium acetate, each containing 2-mercaptoethanol (10^{-3}M). An elution rate of 1ml per min was maintained with a flow inducer (Watson-Marlow Ltd., Bucks., U.K.). 10ml fractions were collected and the absorption at 295nm recorded. The fractions containing the major peak were pooled and freeze-dried. The purified product was dissolved in 40ml of deaerated water containing 500mg of sodium chloride, the solution was adjusted to pH 7.0 with 0.1M-NaOH and filtered, 2-mercaptoethanol was added (to give a solution of 10^{-3}M) and 12ml of calcium chloride solution (containing 10g $CaCl_2.6H_2O$ per 100ml) added. The calcium salt of 5,10-CH_2-H_4PteGlu was precipitated overnight at 4°C by the addition of 200ml of ethanol. The product was filtered, washed with 20ml of 75% aq. ethanol and then 20ml of ethanol. The product was rapidly dried under high vacuum (0.1 torr). The purified product was identified as 5,10-CH_2-H_4PteGlu by u.v. spectra (Fig.5) which showed that the compound was stable in neutral and alkaline solutions and labile in acid. Rf values in 0.1M sodium phosphate buffer, pH 8.0, formic acid in 0.1M sodium formate (2%, v/v) and ethanol-water (7:3, v/v) were 0.72, 0.49 and 0.10 respectively, compared with

0.72, 0.66 and 0.31 respectively for H_4PteGlu.

5-CH_3-H_4PteGlu was prepared by the method of Blair and Saunders[61]. The product showed a small amount of a fluorescent impurity on t.l.c. and the purity was 80-95% by u.v. spectra.

$PteGlu_3$ was a gift from Mr. McArdle, Queen Elizabeth Hospital, Birmingham, U.K. Despite the fact that this had been kept several years in a dil. solution of phenol; the solution showed only one yellow compound on t.l.c. with u.v. spectra similar to $PteGlu_3$.

Chromatography solvents for t.l.c. These were as follows: (a) butan-1-ol—acetic acid—water (4:1:5 by vol, equilibrated for 20h; upper phase); (b) 0.1M-sodium phosphate buffer, pH 7.0; (c) propan-1-ol—aq. 1% (v/v) NH_3 (2:1, v/v); (d) butan-1-ol—acetic acid—water (20:3:7, by vol.). Antioxidant, when used, was 0.5% (v/v) 2-mercaptoethanol for all chromatography solvents. 0.5% (v/v) 2-mercaptoethanol prevented the noticeable decomposition of 5-CH_3-H_4PteGlu, which occurred occasionally when 0.2% was used in t.l.c. solvents.

Liquid scintillators. These were NE220 (Nuclear Enterprises, Edinburgh, U.K.) and a toluene-based scintillator containing 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(5-phenyloxazol-2-yl)-benzene (both from Koch-Light) made up to 1 litre with A.R. toluene.

Radiochemicals (The Radiochemical Centre, Amersham, U.K.) were [2-^{14}C]PteGlu (as the dipotassium salt; sp. radioactivity 31.4 to 55.3mCi/mmol), [3',5',g-^{3}H]PteGlu (potassium salt; sp. radioactivity 1500mCi/mmol), [2(n)-^{3}H]-DL-glutamic acid (aq. solution containing 2%, aq. ethanol; sp.

radioactivity 3800mCi/mmol), [U-^{14}C]-L-glutamic acid (aq. solution containing 2% v/v, ethanol; sp. radioactivity >225mCi/mmol) and [1-^{14}C] hexadecane (sp. radioactivity 1.06 to 1.10μCi/g). Labelled PteGlu was stored at -15°C as the freeze-dried material.

Purity of pteridines and folates was periodically checked by t.l.c. and/or u.v. spectroscopy. When impurities were present in sufficient amounts to interfere with identifications on t.l.c., the material was discarded and new material synthesised and purified. The purity of labelled materials was estimated by densitometry of radioautographs produced by co-chromatography of the material with unlabelled material. The radiochemical purity of labelled materials used in animal experiments was at least 95%.

All glassware, t.l.c. plates, dissection equipment were decontaminated (Decon-75; Decon Laboratories Ltd., Brighton, U.K.) after use. Gauze baskets used for combustion of materials were cleaned by heating to redness for 2 min. prior to use.

METHODS

Radiolabelled compounds were administered to rats, the urine and faeces were collected throughout the experiment and assayed for radioactivity. Urinary folates were purified initially through a florisil column followed by t.l.c. Blood samples, obtained in several experiments, were assayed for radioactivity and were purified directly by t.l.c. At the end of each experiment the animals were sacrificed and balance studies completed by assaying radioactivity in liver and kidney samples. Liver extracts were also assayed for radioactivity and labelled compounds were purified by florisil followed by t.l.c., by ion-exchange or by gel-filtration column chromatography. Microbiological assays of purified folates were done both to aid in identification and to determine their specific radioactivities.

Administration of labelled compounds. Labelled PteGlu was administered in distilled water by stomach intubation. Rats were firmly handled by stretching the skin back over the head so that the mouth was open and the throat unobstructed. A graduated 1ml all-glass syringe, with a curved metal tube designed to pass down the rat oesophagus, was used to administer a volume (about 0.5ml) calculated to give the required dose. Other labelled folates were administered in 0.5ml of sodium ascorbate, pH 6.0, unless otherwise stated. The commercially available solutions of labelled glutamic acid (in aq. 2% ethanol) were diluted to give the required dose and administered in 0.5ml of solution (made up with water). The small amount of ethanol in

these solutions would not significantly alter the results. All animals recovered immediately after the administration.

Urine and faecal collections. These were performed by housing the rats separately in wire-bottomed metabolic cages fitted with a urine/faeces separator (Metabowl; Jencons Ltd., Hemel Hempstead, Herts., U.K.). Some food did, however, contaminate the faeces and was manually removed before the faeces were freeze-dried. Urine samples were collected in subdued light for up to seven days after administration. Urines were collected in acid (0.5ml conc-HCl) or alkali (0.5ml sat.-KOH) with toluene (5ml) in 100ml flat-bottomed flasks, or were collected frozen in tubes (24x200mm) half covered with acetone/solid CO_2 ($-78^{\circ}C$) in a vacuum flask. The tubes prevented urine from freezing inside the cage and yet were covered by sufficient freezing mixture to last 24h. Antioxidants, when used, were 0.5g sodium ascorbate for urine collected in acid and 0.2ml of 2-mercaptoethanol for urines collected in alkali.

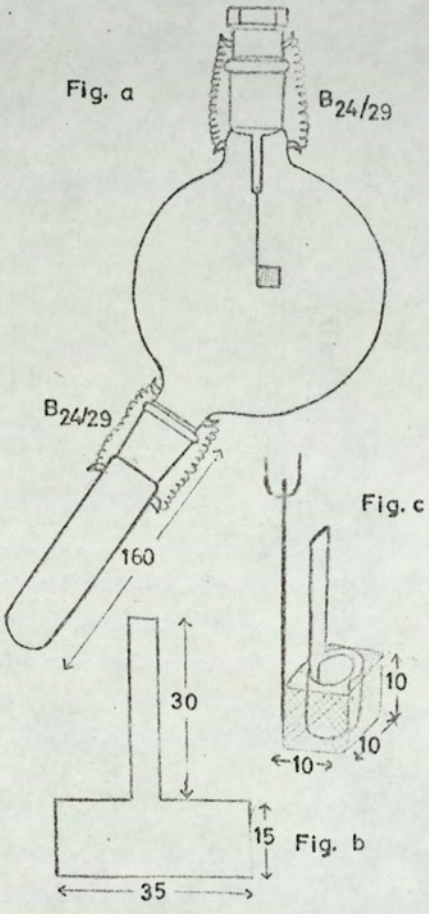
Blood samples. These (up to 0.5ml) were taken from the tail vein. The bottom of a 250ml polythene bottle (with the screw cap removed) was removed and a hole (about 1cm diameter) made in a rubber bung, chosen to fit tightly into the bottom of the bottle. The tail of the rat was fed through the hole in the bung; the rat placed into the bottle and the bung pushed into place. This was found sufficient to restrain without hurting the rats. The tip of the tail was then removed with a new, sterile surgical blade. The end of the tail was then wiped with a tissue wet with heparin (2% aq. solution). Blood was collected in 2 dram vials previously wetted with heparin solution. Blood flow

was induced by gently running the tail (from base to tip) between the thumb and first finger. When sufficient blood had been obtained the rat was freed and placed in a cage. Subsequent blood samples were obtained in a similar way, after removing the newly-formed blood clot. The rats behaved normally, immediately after being freed. Tests on the blood samples were quickly performed before blood clots were formed. Pipettes, wetted with heparin solution, were used and were washed immediately after use.

Rats were routinely killed by placing them on cotton wool above liquid ether in a desiccator. When hepatic folates were to be purified, the rats were stunned by a blow to the head and then decapitated. The extinction of the "leg-jerk reflex" was used as proof of death.

Determination of radioactivity. This was done by liquid scintillation counting. Urines were counted directly in NE220. Blood and tissue samples were assayed by combustion, essentially by the method of Kalberer & Rutschmann [197]. 25ml of a solution of ethanolamine (120ml made up to 1 litre with A.R. toluene) was pipetted into the side arm which was then attached to the combustion flask (Fig.a). The side arm was then dipped into a vacuum flask containing acetone/solid CO_2 mixture. Liver, kidney or faeces were freeze-dried and weighed. Using a pestle and mortar, each tissue was ground to a homogeneous powder. Paper baskets (Fig.c) were formed from ashless filter paper cut to shape (Fig.b) by wrapping the paper around a pencil. The baskets were weighed before and after addition of a sample (about 50mg) of the tissue. The paper basket was then placed inside the Pt-basket (Fig.c). The flask was flushed

with oxygen; the paper wick was lit and the assembly plunged into the combustion flask and secured with springs. After complete combustion (about 2min) the side arm was taken out of the cooling mixture and the ethanolamine solution tipped into the main flask. CO_2 absorption was complete in 1 hour. After this time the ethanolamine solution was tipped back into the side arm; the side arm was removed and 5ml of the ethanolamine solution counted in 5ml of toluene-based scintillator. Combustion was done behind a safety screen and safety procedures (see ref 197) strictly followed. For blood samples, further filter paper was placed in the paper basket sufficient to absorb blood (up to 0.5ml) without the blood reaching the wick. The paper was dried at 110°C for 10min and then the above combustion procedure followed. Direct counting of blood samples in NE220 or after dissolving in Soluene (Packard International, Zurich, Switzerland) was unsuccessful either because samples were not completely dissolved (or decolourised) or low counting efficiencies were obtained. Fresh liver was weighed and a sample (up to 250mg) was quickly weighed into the basket to reduce evaporation losses. The samples were then dried and burnt as for blood samples. Recovery of radioactivity ([2^{14}-C]pteridine in water) carried out with four samples each of about 5nCi, was 96±5%.



Radioactive samples were counted in a automatic liquid scintillation spectrometer (Nuclear Enterprises) operating at 0°C. Total sample counts were at least 5000 counts above background. Counting efficiencies were determined for each sample by an internal standard (about 30mg) of [1-^{14}C]-hexadecane, diluted (about 10 times) accurately (±0.1%) by weight with unlabelled hexadecane (British Drug Houses). Details of the counting conditions are given in Appendix I.

Chromatography on florisil. This was essentially similar to Foxall[198]. Florisil (8g, 60-100 mesh; Koch-Light) was washed with 100ml of aq. 1% (v/v)-HCl, then to neutrality with water, followed by 100ml aq. 14% (v/v)-NH_3, then to neutrality with water and finally with 50ml aq. 1% (v/v)-HCl. This slurry was packed into a glass burette and shielded from light. Samples were applied in (up to 25ml) a solution at about pH 1.0. Elution, designed to desalt samples with the min degradation and with the max recovery, was carried out with 30ml aq. 1% (v/v)-HCl (acid fraction), followed by 30ml of water (neutral fraction) and finally 25 to 30ml of aq. 14% (v/v)-NH_3 (ammoniacal fraction). Antioxidants, when used, were 2% (w/v)-ascorbic acid in acidic solutions and 0.5% (v/v)-2-mercaptoethanol in neutral and alkaline solutions. The distribution and recoveries of radioactivity after applying and eluting [2-^{14}C]PteGlu without antioxidants and 5[^{14}C]CH_3-H_4PteGlu with and without antioxidants are in Table 5. Chromatography of the three fractions obtained after application of [2-^{14}C]PteGlu in water showed the presence of one labelled material which co-chromatographed with unlabelled-PteGlu on

t.l.c. in solvents (a), (b) and (c). When 5-[^{14}C]CH_3-H_4-PteGlu (about 100mμCi, pH 1.0) was eluted from a florisil column, and the acid and alkaline fractions chromatographed by t.l.c., two labelled compounds were present in solvents (b) and (c) and one in solvent (a). 5[^{14}C]CH_3-H_4PteGlu gave similar spots of which one was identified, by co-chromatography, as 5-CH_3-H_4PteGlu; the other was unidentified. When 5[^{14}C]CH_3-H_4PteGlu was applied and eluted from a florisil column and chromatographed by t.l.c. with antioxidants throughout, only 5-CH_3-H_4PteGlu was present. Thus antioxidants prevented the decomposition of 5-CH_3-H_4PteGlu when chromatographed on a florisil column and by t.l.c., and each of the three florisil fractions contained similar compounds. The elution profile of 5[^{14}C]CH_3-H_4PteGlu showed that, on this occasion, urine caused a retention of 5-CH_3-H_4PteGlu possibly due to a buffering action, Fig.6. Elution of urine with NH_3 was therefore carried out until the eluate (up to 30ml) was colourless.

The effect of the changes in pH which occur during florisil chromatography (with antioxidants) was tested on 5,10-CH_2-H_4PteGlu, which is stable in alkali and labile in acid, and on 5,10-CH=H_4PteGlu which is stable in acid and labile in alkali. T.l.c. of the ammoniacal fraction from chromatography of 5,10-CH_2-H_4PteGlu showed five bands: Rf 0.00 (PteGlu), 0.23 (l.blue fluorescence), 0.39 (l.blue fluorescence), 0.46 (purple fluorescence) and 0.53 in solvent (a). The compound at Rf 0.53 was identified as 5,10-CH_2-H_4PteGlu by Rf values and co-chromatography in solvents (a), (b) and (c). T.l.c. of the ammoniacal fraction from chromatography of 5,10-CH=H_4PteGlu showed three bands:

Rf 0.35 (5,10-CH=H_4PteGlu), 0.39 (light blue fluorescence) and 0.62 (light blue fluorescence) in solvent (a). Extensive decomposition of these folates had taken place and florisil purifications were therefore performed as rapidly as possible and the ammoniacal fraction frozen as it was being eluted.

Thin-layer chromatography. T.l.c. plates were made from cellulose (MN 300 or MN 300 u.v.; Macherey Nagel and Co., Düren, Germany) according to the Manufacturer's recommendations using a t.l.c. spreader (A.Gallenkamp and Co. Ltd., London, U.K). In general, larger quantities of material were applied to thicker and larger plates. Over 1mg quantities of freeze-dried material were applied as streaks to plates 1x200x200mm. Smaller quantities were applied to plates 50 or 100mm wide and 0.5 or 1.0mm thick. Precoated films (0.10mm thick; MN 300 u.v., Macherey Nagel and Co.) were used for quantities up to 50µg applied as single spots in volumes up to 20µl. Larger volumes (up to 5ml) were applied to plates as streaks (using an SA 20 streak applicator; Burkard, Rickmansworth, Herts., U.K.). T.l.c. plates were dried in a stream of cold air. All chromatograms were developed to 10cm from the origin, with solvents prepared immediately before use. Compounds were detected by viewing the plates under u.v. light. Fluorescent compounds (down to 2µg/cm^2) were detected under 365nm and absorbing compounds (down to 25µg/cm^2) under 254nm wavelength light. Materials were extracted from cellulose with water by suction through a sintered glass filter funnel, collecting the filtrate in a tube surrounded by acetone/solid CO_2 mixture. The extraction procedure was repeated twice. Antioxidant,

when used, was 0.5% (v/v) 2-mercaptoethanol. Recovery of radioactivity ([2^{14}C]PteGlu) from cellulose was 70±4%. U.v. spectra; recorded on material extracted from chromatograms, showed large peaks due to both the cellulose additive and to the cellulose itself.

The Rf values of the pteridines and folates used during this work are shown in Table 6. The Rf values were determined from several chromatograms and are reported to show that the three solvent systems used were sufficient to separate natural folates and pteridines. Rf values varied with the amount, the thickness of the plate and the solvent preparation. Corrections for variations in the amounts of compounds and thickness of the plate were not attempted since plates were routinely run with standards and compounds were also characterised by their fluorescence or absorption. Most compounds showed variations in Rf of ≈0.1. The variation of Rf with the concentration of PteGlu when chromatographed in acidic systems is given in Appendix II.

Radioautography. This was used for detection and location of labelled compounds on chromatograms. An X-ray film was used as described in Appendix I. The relative amounts of labelled compounds were estimated by scanning the radioautograph with a microdensitometer (MK IIIc; Joyce, Lobel and Co. Ltd., Gateshead, Co.Durham, U.K.). The peaks were cut from the traces, weighed and the relative size calculated.

Chromatography on DEAE-cellulose. The method was that described by Silverman et al.[113]. 16g of DEAE-cellulose (DE 52; Whatman) and 22.5g of Hyflo (Johns Manville, N.Y., U.S.A.) were stirred into a slurry (about 700ml of water)

and a portion packed into a burette. The column was prepared for use by sequential washing with 25ml of 0.5M-NaOH, water to neutrality, 25ml of 0.5M-sodium phosphate, pH 6.0, and water until the eluate was free of phosphate (to sat. $BaCl_2$ solution). The final height of the cellulose column was 19-20cm. Sample volumes (2ml) were adsorbed on the column, followed by washes of 2ml and 1ml volumes of ascorbate solution (ascorbic acid to pH 6.0 with NaOH; final concentration 0.2% w/v). A head(40ml) of the latter ascorbate solution was placed on the column and gradient elution performed by passing 0.5M-sodium phosphate buffer, pH 6.0 containing 0.2% (w/v) sodium ascorbate, drop-wise through this head, from a separating flask, in a closed system. 5ml fractions were collected in tubes containing 0.1ml of 10% (w/v) sodium ascorbate, pH 6.0, using an automatic fraction collector (L.K.B., Produkter AB, Bromma, Sweden). Chromatography was carried out at room temperature and the flow rate was 0.4ml per min. Fractions were stored at $-15^{o}C$ until assayed. [2^{14}-C]PteGlu was eluted with a peak in tube 25 and 5-CH_3-H_4[2^{14}-C]PteGlu in tube 13. Silverman *et al.*[113] and Bird *et al.*[121] recorded peaks at tubes 28 to 29 and 13 for PteGlu and 5-CH_3-H_4PteGlu respectively. Sodium salts were used since potassium salts may interfere with the counting procedures, which was done with 0.2ml of each fraction.

Gel-filtration chromatography. Gel-filtration has been used successfully to separate and determine the molecular weights of both small and macro-molecules. It was therefore hoped that such a valuable aid could be applied successfully to pteridines and folates. Although an en-

tirely adequate theory has not yet been produced to explain the relationship between molecular shape and size to the elution characteristics, the general principle is that smaller molecules enter the pores, present in the solid phase, to a greater extent than larger molecules. Larger molecules are therefore eluted earlier than smaller molecules of a similar type. All molecules larger than the pore size are eluted in the void volume (Vo), that is, in the volume of the 'dead-space' around the solid phase (spheres). Since the mol. wt. of $PteGlu_7$ is 1217, Sephadex-G15 (exclusion mol. wt. 1200) seemed the ideal choice.

A 50ml burette was modified by removing the tap to reduce the dead-space at the bottom of the column. 12g of Sephadex-G15 (Pharmacia, Uppsala, Sweden) was swollen (3h) in 0.1M ammonium acetate containing 0.2% (w/v) ascorbic acid, pH 7.0, and packed into the burette (plugged with glass wool covered with a filter paper). The column was run with a hydrostatic pressure (about 50cm) and, after settling under gravity, the column was topped with a further filter paper. The column was 41.5cm in height and the elution rate was 7.9ml/h. Elution with 0.1M acetate containing ascorbate annuls the ion-exchange effect of the Sephadex (Pharmacia). Fractions (32 drops or 1.08ml each) were collected at room temperature in tubes placed in an automatic fraction collector (L.K.B., Produkter AB). Fractions were stored frozen ($-15^{\circ}C$) until assayed. Table 7 shows the elution volumes (Ve) of compounds (ng to µg quantities) used to standardise the column. Using the elution volume of casein (mol. wt. >100,000) as the void volume (Vo), the constants $K_{av} = V_e - V_o / V_t - V_o$, ($V_t$ is the total

column volume) were calculated for each of the compounds and were plotted against molecular weights (Fig.7).

Glutamic acid and 2-amino-4-hydroxypteridine were eluted much earlier than folates and, in a study of mol.wt., the importance of using molecules of a similar type is apparent. This was done by studying the effect of increasing the no. of glutamic acid groups from zero (pteroic acid) through one (PteGlu) to three ($PteGlu_3$). A plot of the K_{av} values of these three compounds against their molecular weights was not linear (Fig.7). Reduced and substituted derivatives of PteGlu and $PteGlu_3$ were eluted earlier than PteGlu and $PteGlu_3$ respectively, but again the plot of K_{av} against mol. wt. was not linear. A linear regression line (method of least squares) for pteroic acid and folates showed that a trend of decreasing K_{av} against increasing molecular weight, but the correlation (coefficient -0.835) was well below that (-0.99 to -1.0) required for molecular weight determinations. Sephadex-G15 cannot therefore be used to determine mol. wt. of folates and a feasible explanation is that with increases in mol. wt. of folates there are accompanying changes in molecular shape which affect chromatography. Two important points remain. The first is that the pteridines and folates were separated and the Sephadex-G15 provides a purification and identification method which has the advantage over DEAE- cellulose because of its greater capacity. The second point is that since $5\text{-}CH_3\text{-}H_4PteGlu_3$ (mol. wt. 714) was eluted only slightly after casein (mol. wt. >100,000) and that the monoglutamates and triglutamates seemed to be split into families, it may be possible to deduce molecular weights by using

Sephadex with a greater mol. wt. exclusion. To test this a systematic study of folates from monoglutamates up to (say) heptaglutamates is required. Unfortunately such compounds are not yet available and such a study must await their synthesis.

Microbiological assays. Samples to be assayed were freeze-dried in tubes previously sterilised at 130°C for 15min. The tubes were covered (aluminium foil) and stored at -15°C until assayed. Immediately before assay, the samples were dissolved in phosphate buffer, pH 7.0, containing 1% (w/v) sodium ascorbate. The organisms used were Lactobacillus casei N.C.I.B. 8010 grown in Q-Ess medium (gift of B.B.L., Division of Bioquest, U.S.A.), Streptococcus faecalis N.C.I.B. 8043, grown in Difco 0319-15 medium and Pediococcus cerevisiae N.C.I.B. 7837 grown in Difco 0456-15 medium (Difco Laboratories, Detroit, Mich., U.S.A.). Incubation was for 18h at 37°C and the extent of growth was determined turbimetrically. Assays were performed in duplicate at least. Conjugase treatment was done with 0.1ml of conjugase preparation isolated from chicken pancreas[210], incubating at 37°C for 6h in covered tubes and then the solution was frozen until assayed (as above). This conjugase preparation increased the L.casei response to a crude yeast folate-extract.

The Physiological Disposition of Pteroyl-L-glutamic Acid and 5-Methyl-L-tetrahydropteroyl-L-glutamic Acid

SECTION I

Pteroyl-L-glutamic acid was previously isolated from autolysed liver[201], yeast[5], autolysed spinach[6,203] and liver extracts[7,202]. At the time of these isolations little was known of the lability of folate coenzymes and it is now recognised that PteGlu can be formed from folate coenzymes and thus PteGlu was probably an artefact of the isolation procedures. PteGlu is however useful both as a folate derived by decomposition of natural folates and as a model folate. Tritiated-PteGlu has high specific radioactivity and may be used for folate study in man, but is non-specifically labelled[25,184,204,205] and has been criticised on the grounds of possible impurities[8]. The low counting efficiency of tritium and possible tritium-exchange and isotopic effects are other disadvantages. The PteGlu used in these studies was pure and was specifically labelled (at position 2) with carbon-14 which has negligible isotopic effect. 5-CH_3-H_4PteGlu, labelled [2^{14}C], was isolated after administration of [2^{14}C]PteGlu (Section II) and is a coenzyme occurring in liver[206], mitochondria[207], serum[208] and peas[209].

After an oral dose of 320µg PteGlu/kg, assay of the faecal radioactivity showed that 40% of the dose was excreted in 3 days, 4% was excreted in the subsequent three days whilst no radioactivity was detected in a further 3 day faecal collection. Faecal excretion of radioactivity was thus complete in 6 days and faecal excretion studies were done over 6 days. After oral [2^{14}C]PteGlu the mean absorption (dose minus faecal radioactivity) was 25% and 50% at doses of 320 and 22µg of PteGlu/kg (Table 8). The difference in faecal radioactivity at these two doses was

not statistically significant. At the oral dose of 3µg of PteGlu/kg, the absorption is likely to be 50% since the tissue and urinary radioactivity were comparable with those at a dose of 22µg/kg (Table 8). These results show a physiological saturation above 22µg of PteGlu/kg and reflects the saturation kinetics observed *in vitro*[151] and *in vivo*[152]. The faecal excretion (at 22µg/Kg) was higher (Student's *t* test; $<P$ 0.01) than in humans[184] at comparable doses of 40µg of ^{3}H-PteGlu/kg. Although only 4 rats and 3 humans were studied, the differences may indicate a more efficient absorption in man than in the rat. These differences, however, may be due to variations in technique. Anderson *et al.*[184] did not state the purity of the ^{3}H-PteGlu and their subjects were fasted overnight which is likely to increase absorption compared to these administrations which were given between 10.00am and 2.00pm, to animals given food and water *ad libitum*. Another factor which determines the faecal excretion is biliary output. Folate is excreted in human bile[147,170,171] and after oral PteGlu the peak of biliary excretion was 30min after the serum-peak[147], but the fraction of the oral dose which is excreted in the bile has not been determined. The fasting level of biliary folate in human was 10-89ng/ml (*L.casei*) which is 2 to 10 times the serum folate. With a biliary output of about 1 litre per day[171] the excretion (up to 0.1mg/day) is an order of magnitude higher than the urinary output (1-18µg/day). Although it has not yet been established, biliary folate probably does not represent an absolute folate loss since only 300ng/day (*S.faecalis*) were excreted in the faeces[145] and an entero-hepatic circulation has been suggested[171].

No biliary studies have been done in the rat but, by comparison with humans, biliary output must be about 0.5µg of folate per day.

In judging the relevance of PteGlu studies to food folate, it is pertinent to describe the folate form in the diet. Folates are almost universally present in foods. Extraction and assay of food without ascorbate protection showed the richest sources of folate were yeast, liver, asparagus, endive, broccoli, lettuce and spinach and that the last five had little or no PteGlu[211]. However these values are probably low since folate values were higher when ascorbate was present. Four daily diets contained a total of 688µg of folate (L.casei) of which 157µg was 'free' (microbiologically active) folate[116] and low cost diets were similar[117]. These studies were performed without ascorbate protection throughout and some decomposition of the folates was likely to have occurred. The folate content is then likely to be higher than this value but will vary with the diet and its preparation. The normal daily requirement has been estimated at 25 to 250µg of PteGlu[77,83,195,247]. However, in two of these studies [195,247] the diet also contained unknown levels of folate and the total intake was therefore higher. An oral dose of 250µg of PteGlu produced a marked haematological response to a person kept on a diet containing 5µg of folate[77]. Intramuscular injections of 50-75µg produced a good haematological response in patients with megaloblastic anaemia and folate deficiency due to alcoholism. The daily requirement therefore is within the range 50-100µg of PteGlu and the min is often taken to be 50-100µg of PteGlu.

The free-folate concentration in the diet was therefore similar to the requirement of PteGlu. The requirement of other folates, which are likely to occur in the diet in larger quantities than PteGlu, has not been determined.

The contribution of the conjugates to the normal nutrition is much less certain because the content and type(s) of conjugates in foods are unknown. $PteGlu_7$ (a conjugate) was isolated from yeast.

Varying the time and temperature of one extraction technique performed on liver and Portuguese man-of-war, ref.243, conjugates were not isolated when extracts were heated for long periods at a low temperature, whilst a short heating procedure (95°C for 10min) led to the isolation of conjugates. The conjugates were not identified. Lowering the extraction temperature produced decreased amounts of conjugates and increased amounts of free folates. The free folates contained mono-, di-, and tri-glutamates as well as other unidentified materials. The differing results with the different extraction procedures were due to 'liberation' of endogenous conjugase which is denatured at higher temperatures. Since yeast does not contain conjugase this may account for the much earlier isolation of $PteGlu_7$ from yeast[212]. Conjugase is present in pig, dog, rabbit, chicken, turkey, cattle, man, almond, potatoes and other higher plants and the highest levels in animals were found in pancreas, liver, intestinal mucosa, kidney, spleen and brain[8]. Conceivably, then, cooking or autolysis released conjugase or other hydrolysing enzymes which caused rapid decrease in the amounts of conjugates. There

may also be a difference between animal and plant foods. Hurdle et al.[134] showed (though they did not discuss) that conjugates were lower after, than before cooking and, moreover, that cooking egg white, yolk and milk caused a greater loss of conjugates than the loss of total folate; whilst for plant foods (potato, broccoli and porridge) cooking produced a greater loss of free folate than of conjugates. Hydrolysis of folates also occurred on freezing and thawing liver and blood[121]. Folate was also lower in cured and canned foods[8]. Without chemical identifications of dietary folates the literature will remain in its present state of confusion and such a generalisation as "normal subjects must therefore utilise efficiently the polyglutamate derivatives of folate"[8] seems, at least, premature. Little is known of the chemistry of the folate changes which take place during cooking. When 'typical' American diets were autoclaved without ascorbate 11% of the total folate (175μg) was PteGlu[118]. The PteGlu may have been derived by oxidation of H_2PteGlu, H_4PteGlu, by hydrolysis of 10-HCO-PteGlu and, under certain conditions, from 5-CH_3-H_4PteGlu (Beavon, J.R.G. and Blair,J.A., unpublished results). Synthetic $PteGlu_3$ and $PteGlu_7$ were absorbed as PteGlu, but the site of hydrolysis is uncertain. Monoglutamate (including PteGlu) studies are therefore important both as compounds in the diet, as model compounds and are valuable aids in studying the metabolism of folates.

Dietary folates may be altered and synthesised de novo by bacterial metabolism. Xanthopterin was

converted to pteroic acid and 10-HCO-PteGlu, and the folate deficiency in germ-free rats was reversed by inoculation of E.coli, P.vulgaris or A.aerogenes[213]. The nutritional value of such bacterial synthesis in normal metabolism is however probably negligible compared to dietary folate since folate deficiency and anaemia develop on a folate deficient diet[77]. Folate is also rapidly absorbed (Fig.8) from the jejunum and not the ileum[149,150,152]. Antibiotic therapy in one patient increased folate absorption[214] which suggested that bacterial consumption of folate (or reduction in absorption of folate) was more important than bacterial synthesis. With these opposing factors, the results presented here are not due to bacterial metabolism. Even when faecal bacteria were present in the jejunum, serum folate was the normal coenzyme ($5\text{-}CH_3\text{-}H_4$PteGlu) and serum levels were within the normal range[78,146].

Absorption of PteGlu has also been estimated from the rise in serum folate. After oral [2^{14}-C]PteGlu radioactivity was detected after 15min at doses of 30µg/kg and 10min at 320µg/kg (Fig.8). Since systemic blood was assayed, intestinal absorption must have been extremely rapid because the gastric evacuation time is likely to be a minimum of 5min. The results therefore do not preclude some absorption through the stomach wall. Only very low levels of radioactivity were detected in the blood and, at the peak activity, the total blood volume contained some 1% after 320µg PteGlu/kg and 20% with 30µg PteGlu/kg. A similar pattern is found in humans where, using L.casei,

a dose of at least 0.1mg (≈1μg/kg) must be given to produce a significant increase in systemic response. The majority of the dose had therefore either been taken up by tissues or else had been excreted (see Fig.8). Moreover, the amount of radioactivity present in the blood was of the same magnitude despite a ten fold increase in the dose. L.casei response, however, increases (from 1-5ng to 200ng/ml) with a ten fold increase (from 0.5 to 5.0mg PteGlu) in the dose given to humans[8]. Possible explanations are that the dose of PteGlu either displaces tissue (liver) folate into the blood or that the dose is metabolised and is then diluted by the tissue folate. Identification of the cause must await identification and purification of the blood folate (Section II). At doses of 30μg PteGlu/kg two blood peaks were apparent, the first peak occurring within 1 hour and the later (and larger) peak occurred between $1\frac{1}{2}$ to $3\frac{1}{2}$ hours (Fig.8). Similar peaks were found at 320μg PteGlu/kg (Fig.8) when a statistically significant fall (Student's t test, $p<0.01$) in radioactivity (at 82min) was found after the first peak (at 60min) and this was followed by a significant rise in radioactivity (at 115min). In a brief study on humans[176], given 5mg PteGlu, double peaks were detected in systemic blood using S.faecalis. However, a diligent search of other publications failed to confirm the double peaks. This lack of evidence does not mean that they do not occur since the time between sampling was often too large (>30min), small peaks are not likely to be significant above the normal blood level (≈100ng L.casei per ml of

blood) and the data recorded are not sufficiently accurate. Denko[176] concluded that "a more fortuitous time in sampling or continued repetition may have produced more double-maxima curves". The rapid fluctations and double-maxima in systemic blood after oral PteGlu load show that systemic blood folate is complicated because of such variables as absorption, tissue uptake, displacement, metabolism and renal excretion and reabsorption. The earlier blood peak may represent PteGlu and the later peak metabolite(s) formed mainly in the liver. Absorption studies using systemic blood studies are therefore of doubtful significance since they measure metabolism above renal excretion, and Girdwood and Delamore[159] found that in disease, serum folate was "not particularly helpful" in assessing absorption. Portal blood studies clearly overcome these disadvantages, but the technical difficulties involved prevent its use as a diagnostic aid to malabsorption. An improved absorption test was found by assaying systemic folate after an oral dose given up to 3 days after a single "saturation" (preloading) dose of PteGlu[184]. However the dose required to 'saturate' patients is unknown. Furthermore, since systemic blood still represents metabolism of the oral dose, all patients studied must metabolise the preloading dose to the same extent (probably completely) as do normal humans. Sheehy *et al.*[180] indicated an increased metabolism in folate deficiency and Cherrick *et al.*[215] showed a different metabolism in cirrhosis. Girwood and Delamore preferred to compare the urinary excretion after oral with that after intravenous injection[159].

The absorption of other folates has received little

attention. Tritiated-H_2PteGlu and -H_4PteGlu were rapidly absorbed[216] and an intestinal conversion of 5-HCO-H_4PteGlu to 5-CH_3-H_4PteGlu was suggested[217]. These chemically synthesised tetrahydrofolates are mixtures of diastereoisomers at C_6; unlike the natural tetrahydrofolates which are the L-isomers and show full microbiological activity and differ in optical rotation[206] from chemically synthesised compounds. Only the L-isomer of 5-CH_3-H_4PteGlu was taken up by P.cerevisiae[218]. This was difficult to reconcile with the lack of growth of P.cerevisiae with 5-CH_3-H_4PteGlu as substrate (see Table 1.). In mammals it is not known whether a similar specificity occurs or whether the two isomers are interconverted and the interpretation of results obtained with such mixtures are therefore limited. Serum assays after oral H_4PteGlu[216] are unable to differentiate between absorption of the L-isomer only or interconversions of the isomers. Although 5-CH_3-H_4PteGlu has only been isolated from liver, serum, mitochondria and peas, microbiological assays on other sources show an increase response of L.casei above S.faecalis suggesting that 5-CH_3-H_4PteGlu(and its conjugates) occur universally, 5-CH_3-H_4PteGlu is therefore a nutritional folate. The absorption(oral minus faecal radioactivity) of L-5-CH_3-H_4[2-^{14}C]PteGlu was 36% and 45% at doses of 17.2 and 18.8µg /kg respectively (Table 9.) and did not differ significantly from the absorption of 22µg of PteGlu/kg (Table 8.). The absorption of other pteridines can, however, be markedly affected by structural changes. Pteroic acid was poorly absorbed[177] whilst methotrexate, 3'5'dichloromethotrexate and aminopterin were absorbed to a far greater extent than

PteGlu[8]. These folate analogues are bound to serum protein to a greater extent than is PteGlu and, since they are excreted unchanged, an absorption mechanism involving protein binding is suggested. At the intestinal pH, pteroic acid would be present as pteroate and its much lower solubility than that of folate was suggested as an explanation for the poor absorption of pteroic acid[177]. Water transport and pH were also important in PteGlu absorption[151] and an acid microclimate together with solvent drag was suggested[151]. The absorption of folates and their analogues is dependent on several factors and further studies are needed to determine whether the absorption is an active or a passive process.

The majority of the radioactivity of the absorbed PteGlu was distributed between the urine, liver and the kidney (Table 8.). At 320µg PteGlu/kg virtually all the absorbed radioactivity was excreted in the urine and least radioactivity was present in the kidney. At 22 and 3.2µg PteGlu/kg slightly more radioactivity was retained than was excreted and least radioactivity was present in the kidney. Most of the retained radioactivity was therefore present in the liver, the major site of folate when assayed microbiologically. Liver contains up to 20µg of L.casei activity/g[121] whilst kidney, spleen and intestine contain 1.6, 0.6 and 0.6µg of L.casei activity/g respectively [163, 165]. After oral [2^{14}-C]PteGlu, the radioactivity was probably distributed as is the body folate. An interesting finding however was that the ratios of renal/ hepatic radioactivity (about 0.09, 0.07 and 0.15 after doses of 320, 22 and 3.2µg PteGlu/kg respectively, Table 8)

were an order of magnitude higher than the ratio of L.casei determinations (0.007 calculated from L.casei values[163,165] and the weight of rat kidneys and liver). If the specific radioactivities of the labelled folates were similar in the two tissues this would imply a renal concentration mechanism for PteGlu. Immense doses of xanthopterin, 2-amino-4--hydroxypteridine and PteGlu (500mg/kg) given intravenously to rats caused crystalline deposits and renal failure[219]. However, even at this dose (about 350,000 times higher than the daily folate requirement), the renal failure was temporary and reversible and it is extremely unlikely that such a mechanism plays any role at physiological or pharmacological folate levels. Large doses (2mg/kg) of PteGlu given intravenously to rats showed that, after one hour, kidney levels fell but were still above normal 3 days later[163]. After intravenous ^{3}H-PteGlu (29µg/kg) serum levels fell to undetectable-tritium levels in 24h whilst radioactivity was present in the kidney for up to 15 days[220]. Similar findings are presented here, in that, after [2^{14}-C]PteGlu, blood radioactivity fell to undetectable levels after 24h whilst tissue radioactivity was detected after 6 days (Fig.8, Table 8.). Further studies on the reabsorption of PteGlu in the kidney suggested that excess folate was stored in tubular cells by folate binding[199]. Johns and Bertino[221] speculated that if the reabsorption of PteGlu was greater than that of 5-CH_3-H_4PteGlu, this would account for the folate depletion in Addisonian pernicious anaemia, where the levels of 5-CH_3-H_4PteGlu are increased. There is little evidence for this. Furthermore the results reported here show that, after administration, the ratio of radio-

activity present in the kidney / liver (0.22, Table 9.) after 5-CH_3-H_4[2-^{14}C]PteGlu was the same (0.07) as that after PteGlu and therefore such a specific reabsorption mechanism seems unlikely. Such a reabsorption mechanism is also in marked contrast to the renal excretion of other water-soluble substances, such as glucose which is rapidly transported into the peritubular blood and the results do not explain the almost total excretion of folate analogues (methotrexate and aminopterin) which are bound more firmly to proteins[8]. The authors did not study other tissues and it is likely that the renal concentration may represent an increase in total folate after administering PteGlu. The folate content of the kidney is dependent on the folate intake[163]. It is therefore not established that there is any specific concentration mechanism in the kidney and without such a mechanism, the ratio of renal / hepatic radioactivity is a measure of the ratio of folate in these two tissues. This comparison would give a renal folate level of about 10-20µg/g. Grossowicz *et al.*[165] found low (2-16µg/g) and variable hepatic folate but, during extraction, the extent of deconjugation was neither controlled nor determined and it is likely that their procedure gave low values of folate as was the case with hepatic folates. The radioactivity measurements reported here suggest that the recorded literature values[163,165] for renal folate are low and should be redetermined in the light of hepatic studies.

Starvation for 2 or 3 days gave a significantly reduced urinary radioactivity after a large (320µg/kg) oral dose of [2-^{14}C]PteGlu (Table 10). This would suggest a physiological control mechanism which may be dependent on

serum folate levels and/or on folate reabsorption by the kidney. Such a mechanism has been studied in detail and there is a general agreement that after glomerular filtration PteGlu is reabsorbed in the renal tubules. When the concentration was less than 10ng of PteGlu/ml the plasma clearance was less than expected for simple filtration of unbound PteGlu and corresponded to a max reabsorption rate of 0.03µg per minute in humans[25]. Since the normal range is 5-16ng/ml this accounts for low folate excretion. On starvation (or folate free diet) serum folate might be expected to decline rapidly reducing the folate excretion and thereby maintaining body (mainly hepatic) folate. After starvation for two days the excretion of a large dose of PteGlu would be only slightly lower than without starvation but a dramatic fall was noticed. Recent preliminary studies shed light on this apparent paradox, for when serum and liver folate were assayed (L.casei) during dietary folate deficiency of rats, the hepatic folate fell much more rapidly than did the serum folate[222]. This would then account for the much reduced excretion after 2 days starvation, but more work is needed to determine if there is any preferential loss of folate coenzyme(s) and hence the biochemical importance of folate deficiency.

Urinary folate excretion varies from undetectable levels to 18µg per day in humans[129,175,176,187,223]. However these results were dependent on the assay organism. P.cerevisiae gave values of 0.2 to 1µg[132,223,224,226]; S.faecalis from 0 to 5.5µg[129,143,175,176,223] and L.casei 1 to 10µg/day[129,185,187,224]. Assays were often done without any protection against folate decomposition. The

differences recorded may reflect not only variations in sample and growth response of the organisms but also variations in collection and assay techniques. When the urine was stored frozen until assayed slightly higher values (mean 9.5µg of L.casei activity/day) were obtained [187]. Differential microbiological assays of S.faecalis / P.cerevisiae varied from 0.3 to 10. Humans on a poor diet[225], patients with tropical sprue[132] and with megaloblastic anaemia associated with cirrhosis[132] excreted slightly less than normal but patients with megaloblastic anaemia[227] excreted normal levels when assayed by S.faecalis and P.cerevisiae respectively . Higher excretion was recorded in cirrhosis [187], but the values are of scant significance in view of the above variations.

Oral administration of doses of PteGlu causes a temporary increase in folate excretion. The radioactivities excreted following oral doses of 3.2, 22 and 320µg [2^{14}-C]-PteGlu/kg were 11, 12 and 33% of the oral dose in the following 3, 6 and 7 days respectively (Table 8). There was no significant difference in the radioactivity (approx. 6% of the administered radioactivity) appearing in the urine in the 24h period after oral doses of 3.2 and 32µg [2^{14}-C]PteGlu/kg (Sp.radioactivity 55.3mCi/mmol, Table 11), but a marked increase (to 30%; Student's t test, $p<0.001$) when the dose was 320µg PteGlu/kg. [2^{14}-C]PteGlu of sp.radioactivity 31.4 and 50.3mCi/mmol. at doses of 40 and 56µg/kg respectively, showed a similar excretion of radioactivity. In a study on rats given oral PteGlu, a similar excretion was found by assaying the urine with S.faecalis[130]. However this technique probably gave a low folate value since

S.faecalis is not active for all folate coenzymes and no precautions were taken to protect labile folates from decomposition. The radioactivity excreted in the 24h following [2^{14}-C]PteGlu, in these experiments, represented 81% and 31% of the absorbed radioactivity at doses of 320 and 22µg/kg respectively. At 320µg PteGlu/kg a sharp fall in radioactivity occurred after the first day, which represented 90% of the radioactivity excreted in 7 days (Fig.9). Administration of 32 and 3.2µg PteGlu/kg produced a more gradual fall in radioactivity in daily urine collections and the first day urine contained 50% of that excreted in 6 days (Fig.9). When 2 hourly urine collections were made it was found that most of the radioactivity, after a dose of 320µg [2^{14}-C]PteGlu/kg, was excreted in the 2-4h period (Fig.10).The urinary peak therefore coincided with the serum peak of radioactivity (Fig.8). At a dose of 32µg PteGlu/kg the urinary peak of radioactivity varied from 1-6 hours in individual animals and the mean excretion showed a gradual fall throughout the first day (Fig.10). This excretion was in agreement with the excretion pattern observed in humans, in that the largest amount of excreted material, after an oral dose, is excreted within 24 hours [144] and often within 8 hours [143,144,159,174,176]. The levels of material excreted in humans after oral PteGlu vary widely. Anderson et al.[184] using tritiated-PteGlu obtained a similar percentage excretion (6%) at 0.2mg (about 3µg/kg) and higher values (16%) at 40µg/kg in 24h urine collections. When these results are expressed as a percentage of the absorbed radioactivity the excretion (8-19%) for humans was similar to the 13% recorded here for rats. Similar

mean (but widely varying) excretions were found using S.faecalis[228] whilst higher values were found using S.faecalis[143,159,176] and L.casei[144]. None of these workers used antioxidants and the values therefore may be low. Although the total urinary folate was not measured microbiologically in these experiments, by comparison with human studies it seems likely that a higher excretion would have been obtained and this would suggest folate dilution. Two reports have attempted to measure the dilution of PteGlu given to humans. Assay of unpurified urine with S.faecalis and L.casei (after oral [2-^{14}C]PteGlu) suggested that no dilution had occurred in one patient with leukaemia[156] whilst a partial purification of labelled materials (after intravenous [^{3}H]-PteGlu) showed a total dilution (some materials not labelled) in 3 out of 4 normal subjects, 4 out of 5 folate deficient patients and 3 out of 5 vitamin B_{12} deficient patients[161]. These opposing findings are difficult to reconcile. Because PteGlu is not present in mammals any dilution must have occurred by metabolism of at least some of the orally administered PteGlu. To investigate this further, large doses of unlabelled PteGlu were administered orally after [2-^{14}C]PteGlu and the amount of radioactivity displaced into the urine is shown in Table 12. The radioactivity displaced in 24h urine collections (up to three days) was then compared with amounts of radioactivity which are likely to have been excreted without the flushing doses(Table 11). Flushing doses of up to 1.6mg PteGlu/kg, when given 24h or 72h after the labelled dose (2-35µg PteGlu/kg), did not significantly increase the urinary radioactivity in 24h collections up to 3 days (Table 12).

A flushing dose of 1.6mg PteGlu/kg when given after the time of max blood radioactivity($3\frac{1}{2}$h after the labelled dose; see Fig.8) caused a significant (Student's *t* test, $p < 0.001$) increase in radioactivity in the 24h urine and dropped to normal levels on the 2nd and 3rd days. Similar results were found with a large preloading dose of PteGlu (Table12). Administration of 320µg PteGlu/kg at time 0 and 24h later did not produce a summation of label excreted (Table10). This together with the failure to flush significant quantities of radioactivity after 24h suggests that there was little or no PteGlu remaining in the body at this time. The oral [2^{14}-C]PteGlu had therefore either been excreted and/or had been metabolised within 24h. Furthermore, since the flushing dose would have been metabolised to similar compounds as the labelled PteGlu and that significant quantities of radioactivity were not excreted on days 1, 2 or 3 after the flushing dose, it may be concluded that the compounds present in the body after 24h are mainly not those which are excreted in the first 24h urine. This conversion of PteGlu into a storage form (ie. a form not excreted) of folate within 24h could be the result of either metabolism or of storage of PteGlu in a cellular compartment. Metabolism of PteGlu seems the better explanation because different forms of folate are less likely to be displaced than are similar folates in different cell compartments. 5-CH_3-H_4PteGlu is one likely metabolite of PteGlu, but a flushing dose of DL-5-CH_3-H_4PteGlu given 48 hours after [2^{14}-C]PteGlu failed to displace radioactivity. This suggests that there was little 5-CH_3-H_4[2^{14}-C]-PteGlu present at this time and that the storage form of

folate is not 5-CH_3-H_4PteGlu. In studies on humans, intravenously administered flushing doses of PteGlu displaced radioactivity (47% of the radioactivity retained) into the urine 3 days after an intravenous dose of tritiated-PteGlu. The different results may be due to the high specific radioactivity and the adoption of intravenous doses which may cause an unnatural body distribution of label compared to that after oral doses. In agreement with the results reported here, a brief study of patients with chronic lymphatic leukaemia showed less than 1% of an oral dose of [2^{14}-C]PteGlu displaced when the flushing dose was given 4 days later[156]. The types of folate formed from PteGlu and the significance of radioactivity displaced after 3½h and by a preloading dose must await identification of the folate coenzymes (Section II).

The urinary excretion of folate after a load of PteGlu has been used to estimate folate absorption and tissue levels of folate in humans. An improved test for PteGlu absorption is claimed by comparing the urinary excretion of an oral with that of an intravenous dose. 400µg of PteGlu, given intravenously to humans, produced 5-10% (L.casei) of the dose in the following 24 hour urine collection[185], whilst larger doses (5mg) gave 30-52% (S. faecalis) of the dose[175,227,229,230]. When corrected for absorption these levels of excretion are similar to those in rats (Table 11) and suggests that the tissue uptake, metabolism and excretion in humans are comparable to those in rats. Although the excretion after a 5mg test was abnormal in leukaemia[229] and in some patients with megaloblastic anaemia associated with pregnancy[175] and nutrition-

al megaloblastic anaemia[230], Cox et al.[230] studying idiopathic steatorrhoea and Chatterjea[227] studying nutritional megaloblastic anaemia found that the mean excretion was lower than in normals, but the wide variations make this an unsatisfactory test for dietary folate deficiency. In addition to the complications mentioned for oral doses, these tests were not related to body weight which is further complicated in pregnancy where there is a relatively greater increase in body fluid than in the total weight gain[159].

Once diagnosed, folate deficiency is usually easily corrected by administering PteGlu. It should be noted that a more effective treatment should be obtained by using smaller doses (less than 50μg/kg) given more frequently than a single larger dose, since with smaller doses there is a greater percentage absorption (Table 8) and a smaller excretion (Table 11). Larger doses are also potential dangers in that they may mask anaemia due to vit.B_{12} deficiency with the progression of neurological damage. Although PteGlu administration corrects folate deficiency the causative factors (underlying conditions and socio-economic factors) are more difficult to overcome. Clinical assessment and physiological tests alone are neither sensitive nor specific for different types of folate deficiency. Biochemical causes have received less study. Folate-turnover has been measured by the excretion of formimidoyl-glutamic acid (Figlu) after an oral histidine load. Histidine may be metabolised via urocanic acid to formimidoyl-glutamic acid which may be converted to glutamic acid by N-formimidoyl-L-glutamate: tetrahydrofolate 5-formimidoyl-transferase enzyme if there is sufficient folate coenzyme.

Thus in folate deficiency (or, strictly, a lower folate-turnover) the excretion of Figlu after oral histidine is increased. The test could be improved by assaying not only urinary Figlu but also urocanic acid which is dependent upon the amount and rate of conversion of histidine to Figlu and by comparing the excretion before with that after saturation with PteGlu. Further studies are needed to show the origin of Figlu, not all of which is derived from the histidine load. Another biochemical test for folates is based on the formylation of 5-amino-1-ribosyl-4-imidazole carboxamide-5'-phosphate by 10-HCO-H_4PteGlu during purine biosynthesis, when it was suggested that a deficiency of 10-HCO-H_4PteGlu led to the excretion of 5-amino-4-imidazole carboxamide(AIC). However tests of the excretion of AIC after AIC load have not been successful in diagnosing folate deficiency[234].

More direct studies on the types of folates have also been attempted. Leukaemic cells had different folate coenzymes than normal rat tissue[113] but the results are of doubtful significance since the 'normal' folate pattern was not the pattern reported by others. The urinary folate coenzymes were compared in rats fed normal, riboflavin deficient and high methionine diets when, after an intramuscular injection of tritiated-PteGlu, the different folate patterns were compatible with a decreased activity of 5,10-methylene tetrahydrofolate reductase in riboflavin deficiency and high dietary methionine[188]. However in both these reports it is likely that folate decomposition had taken place and until a detailed analysis of the folate coenzymes has been worked out this type of approach must

be inconclusive.

The excretion of folate coenzymes has received little attention. In these studies 5-CH_3-H_4[2-^{14}C]PteGlu was administered in 0.2% (w/v) ascorbic acid solution (pH 6-7) which is sufficient to protect labile folates from oxidation[113]. The urinary excretion of radioactivity in the first 24h after oral 5-CH_3-H_4[2-^{14}C]PteGlu did not vary significantly over the range 0.24 to 18.8µg/kg and was only significantly higher (Student's *t* test, $p<0.01$) than the excretion following [2-^{14}C]PteGlu at 18.8µg 5-CH_3-H_4-PteGlu/kg (Tables 11 and 13). The tissue distribution of radioactivity after oral 5-CH_3-H_4[2-^{14}C]PteGlu (Table 9) was also similar to that after [2-^{14}C]PteGlu (Table 8). The slightly higher values (about 9%) of urinary radioactivity after 5-CH_3-H_4[2-^{14}C]PteGlu than after [2-^{14}C]PteGlu may have been due to the ascorbate. Higher doses of ascorbic acid (up to 40mg/kg) significantly increased (Parker "U" test[231], $p<0.05$) the excretion of radioactivity up to 29% of the dose (Table 13). It is likely that similar increases would have been recorded if ascorbate had been administered with [2-^{14}C]PteGlu since ascorbate was shown to increase the folate excretion (microbiological assays) after PteGlu administration to rats. The reason for the increased excretion caused by ascorbate is obscure but the previous conclusion that ascorbate protected labile folates in the urine does not now seem the complete explanation since the radiochemical assays used here are independent of folate decomposition. In scurvy the folate requirement was increased[102] and a relationship between folate and ascorbate was shown in irradiated rats[99]. Folates have also been

suggested as requirements in ascorbate synthesis[100] and folate deficiency diminished the urinary excretion and tissue concentration of ascorbic acid. The vitamin C requirement in guinea pigs, but not in rats, may account for the lack of folate/ascorbate interrelationships in the guinea pig[101].

On subsequent days (Table 13), the radioactivity excreted after 5-CH_3-H_4[2-^{14}C]PteGlu fell significantly to a level similar to that after [2-^{14}C]PteGlu, which is consistent with the rapid excretion of PteGlu and intravenously administered 5-CH_3-H_4PteGlu[232] and 5-HCO-H_4PteGlu[233]. The lower absorption and higher excretion (neither statistically significant) of radioactivity after 5-CH_3-H_4[2-^{14}C]-PteGlu than with [2-^{14}C]PteGlu produced significantly lower (Student's *t* test, $p<0.02$) hepatic radioactivity (Table 9). The lower absorption may be due to an increased biliary excretion of 5-CH_3-H_4PteGlu than PteGlu. Thus, in general, the physiological disposition of 5-CH_3-H_4PteGlu was similar to PteGlu. A similar conclusion was also reached for 5-HCO-H_4PteGlu[186], and for the plasma clearance of H_2PteGlu and H_4PteGlu[157].

Purification and Identification of Labelled Metabolites of

[2-^{14}C]PteGlu and 5-CH_3-H_4[2-^{14}C]PteGlu

SECTION II

After the administration of [2^{14}-C]PteGlu (32 to 56µg/kg; specific radioactivity 31.4 to 55.3mCi/mmol) to four rats, urines were collected under various conditions, pooled, and then purified on a florisil column. The ammoniacal florisil fraction was then purified by t.l.c.

Collection in alkali without antioxidant and chromatography without antioxidant.

The major radioactivity co-chromatographed with folic acid in three solvent systems, (a) Rf 0.00, (b) Rf 0.35, (c) Rf 0.11. Two other radioactive components subsequently decomposed to folic acid.

Because of the decomposition of the labile folates excreted the author decided to perform all subsequent experiments with antioxidants present throughout (see Materials and Methods).

Collection of urine in acid using antioxidants throughout.

Typical chromatograms of the ammoniacal florisil fraction showed radioactivity at Rf values (0.00)*, 0.28, 0.42 and 0.52 in solvent (a) and corresponding Rf values 0.25, 0.10 and 0.40 in solvent (c). Labelled PteGlu was absent. The compound Rf 0.52 in solvent (a) and 0.40 in solvent (c) was identified by co-chromatography as 5-CH_3-H_4PteGlu in systems (a), (b), (c) and 2% (w/v) ammonium acetate-pyridine (19.1, v/v). The relative amounts of the two major compounds are shown in Table 14.

Radioactive compounds were purified free from any fluorescence or absorption by chromatography in solvent (a) extracted, freeze-dried and the process repeated in

* due to retention of radioactivity at the origin.

solvents (c) and (d). The amounts of radioactive compounds were too small to be detected under u.v. light by using this method.

Collection of the urine frozen followed by freeze-drying.

Antioxidants were used during purification. Chromatography in solvent (a) showed the presence of five labelled compounds Rf (0.00)*, 0.22, 0.31, 0.43 (major radioactivity) and 0.52 identified as 5-CH_3-H_4PteGlu. Labelled folic acid was absent. The major radioactivity was further purified in solvent (c) Rf 0.10, and then in (d) Rf 0.47, and was then free from all fluorescence or absorption.

Labelled 5-CH_3-H_4PteGlu was identified (by its Rf value in two solvents) by a similar purification in all but one of the twelve subsequent experiments. Characterisation by co-chromatography in three solvents was carried out in eight of these experiments.

The relative microbiological activity of the major unidentified compound, Rf 0.42 in system (a) and 0.10 in system (c) and 5-CH_3-L-H_4[2^{14}-C]PteGlu is shown in Table 15. The microbiological activity of the 5-CH_3-H_4PteGlu showed max. activity for L.casei and little activity for S.faecalis, which was nevertheless higher than expected (Table 15) and may have been caused by decomposition during the assay. An L.casei assay on the unidentified material before and after conjugase treatment were exactly the same indicating that this compound is not a heptaglutamate.

Because of the instability of this compound however and its decomposition to PteGlu, even when antioxidant was

present, the identity of this compound was investigated by the addition of folates to the urine collection flask, when, even if decomposition did occur, the radioactivity would corirespond to a pteridine fluorescence of the decomposed folate.

Urine collection in acid with antioxidant with 0.5mg folic acid.

Thin layer chromatography produced radioactivity at the following Rf values:- in solvent (a) 0.41 and 0.49 (5-CH_3-H_4PteGlu) with minor radioactivity at 0.00, 0.22 and 0.30, in solvent (b) 0.7-1.0 and 0.85-1.0 with minor bands at 0.00 and 0.49, and in solvent (c) 0.22, 0.28 and 0.41 (5-CH_3-H_4PteGlu). Folic acid was detected on all chromatograms and was not radioactive. The major compound, Rf 0.41 in solvent (a), was chromatographed in solvent (c), Rf 0.03, and then (d), Rf 0.53, and was then free from fluorescent or absorbing impurities.

Addition of H_2PteGlu (about 1mg suspension in HCl) to the collecting solution (acid with antioxidant) did not distinctly alter the autoradiograph and the radioactive compounds, Rf values 0.42 5-CH_3-H_4PteGlu, 0.07 and minor radioactivity at 0.17 and 0.31 in solvent (c), did not agree with any fluorescence. 7,8-H_2PteGlu was not identified on the chromatograms. The experiment was further modified by collection of the urine frozen and then thawing the urines with a solution of 7,8-H_2PteGlu (0.1M HCl with 2% w/v ascorbic acid containing approximately 1mg 7,8-H_2-PteGlu). Florisil fractionation and thin layer chromatography in solvent (a) produced radioactivity at Rf 0.25, 0.37, 0.48 and 0.60. 7,8-H_2PteGlu was identified as a

broad blue fluorescent band (Rf 0.27-0.45) and did not co-chromatograph with any radioactivity. In solvent (c) radioactivity was present at Rf 0.12, 0.30 and 0.41 ($5\text{-}CH_3\text{-}H_4$-PteGlu). $7,8\text{-}H_2$PteGlu was present at Rf 0.06 free from radioactivity.

Urine collection in acid with antioxidant with 5mg H_4PteGlu.

The chromatogram developed in solvent (a) produced radioactivity at Rf 0.43 and 0.54 ($5\text{-}CH_3\text{-}H_4$PteGlu). H_4PteGlu was not detected. The compound Rf 0.43 was purified free from fluorescence in solvent (c), Rf 0.03.

Urine collection in acid with antioxidant with 0.5mg leucovorin (Ca salt).

The presence of $5,10\text{-}CH{=}H_4$PteGlu distorted the chromatograms. Three radioactive species were present in solvent (c) Rf's 0.10, 0.18 and 0.48 (streaked) -labelled folic acid was absent. The major radioactivity (Rf 0.18) was purified in solvent (a). Decomposition occurred giving radioactivity at Rf's 0.32 and 0.61. A strong white fluorescence ($5,10\text{-}CH{=}H_4$PteGlu) was present at Rf 0.30 and was not radioactive. Both the radioactive compounds were further purified in solvent (b) free from fluorescence.

Radioactive species from a chromatogram developed in solvent (a) were purified free from fluorescence. The isolated $5,10\text{-}CH{=}H_4$PteGlu was not radioactive.

Collection of urine in alkali containing antioxidant and 2mg $5,10\text{-}CH_2\text{-}H_4$PteGlu.

In the chromatogram developed in solvent (a) $5,10\text{-}CH_2\text{-}H_4$-PteGlu (characterised by Rf 0.85 dark absorption under 254n.m. u.v. light)was not isolated. The major radioactive

species was purified in three solvents:- (a) Rf 0.44, (c) 0.07 and (d) 0.50 and was then free from fluorescence or absorption.

Addition of 5-CH_3-H_4PteGlu (2mg) to the collecting solution (acid with antioxidant). The major compound Rf 0.09 solvent (c) could be separated free from fluorescence or absorption and therefore was not 5-CH_3-H_4PteGlu nor any decomposition of 5-CH_3-H_4PteGlu produced during urine collection or purification.

Reduction of unidentified material. Sodium borohydride. The purified labelled material was divided into two, half was reduced, the other half acted as a control (without sodium borohydride). The two solutions were then applied as streaks to two halves of a chromatogram and developed in solvent (a). The autoradiograph showed blackenings at Rf 0.30, 0.47 and 0.62 in both the control and the compound reacted with borohydride. The experiment was repeated on a different sample with similar results.

Catalytic hydrogenation. The autoradiograph showed blackenings at Rf values in solvent (a) 0.43 with minor spots at 0.20, 0.33 and 0.55, in solvent (b) 0.82 with a minor one at 0.70 and in solvent (c) 0.11 and 0.24 with a minor spot at 0.38. The major radioactivity was similar in Rf value to the starting material. The experiment was repeated on a different sample of unidentified compound and the freeze-dried reaction mixture contained 3 radioactive species, none of which co-chromatographed with added 5-CH_3-H_4PteGlu or 5,10-CH=H_4PteGlu.

Purification of the second day urines was performed in exactly similar manner to those of the first day; using

antioxidants throughout. The autoradiographs were essentially similar to those of the chromatograms produced by the first day urines.

Radioactive folic acid was absent. The major radioactive component, Rf 0.48 in (a) and Rf 0.11 in (c) was unidentified. The compound at Rf 0.55 in (a) was identified as 5-CH_3-H_4PteGlu by co-chromatography in solvent (b) Rf 0.83 and solvent (c) Rf 0.41. The experiment was repeated with similar results.

Purification of the third day urines was performed using antioxidants throughout. The autoradiograph showed the presence of similar radioactive compounds and labelled 5-CH_3-H_4PteGlu was identified by its Rf value in solvent systems (a) and (c).

These results show that after an oral dose of 32 to 56µg PteGlu/kg collection in acid and alkali and purification of the first-day urine without antioxidants led to decomposition and only PteGlu was identified (although other labelled materials were present). A similar purification with antioxidants present led to the isolation of labile folates and PteGlu was absent. Collection of urine frozen and in acid gave similar results whilst collection and purification in alkali caused a greater decomposition. PteGlu, isolated from human urine by techniques involving [^{3}H]PteGlu and DEAE-cellulose purification and bioautography, may have arisen by oxidation due to the absence of antioxidants as was found in this work. PteGlu has been isolated after oxidation of H_2PteGlu, H_4-PteGlu and 5-CH_3-H_4PteGlu (Beavon, J.R.G. and Blair, J.A. unpublished results). However collection of urine under

toluene followed by freezing and assay with ascorbate[186] produced a similar S.faecalis and L.casei response and using antioxidants throughout[162] produced labelled PteGlu after administration of [^{3}H]PteGlu. Unfortunately specific radioactivities which would indicate whether or not decomposition had taken place were not recorded by the authors using labelled material. Bioautography (Leeming, R.J., Portman-Graham, H. and Blair, J.A., unpublished results) provides the strongest evidence that PteGlu is excreted in humans given oral PteGlu and suggests a quantitative difference in the rate of metabolism of oral PteGlu between the rat and man.

During the present study two major (representing some 85% of the radioactivity, Table 14) and one minor labelled metabolites were isolated. One of the major metabolites was identified by co-chromatography as 5-CH_3-H_4PteGlu. The other was not identified but was not PteGlu, H_2PteGlu, H_4-PteGlu, 5-HCO-H_4PteGlu, 5,10-CH=H_4PteGlu, 5,10-CH_2-H_4PteGlu or 5-CH_3-H_4PteGlu, nor any artifact formed from these during the isolation. Because both 10-HCO-H_4PteGlu and 5-HCNH-H_4PteGlu are rapidly converted to 5,10-CH=H_4PteGlu, the unidentified compound was not either of these folates. Labelled 2-amino-4-hydroxypteridine and 10-HCO-PteGlu were isolated on two occasions and characterised by chromatography in solvents (a) and (b). These two compounds appeared as relatively small amounts of radioactivity and were derived from other labelled materials. These compounds therefore arose by decomposition of labile folates since other pteridines were absent and the labelled compounds decomposed to PteGlu when antioxidants were not

added. 10-HCO-PteGlu is readily formed by oxidation of 10-HCO-H_4PteGlu whilst 2-amino-4-hydroxypteridine may be derived from H_4PteGlu or tetrahydrofolates which themselves form H_4PteGlu.

Early work suggested an increase in P.cerevisiae activity (citrovorum factor) in the urine after oral administration of PteGlu and in normal urine (Section I) but isolation media often contained no antioxidants and compounds were accompanied by unidentified S.faecalis-active material(s). Without added antioxidants compounds isolated during this work were PteGlu and unidentified materials of undetermined microbiological activity. To account for the citrovorum factor activity, first reported by Sauberlich (1949)[235], two groups of workers proposed that it was 5,10-CH=H_4PteGlu[191,192], which may be derived by rearrangement of 10-HCO-H_4PteGlu. Chromatographic, spectral results and biological response of the isolated material reported by Silverman et al.[191] were identified with 5,10-CH=H_4PteGlu. These results must be viewed with caution since both groups of workers used large doses (up to 175mg given to humans) and Silverman et al.[191] used formic acid (during purification procedures) which reacts with 5-CH_3-H_4PteGlu to give 5,10-CH=H_4PteGlu (Appendix III). It is also possible that, under certain conditions, the 5-methyl group may be oxidised to 5-formyl, in fact, such a conversion is in agreement with the reported[47] presence of 5-HCO-H_4-PteGlu (active for P.cerevisiae) in blood whereas 5-CH_3-H_4PteGlu and its conjugates are the major blood folates[236]. Low conversions to urinary citrovorum factor activity have been reported for PteGlu when given orally[32,125,184],

[226] or subcutaneously[237]. More recent studies[162] showed no activity for P.cerevisiae in human urine which is in agreement with the results presented here. It is therefore unlikely that citrovorum factor is excreted in either humans or rats. Labelled folate conjugates or unconjugated pteridines were not present in the urine, suggesting that little, if any, folate was converted to unconjugated pteridines. This agrees with the earlier observation that orally, subcutaneously or intravenously administered Pte-Glu did not give rise to increased xanthopterin in human urine[238]. Neopterin[239] and biopterin[81,240], both isolated from human urine, may have been derived from compounds in the diet or by synthesis from purines. Pterins occur in micro-organisms, plants, fish and liver, brain and kidney of rat and guinea pigs[8]. The synthesis of pterins from purines (Fig.2) has been demonstrated in amphibia but not in man. Isoxanthopterin previously isolated from urine[241] may have arisen by folate degradation but the origin of xanthopterin[242] is uncertain. Little is known of the occurrence of pterins in mammals and even less is known of their metabolism, but it seems odd that such structurally similar compounds as folates and pterins should occur together and not be interconverted in mammals. It seems unusual that any loss of the ability to synthesise folate during evolution was accompanied by the loss of their interconversion but not by the ability to synthesise pterin[65], or convert it to lumazine[22].

The ratio of the major unidentified material to the 5-CH_3-H_4[2-^{14}C]PteGlu isolated on the first day after an oral dose of 32µg [2-^{14}C]PteGlu/kg, varied from 0.5-7.7

(mean 2.7) to 1.0 in twelve experiments. Both of these compounds were also isolated at 3.2 and 320µg [2^{14}-C]PteGlu /kg and on day 2 and 3 after PteGlu when a similar wide range in ratio of the two metabolites was found. The percentage of radioactivity present on the chromatograms in 5-CH_3-H_4PteGlu was similar at the three doses (about 25%, Table 14) in the first day urine collection, and lower in the second and third days collections. A scan of a typical radioautograph is shown in Fig.11. Due to the wide variations in the label present in the two compounds, any deductions derived from this must be tentative but the relative decrease in radioactivity present as 5-CH_3-H_4-PteGlu may indicate a greater metabolic role for 5-CH_3-H_4-PteGlu than the other material.

The chemical and physical properties of the unidentified compound were further investigated. The compound was not reducible by either borohydride or catalytic hydrogenation and suggests that it was a tetrahydro-form. Microbiological response was similar to that of 5-CH_3-H_4PteGlu (Table 15) and the L.casei response was not increased after conjugase treatment indicating that the compound does not contain more than three glutamic acid residues. Chromatography on t.l.c. was compared to the Rf values of a family of synthetic mono-, di- and triglutamates both unreduced, reduced, formylated(5 and 10) and methylated(N-5) supplied by Salem, A. (Ph.D. thesis, 1971). In solvents: (i) citric acid (5%), adjusted to pH 9 with ammonia (0.88) and saturated with iso-amyl alcohol; aq. layer, (ii) 0.1M-sodium phosphate buffer (pH 7.0), (iii) n-butan-1-ol-acetic acid-water (4:1:1 by vol.), (iv) NH_3 (0.88)-tert-butan-1-ol-

water (1:1:8 by vol.) and (v) formic acid (98% w/v)-ethanol-water (1:15:35 by vol.) the Rf values of the unidentified compound were 0.21, 0.53, 0.42, 0.62 and 0.44 respectively, compared to 0.70, 0.83, 0.54, 0.86 and 0.75 respectively for 5-CH_3-H_4PteGlu. The relative Rf value of the unidentified material showed no resemblance to any of the synthetic folates used by Dr.Salem. Moreover the compound was eluted exceptionally slowly in alkaline solvents (i) and (iv). When chromatographed on DEAE-cellulose the compound was eluted (peak in tube 18) between 5-CH_3-H_4-PteGlu (tube 13) and PteGlu (tube 23). Such an elution profile suggests that the reduced compound is more acidic and is consistent with the observed elution of reduced di- and tri-glutamates. However the elution volume (53ml) from Sephadex-G15 gave a K_{av} of 1.8 showing that the compound was not eluted in the di- and triglutamate region ($K_{av} \approx$ 1.0) and suggested that the compound had a molecular structure similar to a monoglutamate. Mass spectrometry of the material was unsuccessful due mainly to the relatively high level of impurities extracted from thin-layer adsorbent and to the low volatility of pteridines and folates. In an attempt to isolate larger quantities of this material, 5mg of PteGlu was taken by one human volunteer, the following 24h urine was collected frozen and freeze-dried and then purified through a florisil column in a comparable manner to that used for rat urine with added antioxidants. The labelled compound from rat urine was then added and the mixture purified by t.l.c. with antioxidants. Unfortunately the labelled material decomposed on successive chromatography which was required to remove

the excessive amounts of inorganic material. The decomposition may also indicate that this compound was not excreted by humans as more recent results using bioautography suggest (Leeming, R.J., Portman-Graham, H. and Blair, J.A.,unpublished observations). The compound therefore remains unidentified.

Starvation produced radioactive materials at Rf values (Table 16) similar to those excreted from animals given food and water ad libitum. 5-CH_3-H_4[2^{14}-C]PteGlu was identified by co-chromatography in solvents (a), (b) and (c) and the other compounds were unidentified. Furthermore the amount of radioactivity in the compounds was similar to that obtained without starvation, although the specific radioactivities of the major unidentified compound and 5-CH_3-H_4[2^{14}-C]PteGlu were increased by starvation but not by a double administration of [2^{14}-C]PteGlu (Tables 14 and 16).

The radioactivity excreted (in urine collected in acid with 1mg PteGlu) after flushing doses was also purified by florisil chromatography and t.l.c. using antioxidants throughout and the results are shown in Table 17. Flushing doses of 200, 400 or 1600µg PteGlu/kg when given 24-72h after the labelled dose did not produce labelled PteGlu in the urine excreted in the following 24h. The materials excreted showed similar Rf values to compounds excreted without flushing doses and 5-CH_3-H_4PteGlu was identified by co-chromatography in solvent (a) Rf 0.64 in six of these experiments. Minor radioactive materials were present at Rf 0.18, 0.25 and 0.28 in solvent (c) and may have been displaced by the flushing doses or may have been artefacts due to either decomposition or the inaccuracy

in determining the very low levels of radioactivity (see Table 12) present on chromatograms. However, a greater proportion of radioactivity was present as 5-CH_3-H_4[2-^{14}C]-PteGlu than the major unidentified metabolite (Rf 0.08) following flushing doses (Table 17) than was normally excreted on days two and three without flushing doses (Table 14). This suggests that the body contained radioactive folates, 5-CH_3-H_4PteGlu or compounds converted into 5-CH_3-H_4PteGlu, in greater amounts than the unidentified material. Flushing doses of 1600µg PteGlu/kg given 3½h before or 3½h after labelled [2-^{14}C]PteGlu produced labelled PteGlu at Rf 0.14 in solvent (c) and represented 49 and 45% of the total radioactivity respectively. The labelled PteGlu was purified in solvent (a) and then co-chromatographed in solvent (a) Rf 0.00, (b) Rf 0.46 and (c) Rf 0.12. The labelled PteGlu was not derived by decomposition since similar collection and purification procedures did not cause decomposition to PteGlu. The specific radioactivity of the PteGlu was not determined and would have been of only limited significance since PteGlu was added to the urine and the dilution produced by the flushing dose cannot be determined since neither the absorption nor the extent of metabolism of the flushing dose were known. 5-CH_3-H_4[2-^{14}C]PteGlu, Rf 0.4 solvent (c), was also flushed by these doses of PteGlu but the amount of the unidentified material (Rf 0.08) was difficult to determine because of contamination with labelled PteGlu. However, chromatography of labelled PteGlu in solvent (a) did produce labelled material (Rf 0.38) which did not correspond to any fluorescence or absorption and was not

therefore a decomposition product of PteGlu. A flushing dose of 5-CH_3-H_4PteGlu produced no labelled PteGlu but produced 5-CH_3-H_4[2^{14}C]PteGlu and the unidentified compound (Table 17). The ratio of 5-CH_3-H_4PteGlu to the unidentified material was higher than before the flushing dose, (Tables 14 and 17) suggesting a preferential flushing of 5-CH_3-H_4PteGlu or that more of the retained radioactivity was present as 5-CH_3-H_4PteGlu than the unidentified compound at this time.

After oral 5-CH_3-H_4[2^{14}C]PteGlu the radioactivity excreted in the urine, collected in acid with ascorbate, was purified with antioxidants present throughout. When administered in 0.2% (w/v) ascorbate solution the major compound excreted was 5-CH_3-H_4[2^{14}C]PteGlu (Table 18) and represented some 60% of the total radioactivity excreted after oral doses of 0.24 to 19µg/kg. At a dose of 84µg/kg the radioactivity represented 80% of the excreted radioactivity. At 0.24µg 5-CH_3-H_4PteGlu/kg a significant proportion of the radioactivity excreted was present at Rf 0.08 in solvent (c) whereas very little of this labelled material was present at higher dosages and may have been produced by 'tailing' of radioactivity from a compound present at Rf 0.25 (Table 18 and Fig.11). Although the material at Rf 0.25 was not identified it had an Rf value similar to the minor compound excreted after PteGlu administration. This relatively low conversion of 5-CH_3-H_4-PteGlu compared to the higher conversion of PteGlu into metabolites is likely to be due to a difference in specific radioactivity of the administered folates. The PteGlu administered was not only of higher sp. radioactivity but

is not diluted by body folate whereas the 5-CH_3-H_4PteGlu was of lower (about 2.5 times less) specific radioactivity and would have been further diluted by the body 5-CH_3-H_4-PteGlu and excreted as such, without the excretion of other labelled metabolites. The excretion of other metabolites at a dose of 0.24µg 5-CH_3-H_4PteGlu/kg is explained by a slower excretion of this lower dose (as was the case with [2-^{14}C]PteGlu; see Section I) giving more time for metabolism. Administration of 5-CH_3-H_4[2-^{14}C]PteGlu in 1 or 4% ascorbate (Table 18) increased the excretion of metabolites of 5-CH_3-H_4[2-^{14}C]PteGlu, and they had similar Rf values to the unidentified metabolites excreted after [2-^{14}C]PteGlu administration (Table 14).

Purification of blood samples obtained after [2-^{14}C]-PteGlu administration was carried out on t.l.c. applying 10µl of blood collected at times up to 17h after the administration and developing the chromatograms in solvent (b) with antioxidant. After exposure for 3 months, the radioautograph showed the presence of two labelled materials (Rf ≈0.5 and ≈0.85) when blood was taken from 30min to 3½h after oral administration of 320µg [2-^{14}C]PteGlu. There was no blackening at Rf 0.85 in blood taken at 10min and no blackening at Rf 0.5 in blood taken after 3½h. On the same chromatogram the administered [2-^{14}C]PteGlu had an Rf value of 0.48 and it is therefore likely that the compound at Rf 0.50 in blood samples was [2-^{14}C]PteGlu. The material at Rf 0.85 was probably a tetrahydrofolate metabolite (see Table 6) and is likely to have been one or a mixture of the compounds excreted in the urine ie 5-CH_3-H_4PteGlu or the unidentified materials. From a micro-

densitometer scan of the radioautographs the percentages of the radioactivity present as the two materials were calculated for each of the blood samples. These percentages were then combined with the radioactivity per ml of blood (Fig. 8) to calculate the amount of radioactivity present as the two materials and these were then plotted against the time of sampling (Fig.13). The labelled PteGlu (Rf 0.50) rose after 10min, remained approximately constant for over an hour and then declined slowly to an undetectable level at 3½ hours. Because there is no PteGlu present in rat tissues and the amount of PteGlu absorbed over this period from the diet is negligible (see Table 3) compared to the labelled dose(80µg of PteGlu), the specific radioactivity of the [2^{14}-C]PteGlu isolated from blood was the same as the oral dose (55.3mCi/mmol). The peak of serum radioactivity (2mµCi; Fig.13) therefore represented only 15ng of PteGlu/ml of blood after an oral dose of 80µg of PteGlu. Most of the radioactivity was present as metabolite(s) which showed a peak in systemic blood slightly before 2 hours. The amount of folate present in the metabolite(s) is likely to be higher than PteGlu because the urinary metabolites showed lower sp. radioactivities than the oral dose (Table 14). The specific radioactivity of the metabolite(s) could not be determined since materials extracted from the thin-layer chromatogram failed to stimulate the growth of L.casei showing that the folate levels were below microbiological detection (min. of 2ng/ml in the assay medium which corresponded to 200ng of folate/ml of blood). The small plateau of radioactivity (of Rf value 0.85) which occurred between 30 and 90 min

(Fig.13) and preceded the major blood peak may have represented a different folate composition from the major peak, although this was not further investigated because of the low levels of radioactivity. Compounds excreted in the urine were not examined over this period.

Purification and identification of hepatic folates has previously presented difficulties and the results have caused confusion in the literature. Determination after the administration of materials is further complicated by the changes occurring because of the negative radiochemical balance. Extraction prior to purification of folates usually relies on homogenisation and deproteinisation by heating. However, if the temperature is not sufficiently high, and the deproteinisation procedure extended, loss of folate conjugates occurs due to the action of conjugase enzyme[121]. p-Chloromercuribenzoate, a conjugase inhibitor, and acetone, which precipitates conjugase[8], were therefore used with antioxidants in a comparative study of extraction procedures as follows:-

method (i).Freshly excised liver (7-15g) was homogenised in 25ml of water containing 2% (w/v) ascorbic acid. The protein was then precipitated by adding 1g of trichloracetic acid. The precipitate was centrifuged, the supernatant decanted off and the precipitate washed with 5ml of 3% (w/v) trichloracetic acid. The supernatants were combined.

method (ii).Freshly excised liver was homogenised in 25ml of hot (90-100°C) water containing 2% (w/v) ascorbic acid and 0.1% p-chloromercuribenzoate (dissolved in a small volume of ammonia). The mixture was centrifuged and

protein precipitated by the addition of acetone. The mixture was centrifuged and the volume of the supernatant reduced by rotary evaporation (at about 10°C).

method (iii). Freshly excised liver was homogenised as in (ii) and the protein precipitated with 2ml of conc. HCl, centrifuged and the supernatant decanted.

method (iv). Freshly excised liver was homogenised in 100ml of hot water made 2% (w/v) with ascorbic acid and 0.1% with p-chloromercuribenzoate. The mixture was centrifuged and the protein precipitated by the addition of acetone. The mixture was centrifuged, the precipitate washed and the combined supernatant were freeze-dried.

method (v). The freshly excised liver was placed in a boiling-tube with a B_{34} cone resting in an ice-water mixture. To this was added 12ml of a cold solution consisting of 2.2ml of 10% ascorbate solution, pH 6.0, plus 9.8ml of water. The mixture was homogenised for 30sec with a metal-blade homogeniser attached to a fast stirrer. The homogeniser was then quickly removed, the test-tube stoppered and heated in a water bath at 95°C for 10min. After this time the contents of the tube were 90°C. The test-tube and contents were cooled in ice-water, diluted with 15ml of a cold solution of 1% ascorbate solution, pH 6.0 and rehomogenised for 15sec. After centrifuging, the supernatant was decanted. The method differed from that of Bird *et al.*[121] only in the type of homogeniser used and that the homogenate was not sub-divided.

All aqueous extracts were stored frozen for a minimum period until used. For purification of radioactivity by t.l.c., 20ml of the extract was acidified to pH of about

1.0 with conc. HCl, centrifuged to remove any precipitate and the extract chromatographed on a short florisil (5g) column made in a burette. The radioactivity was eluted as were urines(see Methods) with solutions containing anti-oxidants. The percentage(mean ±S.D. for four experiments) of the radioactivity recovered in the acid, neutral and ammoniacal fractions were 17.25±15.48, 3.20±2.20 and 79.55 ±15.60 and the recovery of radioactivity was 78.00±16.39%. These were similar to the distribution and recovery of radioactivity of synthetic and urinary folates (Table 5).

A comparison of the recovery and of the Rf values of materials extracted by different procedures was carried out after the oral administration of 80µg of [2-^{14}C]PteGlu to rats, and the livers removed after 24h. The recoveries of radioactivity were 51 to 89% when determined once for each of methods (ii) to (v) and are unlikely to be significantly different (Table 19).

T.l.c. of the extracts was performed with and without added PteGlu on the same chromatograms. Radioautographs did not show blackening corresponding to either the Rf value or the dark absorption (under 254n.m. light) of PteGlu, and therefore labelled PteGlu was not present in the liver 24h after administration of [2-^{14}C]PteGlu. The higher recovery of radioactivity using method (iv) with the addition of PteGlu before homogenising (Table 19) may therefore suggest that the addition of PteGlu reduced folate losses. Such losses may have been caused by protein binding (or co-precipitation) of folates. On the chromatograms developed in solvent (c), the greatest amount of radioactivity was present as a band at Rf 0.04 to 0.08 in

each of the extraction methods used (Table 19). Therefore these materials could not be distinguished by t.l.c. in solvent (c) and may reflect the similar nature of each of the materials extracted by the extraction methods used. Precipitation of protein with acid, methods (i) and (iii), produced a greater proportion of the radioactivity in materials other than that at Rf 0.04 to 0.08 than did protein precipitation with acetone or heating, methods (ii), (iv) and (v). Precipitation of protein with trichloracetic acid, method (i), produced the largest number of labelled materials detected on the chromatogram (Table 19). After precipitation of protein with acetone, rotary evaporation, method (ii), produced more labelled materials than did freeze-drying, method (iv). Thus prolonged extraction procedures or the use of strong acids during extractions produced more labelled materials than shorter procedures and the use of acetone. Furthermore, since the percentages of radioactivity extracted were the same for the different methods, the compounds produced only by longer extraction procedures and with acids must have been derived by changes in other labile materials. P-chloromercuribenzoate, which inhibits all the enzymes producing folate changes yet tested[8], had little effect on the materials isolated and therefore the changes produced probably arose by chemical decomposition caused by longer extraction procedures or the use of strong acids. These deductions therefore led to the use of method (v) for further experiments on hepatic folates.

Extraction of hepatic radioactivity 6h, 24h and 10 days after an oral dose of [2-^{14}C]PteGlu and chromatography

in solvent (c) showed that most (74 to 85%) of the radioactivity had an Rf value of 0.04 (Table 19). Attempts to increase the incorporation of radioactivity into hepatic folates by starvation of animals, prior to administration of [2-^{14}C]PteGlu, produced a similar percentage (84 and 89%) of radioactivity in the material at Rf 0.04 (Table 19). This material then probably represented the largest amount of folate in the liver but speculation about the identity of this material from this one chromatographic property is unlikely to be fruitful. This labelled material was extracted from the t.l.c. and chromatographed on a DEAE-cellulose column and fractions eluted were assayed for radioactivity (Fig.14). The elution of synthetic folates were not tested in detail but were eluted from these columns slightly earlier than synthetic folates used by others (Table 20) using similar columns and elutions. The elution of radioactivity of the major band from t.l.c. shows that several (>8) compounds were present and the shape of the peaks indicates that mixtures may be present in the tubes showing that there had been an incomplete separation of radioactivity. Most of the radioactivity was eluted in tubes after 17 and peaks were identified at 20, 26, 29, 32 and 38 (Fig.14). The original purification by t.l.c. in solvent (c) would separate monoglutamates which have higher Rf values (see Table 6). The peaks at 6, 8 and 15 were either not monoglutamates or were formed by decomposition during handling. Without purification by t.l.c. the liver contained a greater proportion of material in tubes 10 to 20 (Fig.15), a region containing some mono- and triglutamates (Table 20). To further characterise the

radioactivity, tubes were assayed by *L.casei* before and after conjugase (Table 21). Most of the peaks showed some increased growth after conjugase treatment and a 2-fold increase was regarded as within the errors in microbiological determination and this error may be an increase by errors in radioactivity determinations, particularly when low levels were present. Tubes 18 to 38 did, however, show a marked increase in growth after conjugase treatment (Table 21) and were therefore labelled conjugates. The radioactivity present as conjugates was some 70% of the total radioactivity and was similar to the value (75%) for liver conjugates using microbiological assays before and after conjugase[121]. It thus appears that under these conditions the oral [2^{14}-C]PteGlu had been metabolised and the radioactivity distributed throughout the liver folates, which were mainly conjugates. When another animal was starved for 3 days before administration of [2^{14}-C]PteGlu and the liver extracted after a further 6 days, a greater proportion of the hepatic radioactivity was present in tubes after 17 (Fig.16) compared to the distribution of radioactivity after 24h (Fig.15). This apparent increase in conjugates may have been caused by further synthesis of conjugates after 24h and/or to the excretion of labelled monoglutamates.

Investigation of the metabolic pathway(s) from PteGlu to hepatic conjugates was attempted by isolation of hepatic folates extracted at different time intervals after the administration of [2^{14}-C]PteGlu. 10 days after the administration of [2^{14}-C]PteGlu, the liver radioactivity showed a similar chromatographic pattern (Fig.17) to that with

prior starvation of animals (Figs.15 and 16). The major radioactivity was present in tubes 18-20 with appreciable radioactivity in latter tubes up to 40. Liver extracted 36h after [2-^{14}C]PteGlu contained radioactivity which was distributed between two broad peaks (tubes 10 to 19 and 20 to 35; Fig.18) with little radioactivity at tubes 18 to 20 characteristic of liver extracted after 6 and 10 days but not after 24h. The distribution of radioactivity eluted from a DEAE-cellulose column (Fig.19) from a liver extracted 6h after oral [2-^{14}C]PteGlu was similar to that (Fig.18) present after 36h. Thus extraction of liver folates at times up to 10 days after the administration of [2-^{14}C]PteGlu showed that some conjugates were formed after 6h whilst others were formed at a much slower rate, but the determination of the pathway(s) involved must await the characterisation of hepatic folates. The radioactivity (30%) eluted before tube 17 (Fig.15) probably consists of 10-HCO-H_4PteGlu (tube 8) and possibly 5-HCO-H_4PteGlu (tube 13) with little 5-CH_3-H_4PteGlu (tube 13), which would have been separated (Rf 0.40) from most of the radioactivity (Rf 0.08) by t.l.c. in solvent (c). Most of the radioactive non-conjugates however must represent other unidentified materials. These may include triglutamates which were isolated from liver[142] and Portuguese man-of-war[243] and which are active for L.casei[30]. Little further evidence is available about the conjugates.

Using a similar extraction and purification procedure liver folates showed a much greater growth with L.casei than with S.faecalis or P.cerevisiae before and after conjugase was obtained and it was concluded that most of the

derivatives were reduced and methylated derivatives of PteGlu[85,113,121]. The results presented earlier in this thesis, however, showed that a natural folate which was active only for L.casei was not 5-CH_3-H_4PteGlu and therefore those conclusions based only on differential microbiological assays should be viewed with caution until substantiated by other methods. With controlled autolysis during extraction procedures the amounts of conjugates were lower[121,243] and may account for the low levels of conjugates reported for rats[163,164,165] and the absence of conjugates in leukaemic cells of the mouse, mouse liver[113] and in human liver biopsy material[166]. Autolysis has caused further confusion since extracts of liver formed after uncontrolled autolysis had a relatively different growth response to the three organisms[164] than had liver[121] or blood[236] extracted without autolysis and then treated with purified conjugase before assays were performed. Thus autolysis is unsatisfactory when determining either the folate levels or the types of compounds present and the procedure used in this work were designed to minimise any autolysis.

An increased microbiological response, after prior treatment of folates with conjugase, was also found in red blood cells[236], marine algae[244], bacteria[54] and yeast[141]. Conjugates therefore probably occur universally. Early work on yeast folates led to the isolation and synthesis of $PteGlu_7$[141]. More recent work[30] showed that yeast contained five conjugates which were separated by and eluted with a stronger buffer than triglutamates from an ion-exchanger. After conjugase treatment these materials had

differing responses to L.casei, S.faecalis and P.cerevisiae. By comparison with the earlier isolation of $PteGlu_7$ the authors assumed that all five conjugates were heptaglutamates although they stated that "the exact degree of conjugation of yeast folates is unknown" and that "more substantial evidence was being sought". No such evidence has appeared. Indeed the occurrence of even higher folate polyglutamates in yeast was indicated by the isolation of p-aminobenzoylglutamates containing 10 to 11 glutamic acid residues[245]. Four lines of evidence, namely behaviour on ion-exchange column, microbiological assays before and after conjugase treatment and the isolation of $PteGlu_3$, suggest that liver conjugates are similar to yeast conjugates and are therefore polyglutamates. However, neither the extent of reduction, substitution nor the extent of conjugation of liver polyglutamates has been determined. It was therefore decided to investigate the extent of conjugation of rat liver folates by combining the previously established method of producing labelled conjugates together with the administration of labelled glutamic acid. It was then hoped to isolate doubly-labelled conjugates from which the extent of conjugation could be accurately determined.

High sp. radioactivity tritiated-glutamic acid was administered together with [2^{14}-C]PteGlu by stomach intubation to one rat. The liver was removed after 24h, the radioactivity was extracted and the extract chromatographed on a Sephadex column (Fig.20). Most of the pteridines (^{14}C-labelled materials) were eluted at tubes less 20, indicating that they had a molecular weight greater than mono-

glutamates. Small amounts of ^{14}C-labelled materials were eluted up to tubes 50 and this confirms the ion-exchange elution pattern (Fig.18). The major labelled metabolite found in the urine was absent from the liver extract. Tubes containing the major carbon-14 labelled and tritium labelled materials were clearly separated. Furthermore, the tritium/carbon-14 ratios, which indicate the extent of folate conjugation, were not constant throughout any of the peaks. The presence of both tritium and carbon-14 in the same tubes was therefore due to incomplete separation of these labelled materials and not to doubly-labelled compounds. The experiment was repeated using a larger amount of glutamic acid (4mg/kg) but again doubly-labelled materials were not obtained (Fig.21). Because of the possible lability of the tritium at position 2 (nominally) of the glutamic acid, it was conceivable that this labelling may have been lost before conjugate synthesis. The last experiment (shown in Fig.21) was therefore modified by using ^{14}C-labelled glutamic acid and tritiated-PteGlu. The liver extract was chromatographed on a DEAE-cellulose column and, despite the difficulty in assaying carbon-14, the fractions did not contain doubly-labelled materials (Fig.22). In the above experiments glutamic acid was not isolated from the liver extract and it was possible that it had been excreted or metabolised sufficiently before any incorporation of PteGlu into the liver conjugates had taken place. The experiment was therefore modified by administering tritiated-PteGlu 3h before ^{14}C-glutamic acid (4mg/kg). The liver was removed after 24h, the radioactivity extracted and chromatographed on a Sephadex

column (Fig.23). No doubly-labelled materials were obtained. Urinary folates did not contain doubly-labelled materials when chromatographed on a Sephadex column (Fig.24) or when materials were purified through a florisil column and then by t.l.c. (Table 22).

Therefore, under the above condition, the de novo synthesis of hepatic folates was not accompanied by the incorporation of labelled glutamic acid. However, despite attempts to increase the administered glutamic acid to a level which would not have been completely metabolised within 24 hours, glutamic acid was not isolated from the homogenates. It was not, therefore, ruled out that the label administered as glutamic acid was absorbed as such or that the glutamic acid was metabolised at a much greater rate than the synthesis of conjugates from PteGlu. Both of these seem unlikely since the label in both labelled (^{3}H and ^{14}C) forms of glutamic acid was absorbed and most of the label as folate was converted into conjugates within 6 hours. Two deductions are possible. The first is that the conjugates do not contain glutamic acid and emphasises the tentative nature of the present suggestion that conjugates are peptides containing glutamic acid residues. The second is that, if conjugates are polyglutamates, then the glutamate residues were derived from a source other than glutamic acid itself. The explanations of these conclusions however will remain speculative until the biosynthesis and structure of hepatic folates are determined.

The Metabolic Fate of Folates and Related Compounds in the Rat

SECTION III

Our knowledge of the fate of folates *in vivo* is limited largely by our lack of identification of the natural forms of folates. The approach, described previously, was designed to clearly characterise the intermediates and the results were both disappointing and rewarding. The identification of folates was minimal, but, even with this limitation, several deductions can be made and will be considered in this section.

Reports disagree about the absorption of folates. Different authors have concluded that PteGlu is absorbed either with [125,154,216] or without[151,155,156] metabolism. Some misunderstanding may also have occurred because "absorption with (or without) metabolism" can be interpreted differently. The work presented earlier established that, in the rat, metabolism of PteGlu is not a prerequisite for absorption. The relatively high metabolic rate of the intestine may however cause some metabolism. This conclusion was derived from assays and chromatography of blood samples, the effects of flushing doses of PteGlu and purification of urinary folates.

When doses of labelled PteGlu (ranging from physiological to large doses) were administered orally, the radioactivity in the urine continuously declined and PteGlu was not excreted in the urine collected for up to 7 days. More abrupt changes occurred in the radioactivity of blood samples and two peaks of radioactivity were present in systemic blood in the 24 hours after administration. After a dose of 320µg of PteGlu/kg the earlier (minor) of these peaks contained PteGlu and the PteGlu declined slowly to an undetectable level at $3\frac{1}{2}$ hours after

administration. A large flushing dose of non-labelled PteGlu, given (orally) shortly after the complete absorption of the labelled dose, produced an increase in radioactivity and labelled PteGlu in the urine. Shortly after the administration of a large preloading dose, some of a labelled PteGlu dose was excreted in the urine as PteGlu. Some of the labelled dose was therefore absorbed as PteGlu. Unfortunately, using these systemic folate assays, it was impossible to obtain an accurate estimate of the extent of metabolism, if any, during absorption, because of the rapid metabolism after absorption (which will be discussed later). Cohen[154], using differential microbiological assays was able to show that metabolites (5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu) accumulated on the serosal side of everted-sac preparations, and, although he concluded that this was due to metabolism of the mucosal PteGlu, the possibility of diffusion of endogenous folate was not ruled out. Baker *et al.*[125], using bioautography, showed that, after oral administration of PteGlu to humans, the *L.casei* assays of systemic blood were much higher than *S.faecalis* assays; whilst after parenteral administration both *L. casei* and *S.faecalis* assays were increased. They concluded that the most likely place for metabolism was during absorption. Although these radiochemical determinations showed only very low levels of PteGlu (some 0.02% of a dose of 320µg PteGlu/kg) in systemic blood, a comparable amount in the human studies of Baker *et al.* would have produced a large microbiological response. Their lack of *S. faecalis* growth may therefore suggest absorption with metabolism, however their results were confusing because

they obtained an increased S.faecalis activity in the urine. When the experiment was repeated PteGlu was present in systemic blood (Blair, J.A. and Leeming, R.J., unpublished results). Thus it would appear that PteGlu can be absorbed without metabolism in both the rat and in humans. The absorption of naturally-occurring folates, however, may be different. The absorption of tritiated-H_2PteGlu and -H_4PteGlu in humans caused an increase in growth-promoting activity for L.casei but no increase for S.faecalis (which grows with H_2PteGlu and H_4PteGlu) in systemic blood and the authors concluded that metabolism to 5-CH_3-H_4PteGlu had occurred during absorption[216]. However, the peak of radioactivity occurred significantly (in my view) earlier than the peak of L.casei growth. This suggests that folate of high sp. radioactivity was absorbed before it was converted to a folate of low sp. radioactivity when assayed with L.casei. Such results are consistent with the absorption of some of the H_2PteGlu and H_4PteGlu without metabolism. Further studies are required with H_2PteGlu and H_4PteGlu to investigate this and to determine the metabolites. The results presented previously showed that the natural diastereoisomer of 5-CH_3-H_4PteGlu was excreted largely unchanged and suggests that it was absorbed as 5-CH_3-H_4PteGlu. It has been suggested[125,217] that the absorption of 5-HCO-H_4PteGlu (determined by microbiological assays) was accompanied by metabolism to 5-CH_3-H_4PteGlu, but evidence in this laboratory (Beavon, J.R.G. and Blair, J.A., unpublished results), using 5-HCO-H_4[2-^{14}C]PteGlu, suggested the converse was the case. Both these studies were done using racemic mixtures. Since only the L-isomer

is microbiologically active, these conclusions may reflect differences in absorption and/or metabolism of the two isomers. Few studies have compared the two isomers. One such investigation[218] reported (with esoteric argument) that the L-isomer was taken up preferentially by P.cerevisiae but the importance of this mechanism requires further studies. It would appear then that folates in mammals can be taken up without metabolism. Furthermore, there would be little advantage in an intestinal conversion of all food folates to $5\text{-}CH_3\text{-}H_4$PteGlu (except in certain cases of liver disease).

At physiological doses only small amounts of folate were excreted whilst at larger doses the percentage excreted increased suggesting that there was a saturation level of folate conversion in normal nutrition and that the dietary excess was excreted. Dietary levels of folates may be stored to a certain (unknown) degree. Chromatography of urine and blood samples gave rise to an apparent anomaly in that, PteGlu was present in the blood during the maximum excretion of folate but was not itself excreted. The probable explanation is that the concentration of PteGlu (15ng/ml) did not reach the maximum kidney reabsorption rate (for which the level in dogs was 0.04µg of PteGlu per 10g of kidney per single pass[199]), even after a dose of 320µg of PteGlu/kg. It was also noticed that the rate of disappearance of PteGlu from the blood (about 0.2µg/kg/h) was much less than its rate of metabolism (>20µg/kg/h over the same period) and suggests that the rate of disappearance of PteGlu from the blood was reduced possibly by a reversible protein binding. PteGlu was ab-

sorbed onto milk-[187] and serum-proteins[25,157,199]. There is however little information on the rate of formation or of breakdown of these complexes although "both rates are presumably high"[8]. There is no evidence to show that the binding is specific for either the folate or the protein and may involve weak ionic, hydrophobic or hydrogen bonds or the formation of charge transfer complexes. More recent studies[187] on the physiological role of protein binding of natural folates (studied with 5-CH_3-H_4-PteGlu) showed that there was no relationship between the free folate and the folate excreted in several diseases whilst in pregnancy the free folate was increased but the excretion was decreased. The physiological importance of protein binding is therefore unknown.

The excretion of radioactivity up to 10 days after doses of labelled-PteGlu and -5-CH_3-H_4PteGlu suggested a half time turnover of 10 to 100 days (depending on the dose). Even considering the low radioactivities (and associated errors) involved this was a much lower turnover than that using tritiated PteGlu[87]. Dr. Thenen's use of tritiated PteGlu may be affected by tritium exchange reactions but a further comparison must await publication of her results. In these experiments the compounds excreted (5-CH_3-H_4-PteGlu and others) represented only about 10% (see below) of the liver folates and therefore the half-time turnover is a measure of the turnover of 5-CH_3-H_4PteGlu and other folate monoglutamates. A further complication is that some of the radioactivity would follow the turnover of red blood cells or the movement from one folate compartment to another and therefore the usefulness of such a determin-

ation is doubtful.

Investigations of the biochemical pathways is dependant on characterisation of folate intermediates. Few *in vivo* studies have been done in mammalian systems and identification of folates has been sporadic and sparse. Biochemical pathways have largely been determined *in vitro* on enzyme extracts, homogenates or in non-mammalian systems. Identification of folates in mammals has almost entirely been done by comparing microbiological assays with the response to synthetic folates. However, not enough is yet known about folates for microbiological assays, on their own, to be used for folate identification and were only used, in this study, to confirm identifications and to determine specific radioactivities. Methods of separation and identification have now been developed and the next era will hopefully be characterisation of folates. Radiochemical approach was the most useful technique used here with t.l.c., and was particularly useful for monoglutamates and simple pteridines but preliminary studies with DEAE-cellulose and Sephadex produced only marginal advances for liver folates. However some interesting ideas were forthcoming.

The urinary compounds excreted after a dose of PteGlu were largely 5-CH_3-H_4PteGlu and an unidentified compound. Sp.radioactivity determinations at different doses suggested that these were both naturally excreted folates in the rat. The identification of 5-CH_3-H_4PteGlu as a urinary folate is consistent with more recent microbiological assay of urine and with serum 5-CH_3-H_4PteGlu, identified by bioautography of human serum[168], and of the increased *L.casei*

response with the serum of other species[8]. The excretion of two folates is confusing, since 5-CH_3-H_4PteGlu was by far the major metabolite in humans. The two compounds were however excreted on subsequent days after administration and were not therefore due to overloading of folate in the body. The second compound was unidentified although it may be of worthwhile interest to speculate that the compound was 5,10-C(COOH)=H_4PteGlu or the rearrangement, 10-CO(COOH)-H_4PteGlu and reduced, 5,10-CH.(COOH)-H_4PteGlu derivatives. Chromatography of these compounds compared to other synthetic folates would be influenced by the additional carboxyl group, which would cause a reduction in Rf value in alkaline solvent systems and would only be eluted by stronger buffers on ion-exchange chromatography. Similar properties are shown by di- and triglutamates. Similar chromatography was exhibited by the unidentified compound yet gel-filtration suggested that the compound was a monoglutamate. Biochemically a link between folate and ascorbate exists. Ascorbate increased the excretion of PteGlu in the rat but not in the guinea pig[101]. The results presented here suggest that ascorbate increased the excretion of the unidentified material when 5-CH_3-H_4PteGlu, a natural folate, was administered. A similar study in humans using PteGlu showed that the compound was not excreted (Leeming, R.J. and Blair, J.A., unpublished results) suggesting a difference between the rat and humans. In both humans and guinea pigs ascorbate is a vitamin, whilst the rat can synthesise ascorbate for which a folate requirement has been suggested[100]. Unfortunately, little is known of the function, synthesis and

metabolism of ascorbate, but most of the oxalic acid and glyoxylic acid excreted in the urine is derived from ascorbate. Glyoxylic acid condenses with H_4PteGlu giving 5,10-CH(COOH)-H_4PteGlu which is readily oxidised to 5,10-C(COOH)=H_4PteGlu[51]. These reaction products are analogous to those formed by the condensations of formaldehyde and formic acid with H_4PteGlu and enzymes catalysing these reactions have been isolated[8]. This hypothesis, then, explains the observed link between ascorbate and folate in the rat as a branch point between one- and two-carbon derivatives of H_4PteGlu. The sp. radioactivities of materials isolated in the urine are consistent with this hypothesis (discussed later).

Methods were thus developed which would both lead to the isolation of metabolites without decomposition and would distinguish them from PteGlu. The methods were then used to study both the extent and pathway of metabolism. Urinary and hepatic folates isolated 24 hours after the administration of 320µg PteGlu/kg, did not contain PteGlu showing a rate of metabolism of at least 4µg of PteGlu/kg/hour. Furthermore, since most of the absorbed radioactivity was excreted in 5 hours, the rate of metabolism of PteGlu was at least 20µg/kg/hour. Flushing doses also confirmed this, whilst a dose of 1.6mg of PteGlu/kg was not completely metabolised under the same conditions. Since the metabolism involved reduction, the rate of metabolism of reduced folates is likely to be higher than for PteGlu. This rate of metabolism is much greater than that required to convert the dietary intake of folates into body folates.

One of the metabolites was 5-CH_3-H_4PteGlu. Earlier

results also suggested that PteGlu was metabolised to 5-CH_3-H_4PteGlu in humans[86] but later observations, using tritiated PteGlu, suggested that the urinary 5-CH_3-H_4-PteGlu was derived by displacement[161]. The displacement was shown in only 10 out of 14 subjects and should be viewed with caution because other metabolites and folate decomposition products were present. Work with [2-^{14}C]PteGlu in humans suggested that metabolism had taken place, although the metabolites were not identified[156]. The rate of metabolism of PteGlu in humans has not been determined. In these experiments most of the retained radioactivity was present in the liver and, since all the known folate coenzymes occur in the liver, it seems reasonable to suppose that most of the metabolism took place in the liver.

Using labelled-PteGlu enables one to carry out dilution analyses and thus estimate the extent of metabolism and folate pool sizes, assuming that the labelling is completely and evenly distributed throughout the folate pool. Although this assumption was so for the final distribution and for subcellular fractions[246], it is clearly not true for the *in vivo* physiological experiments used here and approximations must be made for rates of absorption, metabolism and excretion of the label (discussed above). The labelled 5-CH_3-H_4PteGlu excreted on day 1 after oral doses of 3.2, 32 or 320µg of PteGlu/kg showed that the absorbed dose had been diluted by 100-200µg of folate. Thus the absorbed dose had (on average) been metabolised and distributed into half the total hepatic folate (20µg/g), determined by *L.casei*[121]. The total folate pool however is likely to be larger since, during synthesis of

hepatic folates, radioactivity was being excreted without being metabolised (with accompanying dilution) further. This was confirmed by analysis of liver folates which showed a further (5 fold) dilution when assayed 24 hours after administration. The specific radioactivity of 5-CH_3-H_4PteGlu isolated on day 2 after administration was lower than on day 1 suggesting that the compound represented only a small part of the liver folate and was diluted further by the relatively low folate intake (≈5μg) compared to the total hepatic folate (≈300μg). Flushing doses produced a further (10 fold) dilution establishing that the compounds excreted represented only a small pool of the total folate since the absorbed flushing dose (about 100μg of PteGlu/kg) would have produced only a slight (<1.5 fold) dilution if it were distributed throughout the body folate. Thus orally administered PteGlu was rapidly metabolised into a normally small folate pool and this was further metabolised into the major folates within 24 hours. The folate pool contained two metabolites, 5-CH_3-H_4PteGlu and the unidentified material, which were excreted.

The dilution of the unidentified compound was dependent on the dose and dilutions were less than the 5-CH_3-H_4PteGlu. The compound excreted in the first day urine was diluted by about 5 to 200μg of folate, depending on the dose. This was unexpected and may reflect the differing extents of further metabolism of the oral dose. The compound was derived from PteGlu and was extensively diluted by a flushing dose of PteGlu. However, since the compound was not diluted further on day 2 the compound, once formed, does not seem to be metabolised into other folates (or at

least those folates which are formed from 5-CH_3-H_4PteGlu) and was not extensively formed by natural folates in the diet. Thus it appears that this compound was formed readily from PteGlu, was present in much smaller amounts and had a relatively lower turnover than 5-CH_3-H_4PteGlu. The amount of the compound excreted and present in the tissues may be exaggerated by using PteGlu and indeed only a small amount was formed from 5-CH_3-H_4PteGlu (Section II). It may be then that the naturally excreted compound was 5-CH_3-H_4PteGlu since it is difficult to reconcile the excretion of two folates. These dilution results are therefore consistant with H_4PteGlu being the branch point (see above) and hence the only other folates likely to give extensive amounts of the unidentified compound would be H_2PteGlu and H_4PteGlu. Due to their lability, however, they are unlikely to be present in the diet.

Thus, studies on the distribution and metabolism of PteGlu suggested that the liver contained a small pool of folates, containing 5-CH_3-H_4PteGlu, but that the major folates were other derivatives which were not excreted. Other techniques were then used to investigate the pathways of metabolism, the size of the pool and the nature of the conjugates.

The physiological distribution of 5-CH_3-H_4PteGlu was similar to PteGlu, the biochemical distribution however seemed different. 5-CH_3-H_4PteGlu was largely excreted as such. The dilution (about 5 fold) of the 5-CH_3-H_4PteGlu excreted on day 1 was much lower than that (about 70) derived from PteGlu and suggested that the body level of 5-CH_3-H_4PteGlu was 5 to 20µg compared to 100-200µg of

total folate. The dilution which would have been incurred by the serum level of 5-CH_3-H_4PteGlu would have been negligible because the level (62ng/ml) of 5-CH_3-H_4PteGlu in the serum is very small compared to the doses used. The dilution was therefore mainly caused by the liver folate and the results indicate that only 10% of the total hepatic folate was 5-CH_3-H_4PteGlu. An approximate measure of hepatic 5-CH_3-H_4PteGlu can be obtained by comparing the L.casei assays above S.faecalis and P.cerevisiae assays. Unfortunately the relative growth of these three organisms is dependent on the extraction and assay procedures and values of 5-CH_3-H_4PteGlu have varied from 0 to 100% of the total folate which, in turn, has varied from 0.1 to 20µg/g of liver[8]. However, I consider (for reasons which are discussed earlier) the best estimate was that reported by Bird et al.[121] who suggested that 75% of the total hepatic folates were conjugates and of the non-conjugates, 67% was 5-CH_3-H_4PteGlu. This suggests that the level of 5-CH_3-H_4PteGlu was 17% of the total folate, slightly higher than the value reported here from dilution studies. This dilution analysis could be extended to the study of 5-CH_3-H_4-PteGlu levels and metabolism in diseases and should be a useful tool to investigate the methyl-trap hypothesis in vitamin B_{12} deficiency.

Little information was forthcoming on the pathways of metabolism. PteGlu was metabolised into liver folates within 6 hours, but only low levels of radioactivity were present in monoglutamates when extracts were taken from 6 hours to 10 days. This suggested that the percentage of folate present as monoglutamate was low and that this

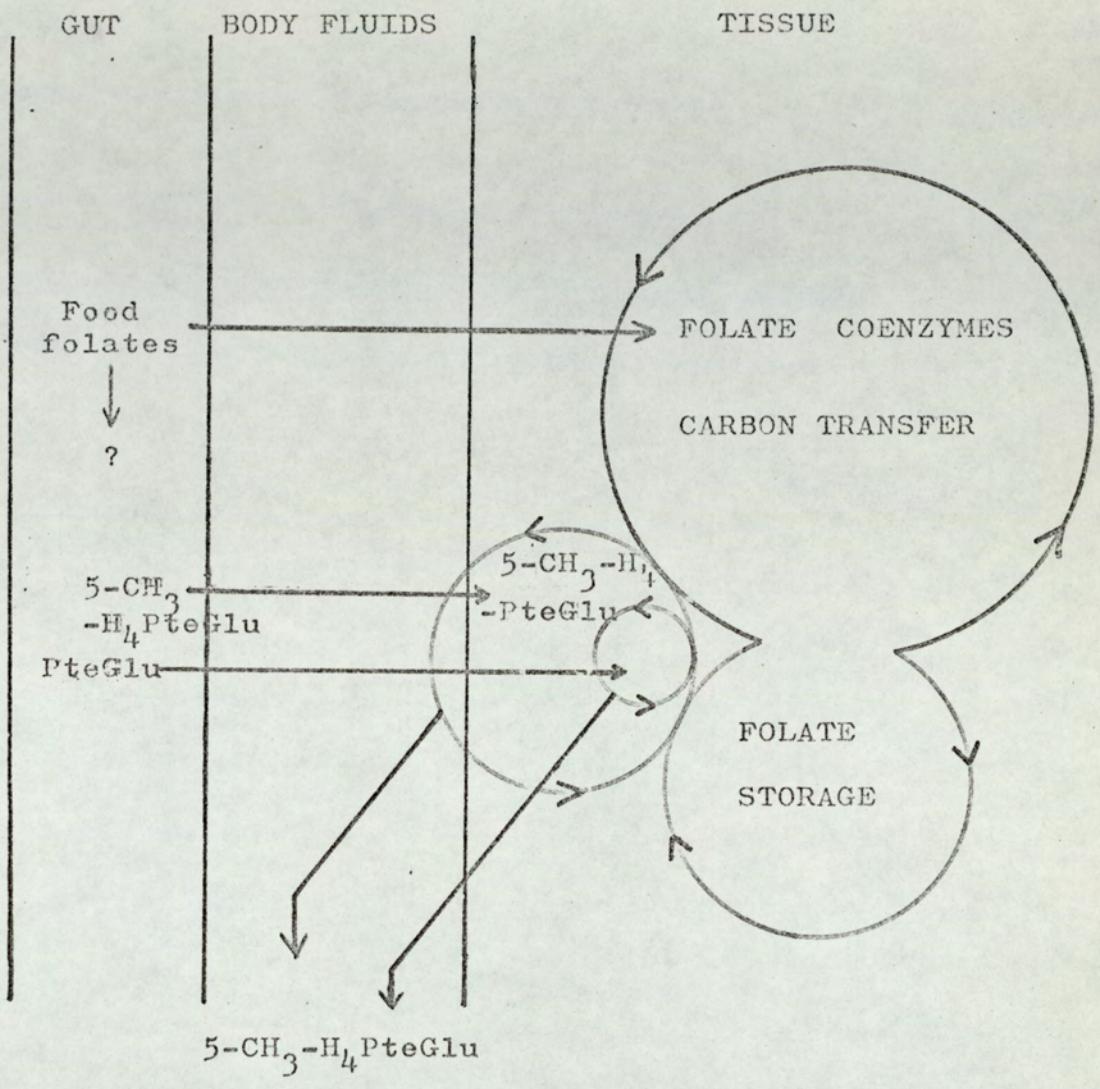
could be accounted for largely by the 5-CH_3-H_4PteGlu. The formation of 5-CH_3-H_4PteGlu from PteGlu could not be determined because of the low levels of monoglutamates and the rapid metabolism. It may involve reduction, condensation with formaldehyde and then further reduction. If 5-CH_3-H_4PteGlu proves to be the only natural monoglutamate in the mammalian liver, then a suitable role as a transport folate seems predictable since 5-CH_3-H_4PteGlu is also exclusively present in mammalian serum and urine. The role of 5-CH_3-H_4PteGlu as a transport folate would be advantageous over other reduced monoglutamates. 5-CH_3-H_4PteGlu is more stable than H_2- and H_4PteGlu and is not affected by small (physiological) changes in pH of the medium or changes in different buffers as are other reduced monoglutamates.

Less is known of other forms of folate. Our knowledge of the chemistry of conjugates has advanced only slightly since the late 1940's and conjugates are still referred to as storage forms of folate. The term storage can, however, be misleading since it implies that they are metabolically inactive. Because we do not know the function of conjugates does not imply that they are inactive. Indeed in all cases tested, purified enzymes utilise di- and triglutamate forms of folate either equally or more effectively than monoglutamates and H_4PteGlu$_7$ can act as substrate for 10-formyl-synthetase[8]. On the question of storage, it is interesting to see that bacteria and plants, which can synthesise folates, contain conjugates and suggests that, in these organisms, the conjugates are not merely storage forms. The conjugates in different organisms, of course,

may not be the same.

Work in this thesis showed that several forms of folates existed and that there were several forms of conjugates. If conjugates were the storage forms, the question to be asked is "Why are there several forms of a storage folate?". This work also showed that, in vivo, only a small part (about 10%) of the total hepatic folate was present as 5-CH_3-H_4PteGlu and that, in homogenates, only small amounts of other monoglutamates were present and that these may have been derived by the decomposition of other folates.

It would therefore appear reasonable to suggest that the coenzyme forms of folates were not monoglutamates. The tissue pattern would then appear as summarised on the next page. In the tissue the major forms of folate are shown as a coenzyme pool and also involving storage folates which are represented as being of a much lower concentration than the true coenzyme forms. The size of the two pools and their chemistry are as yet unknown. Because the structures are unknown at present the advantages of these forms of folate over monoglutamates cannot be estimated but may be a structural one. The structural necessity of conjugates (suggested by ionophoretic studies as containing 5 or 6 glutamic acids) in T_4 bacteriophage was suggested since the removal of one of the 6 glutamic acid molecules completely inactivated the phage. Nothing is yet known of the biosynthesis of conjugates.



"Ne frustra vixisse videat"

One of the last remarks of Tycho Brahe (1546-1601).

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Fig.1 Enzymatic Interconversion of Folates

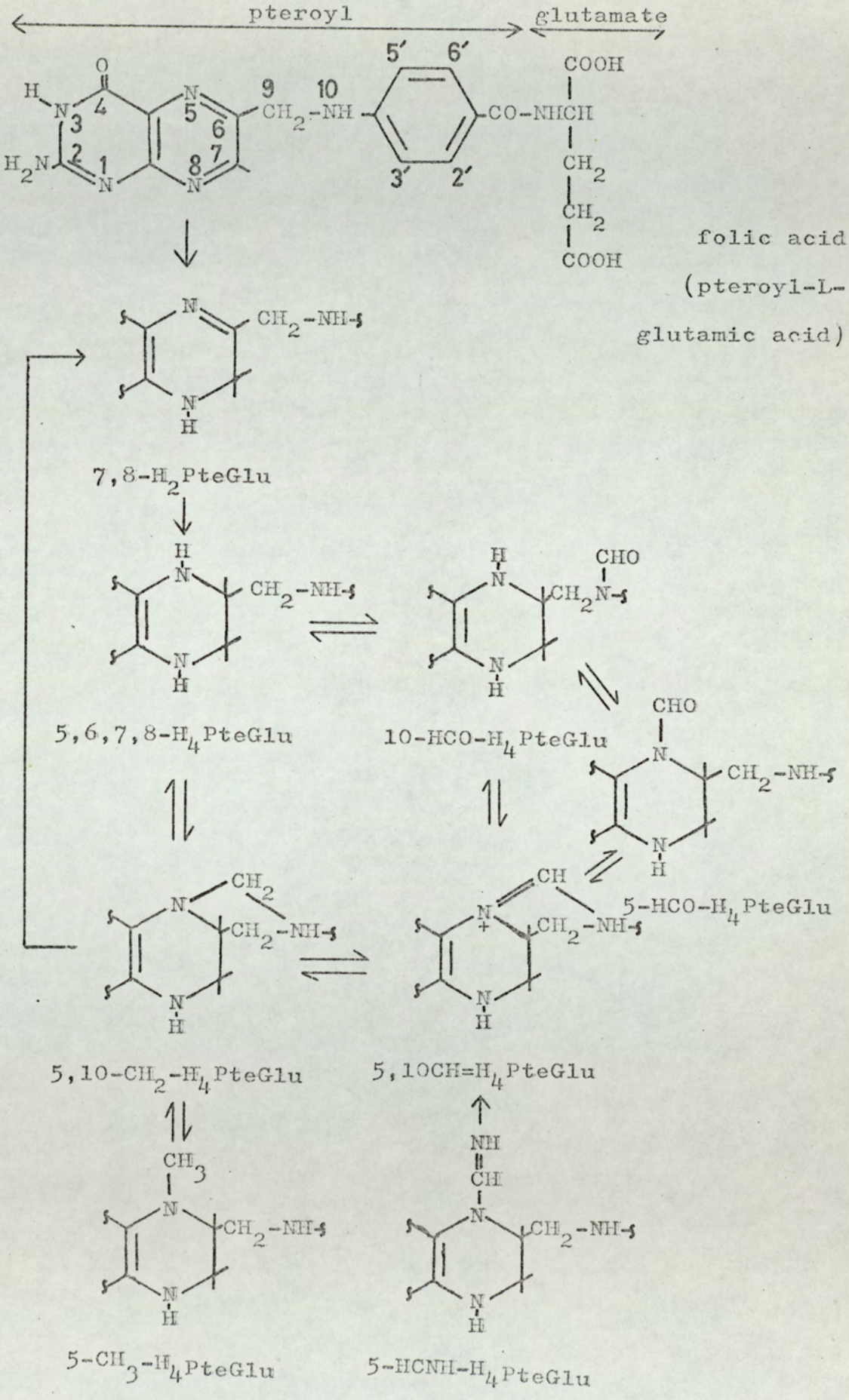


Fig.2 The Biosynthesis of Pteridines and Folates

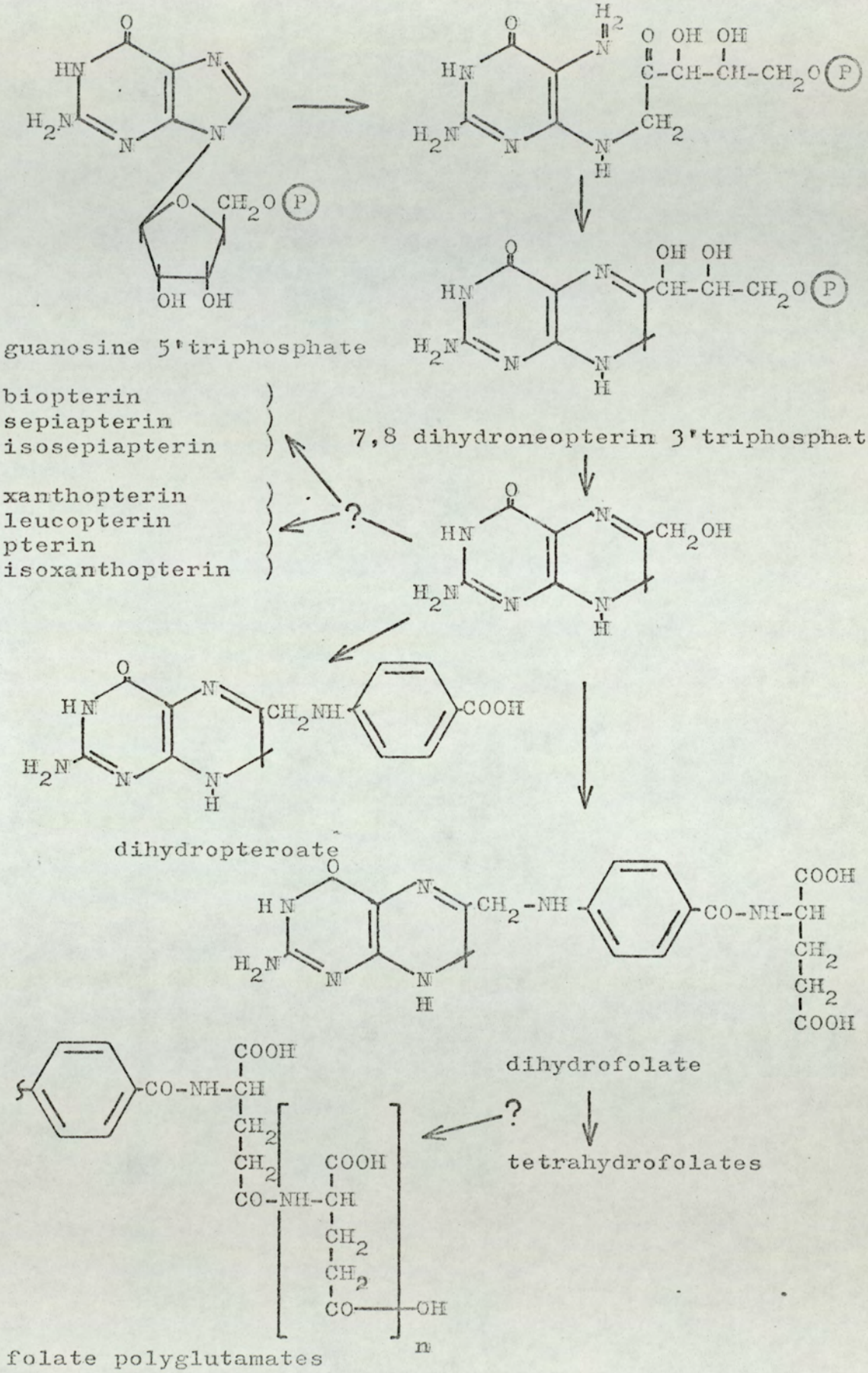
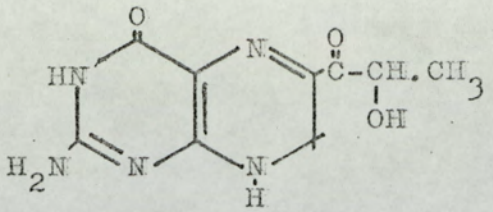


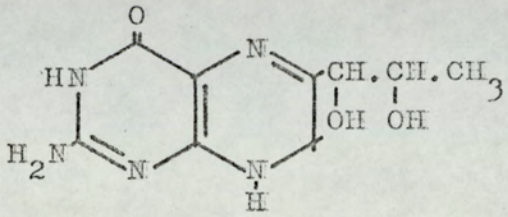
Fig.3 Interconversion of Pteridines

(a) Reduction



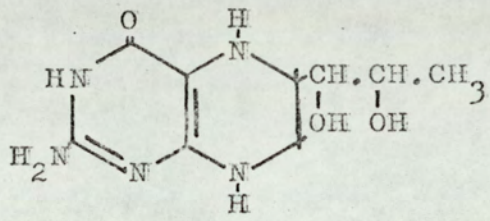
sepiapterin

↓ sepiapterin reductase



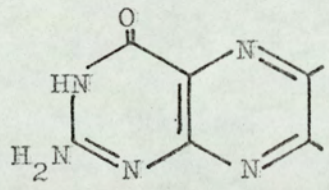
dihydrobiopterin

↓ dihydrobiopterin reductase



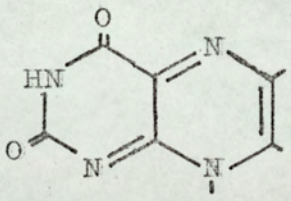
tetrahydrobiopterin

(b) Biosynthesis of lumazines and flavins

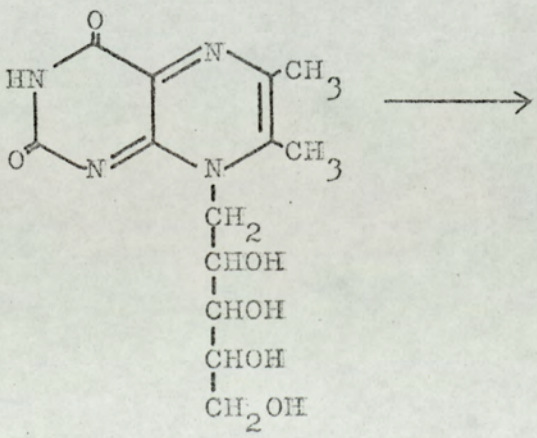


pteridine

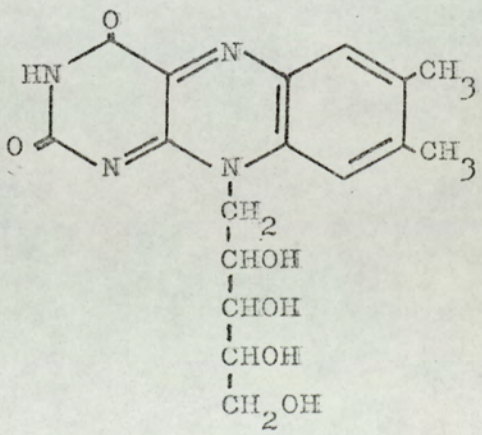
deaminase →



lumazine



5,6, dimethyl, 8 ribityl-lumazine



riboflavin

Fig.4 The role of folate in megaloblastic anaemias

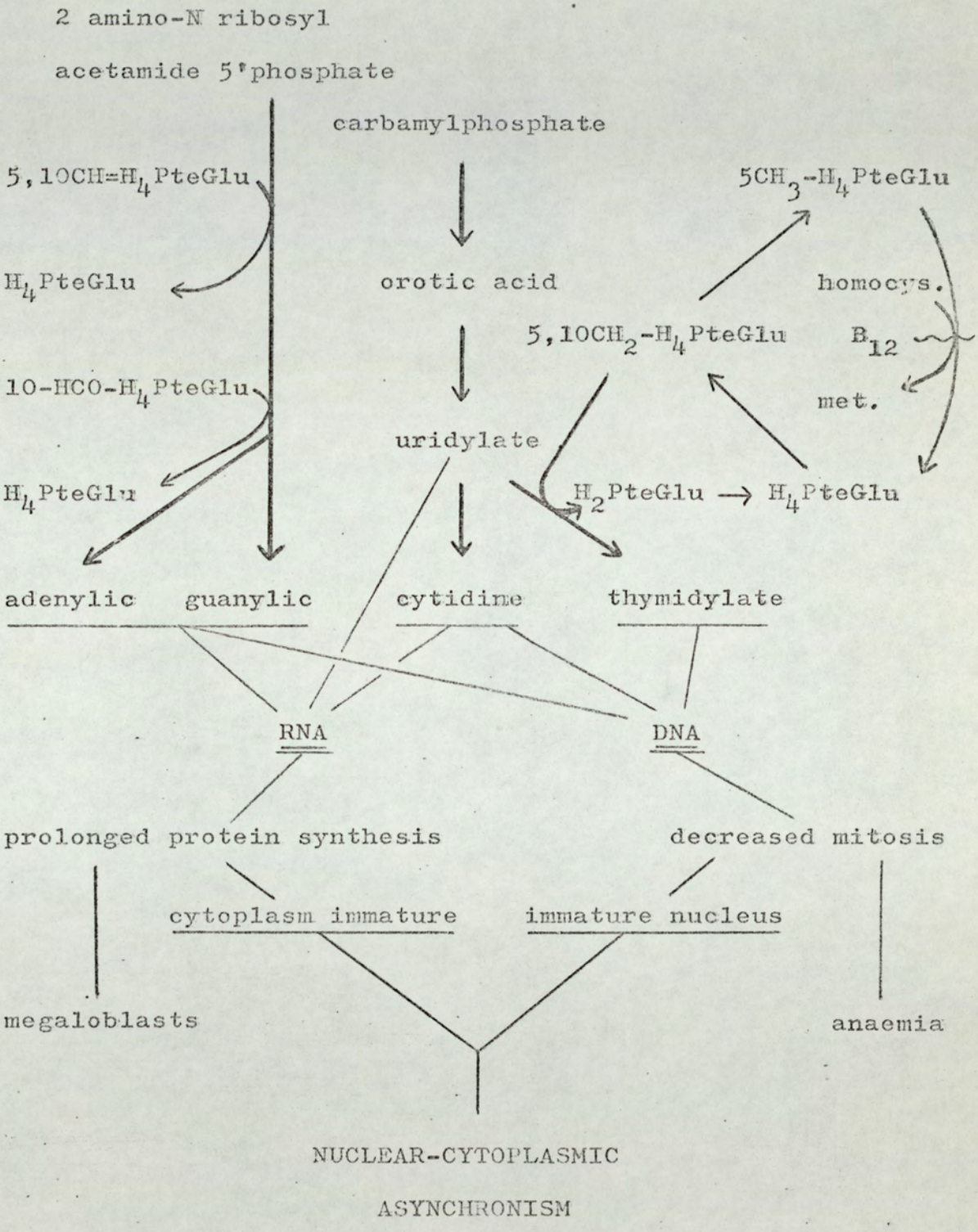
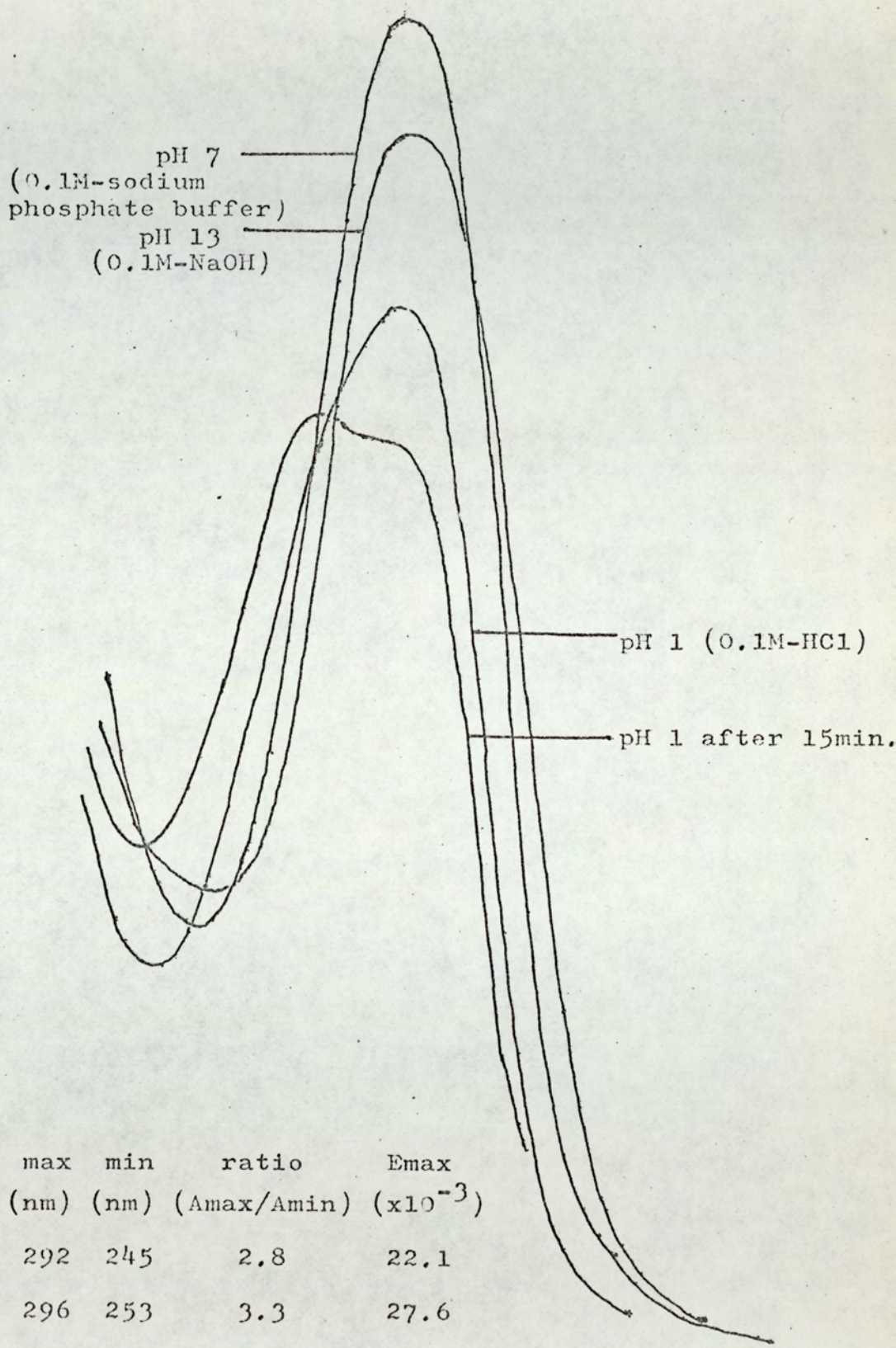


Fig.5 5,10-CH_2-H_4PteGlu prepared by a modification of Blair and Saunders u.v. spectral data.



pH	max (nm)	min (nm)	ratio (Amax/Amin)	Emax ($x10^{-3}$)
1.0	292	245	2.8	22.1
7.0	296	253	3.3	27.6
13.0	297	253	2.7	25.4

Fig.6 Elution profile of 5-[2^{14}C]CH_3-H_4PteGlu from a florisil column.

Application and elution were as described in the text.

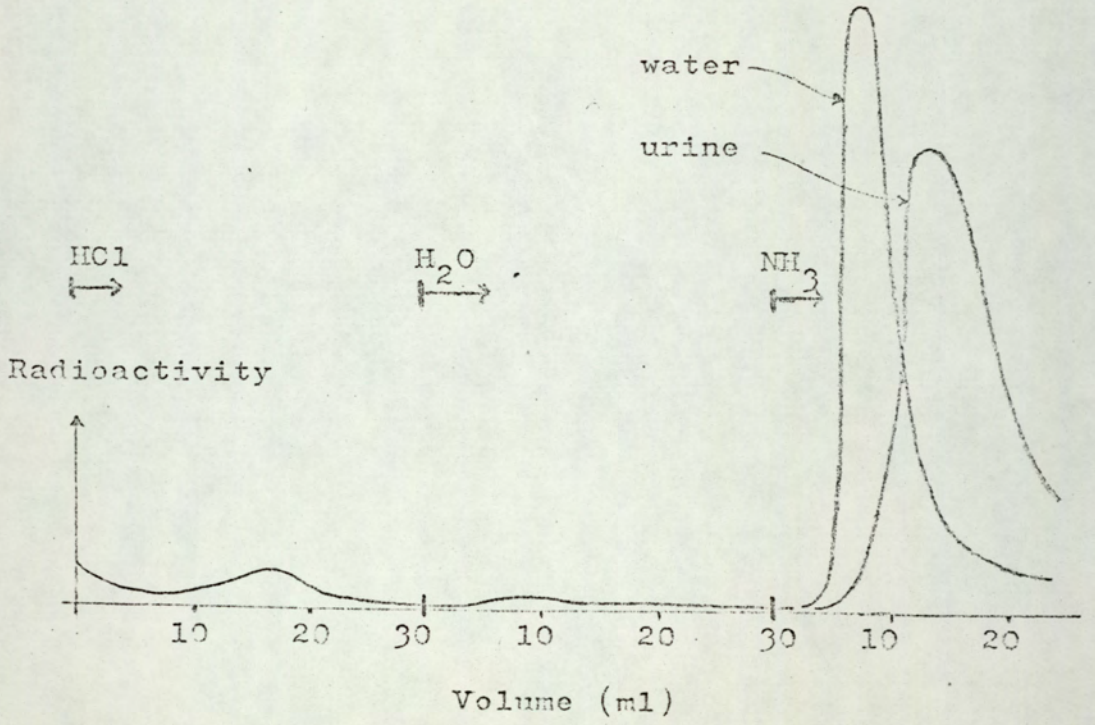
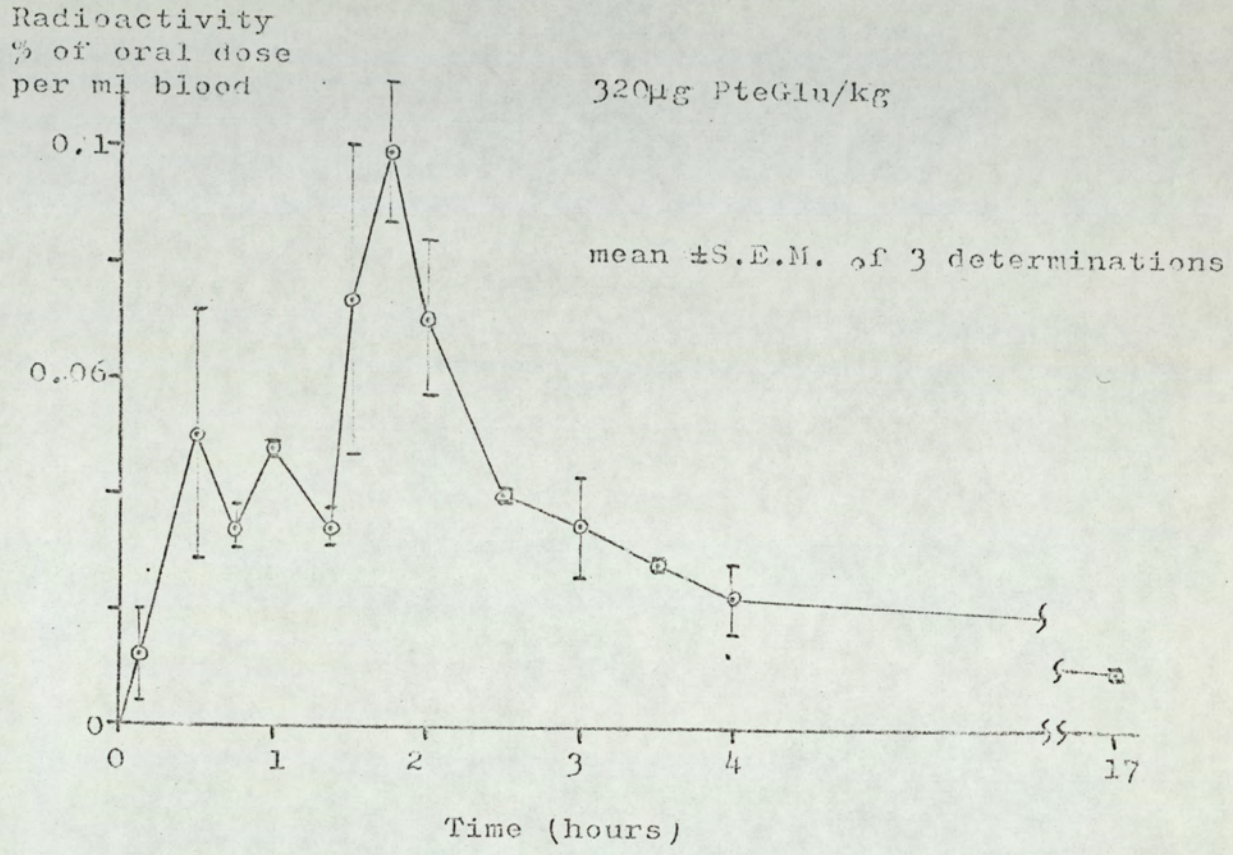


Fig.8 Radioactivity in blood after oral doses of [2^{14}-C]PteGlu.

PteGlu was administered by stomach intubation and blood samples were withdrawn from the tail vein. Radioactivity was determined by combustion followed by liquid scintillation counting.



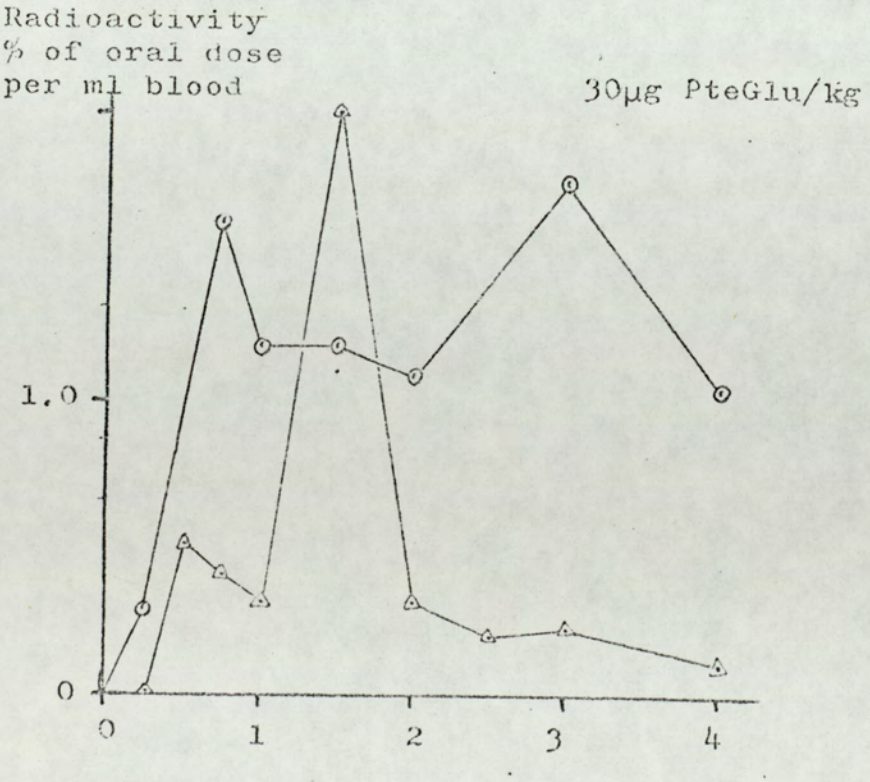


Fig.9. Distribution of radioactivity in the urine after oral doses of [2-^{14}C]PteGlu.

The total radioactivity excreted (expressed as the mean ±S.D. for four animals) at doses of 320 and 32µg PteGlu/kg were 32.6 ± 4.5 and 5.40 ± 2.12 respectively.

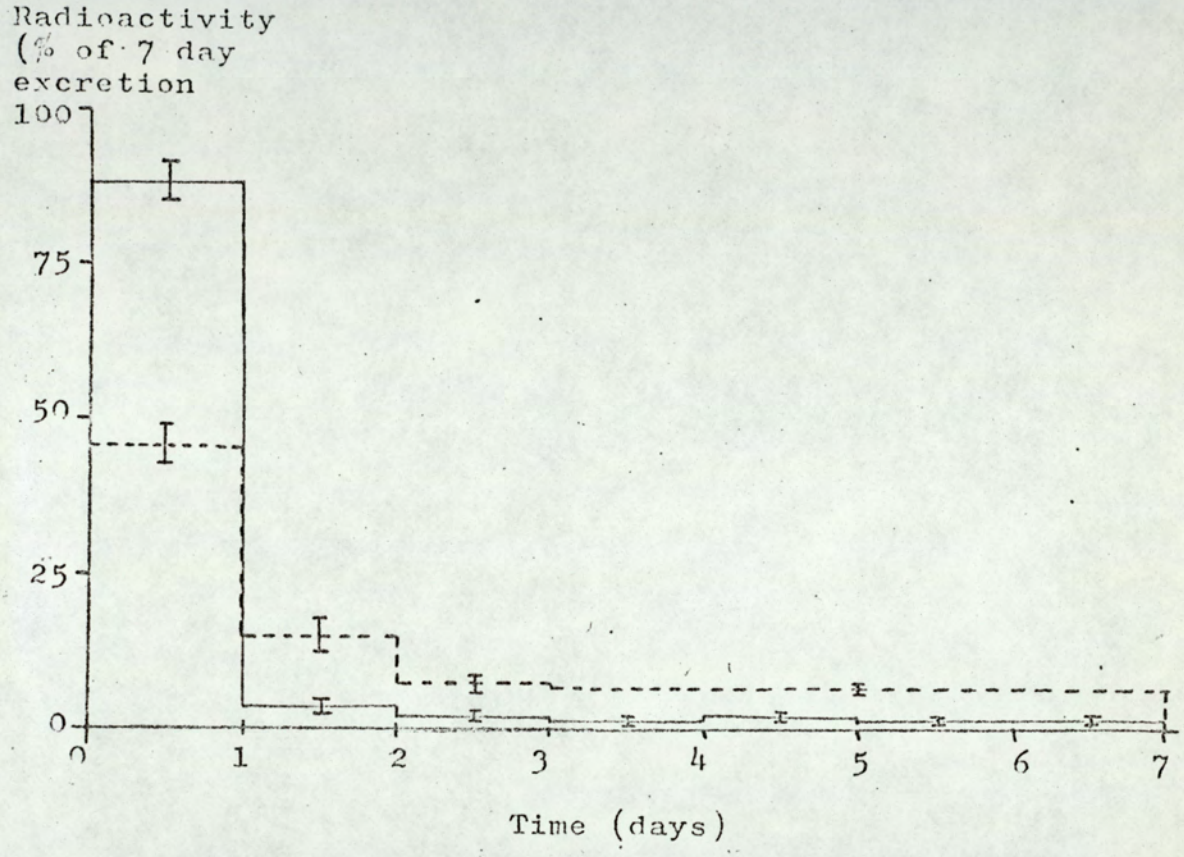
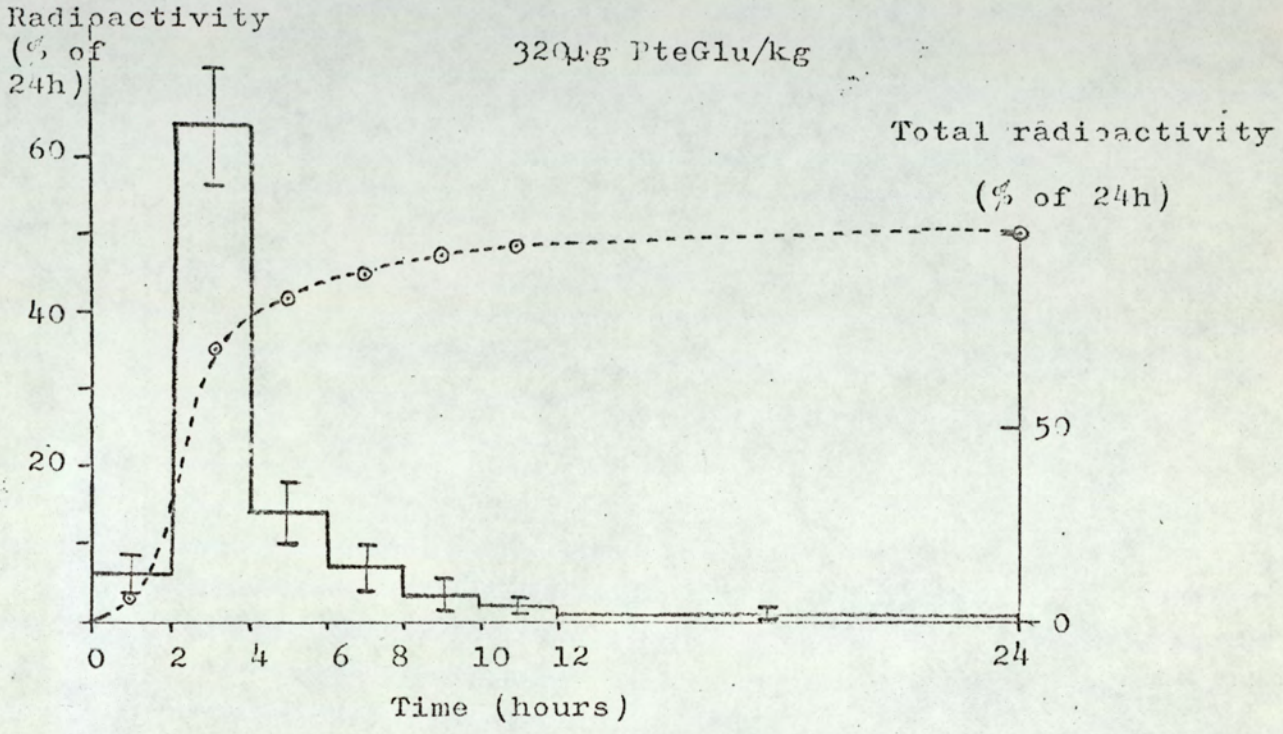


Fig.10 Excretion of Radioactivity after oral [2^{14}-C]PteGlu.

Results are expressed as the percentage mean (±S.E.M., histograms) of an oral dose excreted and the percentage (total, dotted) of the excreted dose from four rats (320µg/kg) and from five rats (32µg/kg).



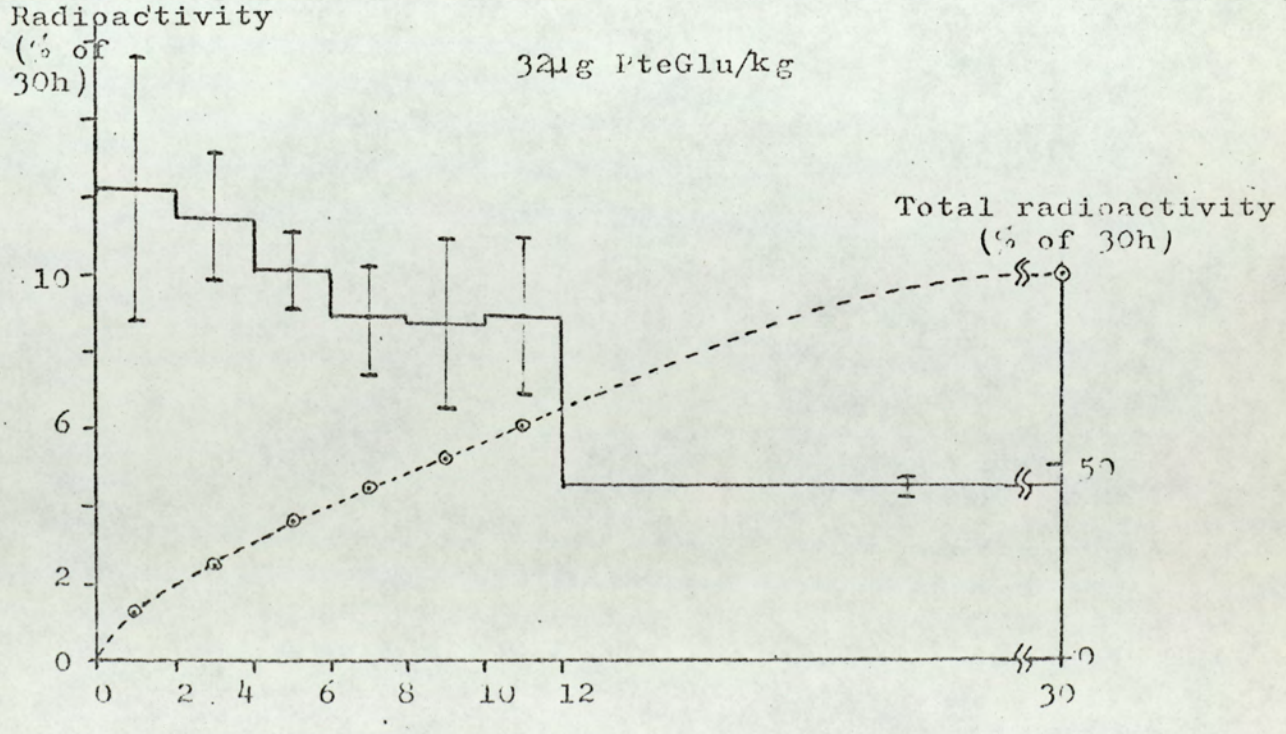


Fig.11 Urinary metabolites after oral folates

Chromatography of first day urines after the administration of labelled folates. Chromatograms were run in solvent (c) and radioautographs produced. Radioautographs were scanned with a microdensitometer.

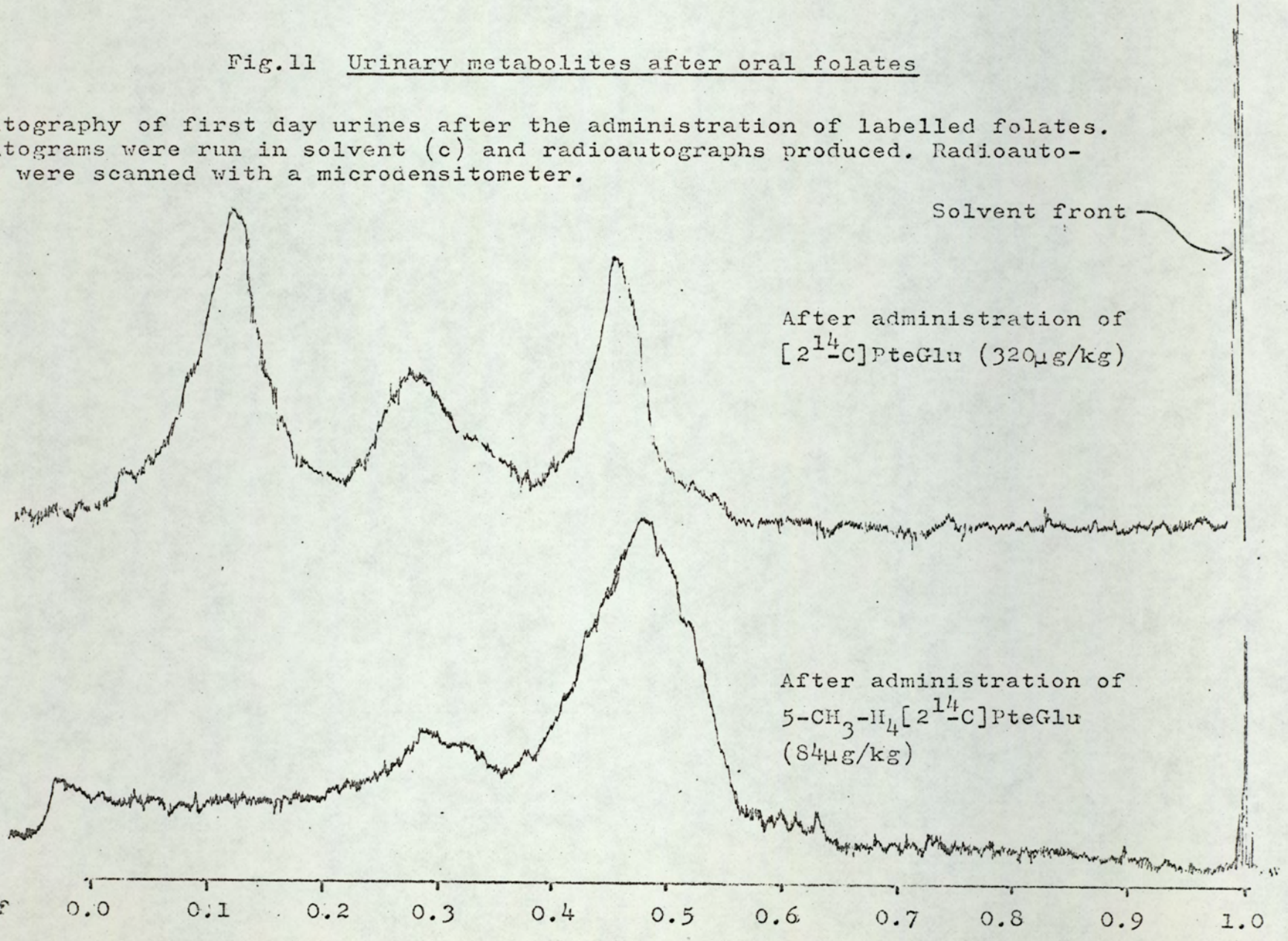


Fig.12 · Distribution of radioactivity on t.l.c. after administering 5-CH_3-H_4[2^{14}-C]PteGlu

0.24µg 5-CH_3-H_4[2^{14}-C]PteGlu/kg was administered to four rats, the urine collected, purified through a florisil column and then on a t.l.c. developed in solvent (c). Equal portions of the t.l.c. were scraped off, extracted with 1ml water and each extract counted in 10ml NE 220 scintillator.

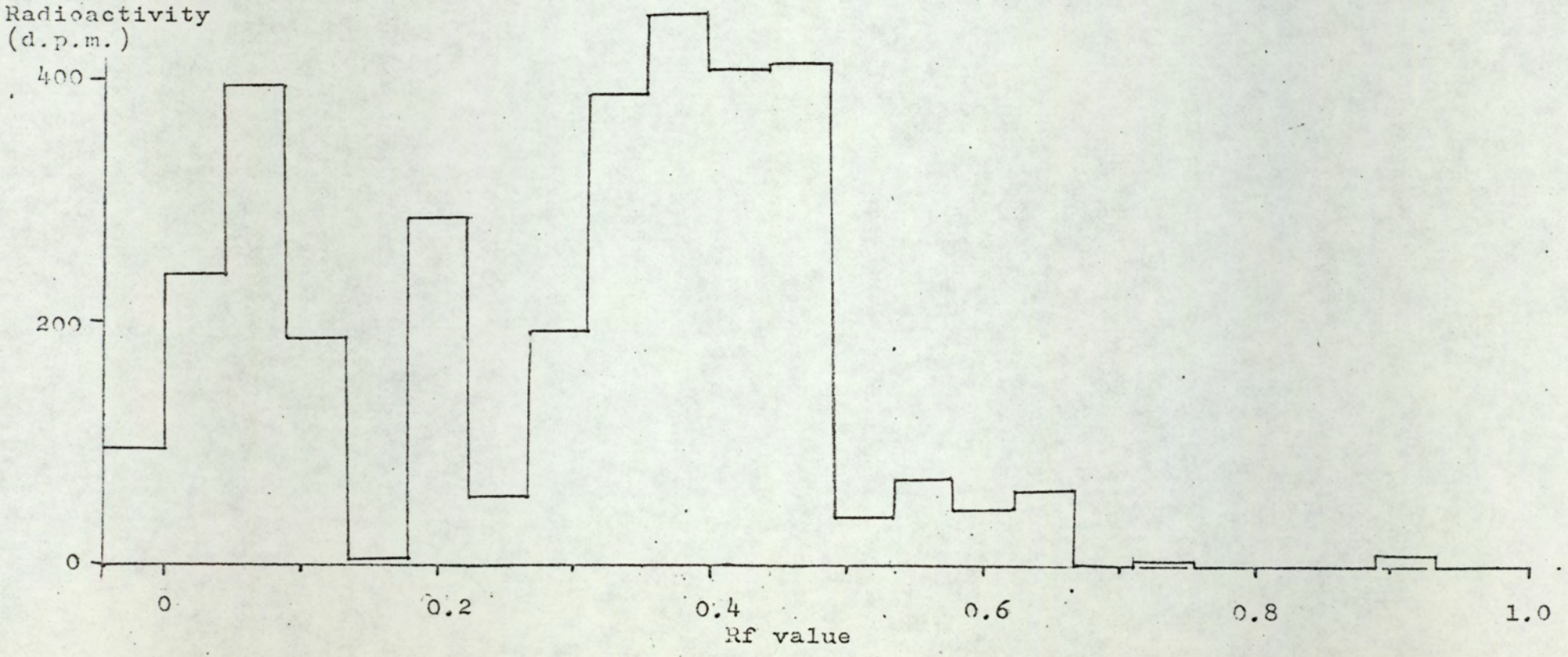


Fig.13 The two materials present in systemic blood following [2-^{14}C]PteGlu administration, which were separated by t.l.c.

The results are expressed as the amount of radioactivity (calculated by densitometry and liquid scintillation counting) present when blood samples were chromatographed in solvent (b).

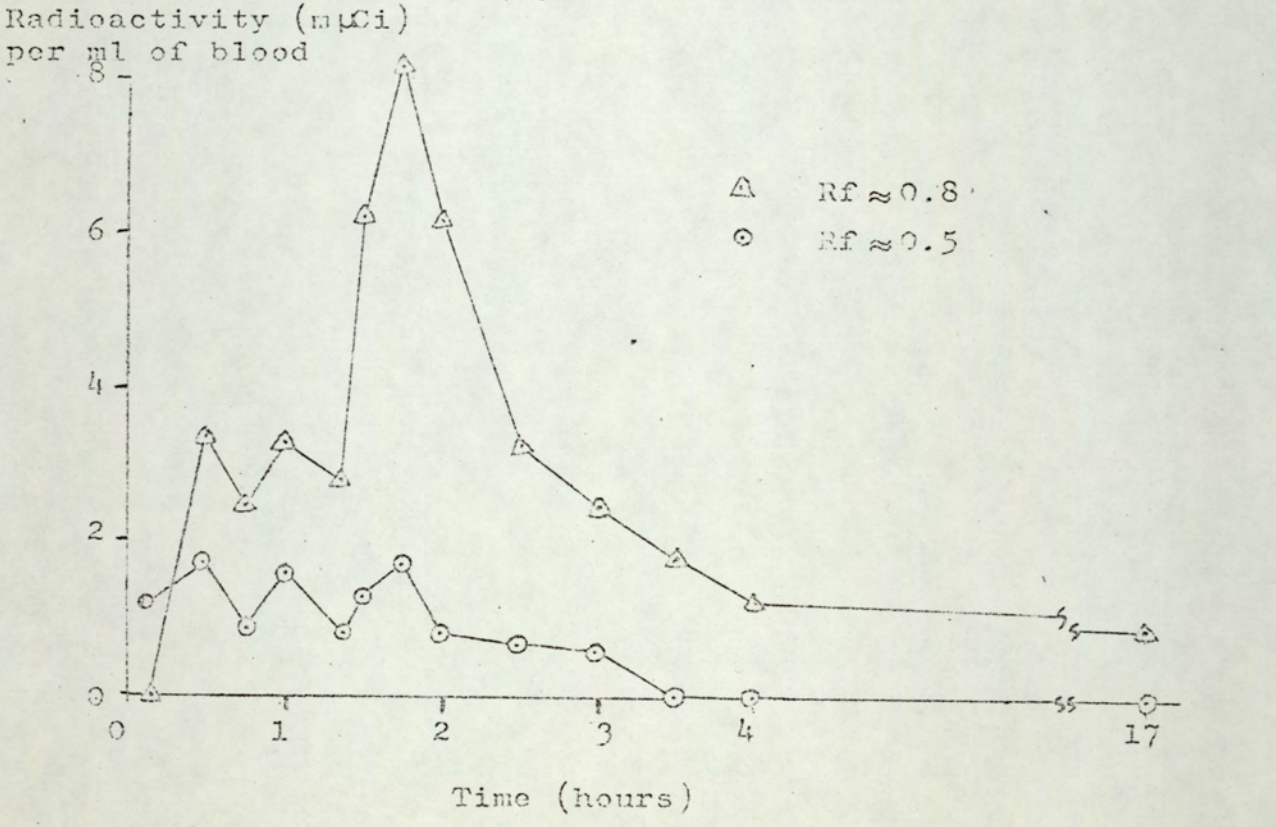


Fig.14 Ion-exchange column chromatography of radioactive material extracted from liver and previously purified by t.l.c.

10μCi (80μg) of [2^{14}-C]PteGlu were orally administered to one rat previously starved for 3 days and the liver removed after a further 24h. Radioactivity extracted was 85%. The extract was chromatographed by t.l.c. on cellulose and the major radioactivity (Rf 0.00 to 0.08) extracted and chromatographed on a DEAE-cellulose column (see Methods section). 5ml fractions were collected and assayed for radioactivity. The recovery of radioactivity was 125% of the 8.3mμCi applied.

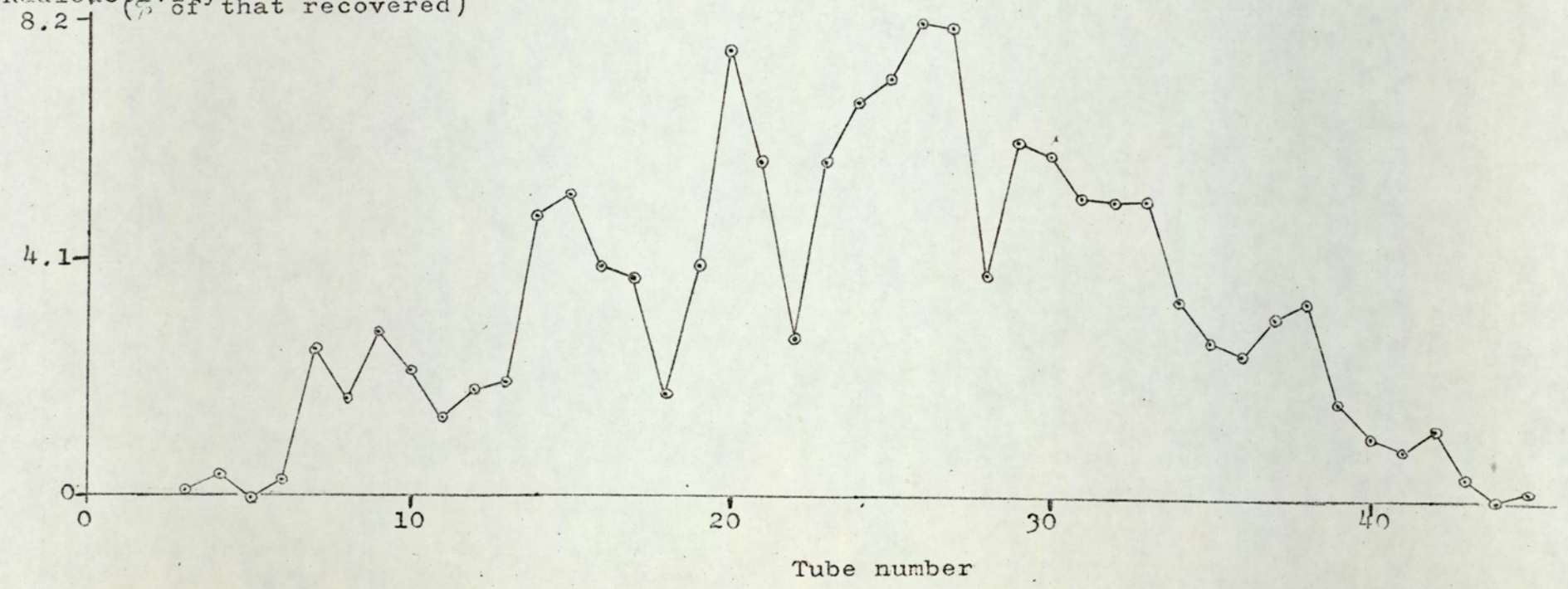


Fig.15 Hepatic radioactivity after oral administration of labelled-PteGlu.

10μCi (80μg) of [2^{14}-C]PteGlu were administered to one rat previously starved for 3 days and the liver removed after a further 24h. Folates were extracted by method (v) and the radioactivity extracted was 83%. The extract was chromatographed on a DEAE-cellulose column (see Methods section), 5ml fractions were collected and assayed for radioactivity. The recovery of radioactivity was 110%.

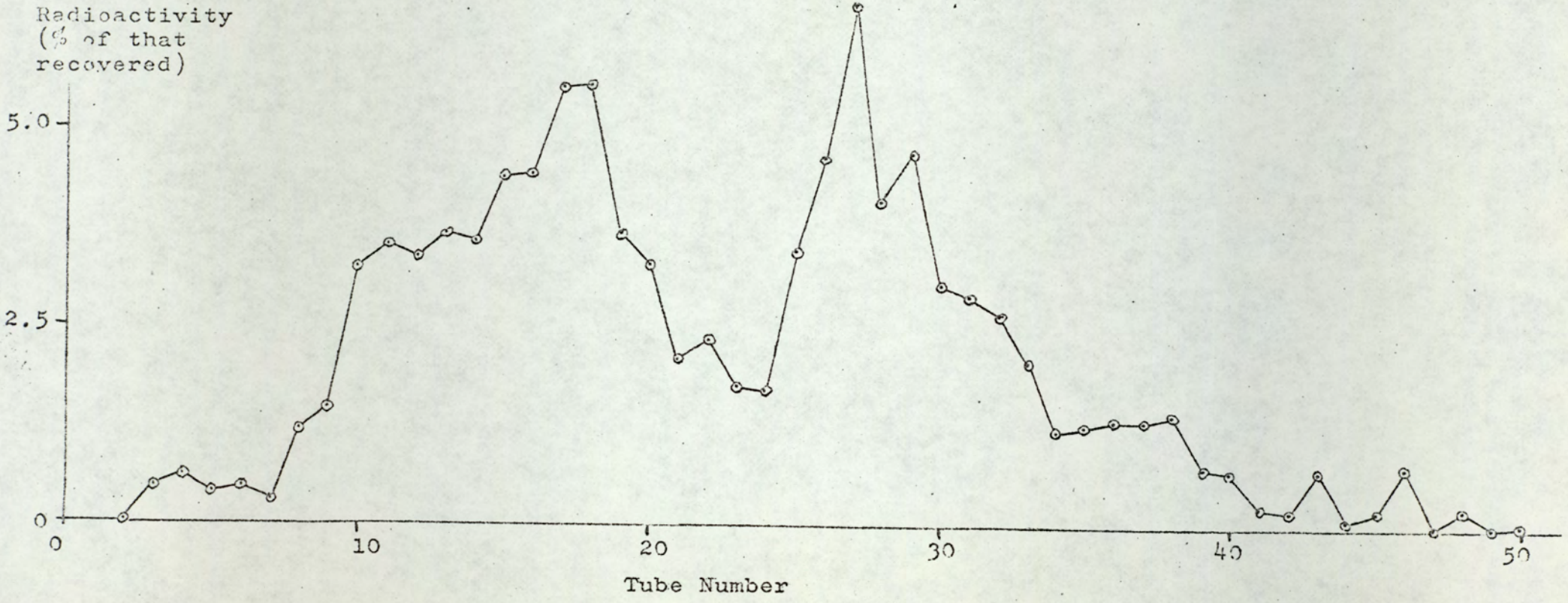


Fig.16 Hepatic radioactivity after oral administration of labelled-PteGlu.

10μCi (80μg) of [2^{14}C]PteGlu were administered to one rat previously starved for 3 days and the liver removed after a further 6 days. Folates were extracted by method (v) and the radioactivity extracted was 88%. The extract was chromatographed on a DEAE-cellulose column (see Methods section), 5ml fractions collected and assayed for radioactivity. The recovery of radioactivity was 92%.

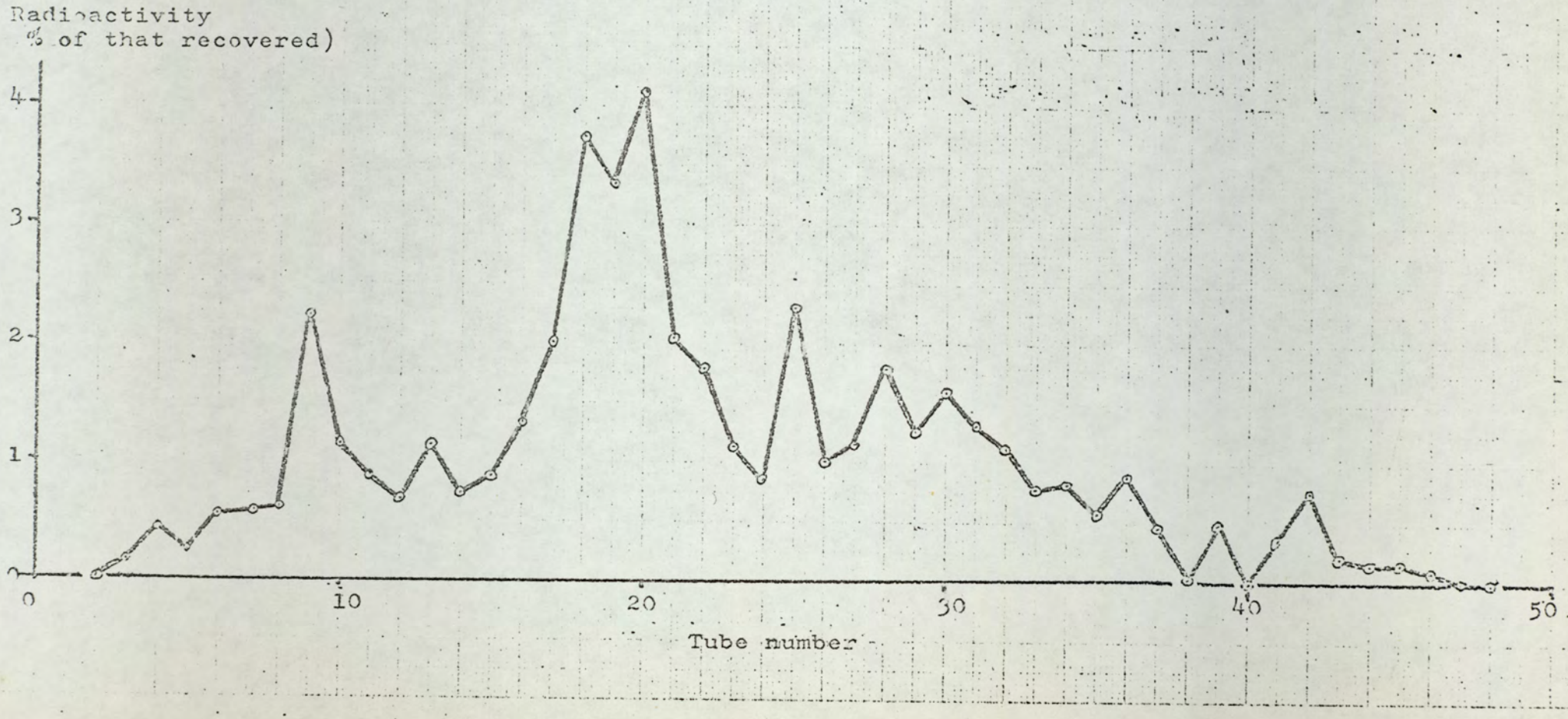


Fig.17 Hepatic radioactivity after oral administration of labelled-PteGlu.

10μCi (≡80μg) of [2^{14}-C]PteGlu were administered to one rat and the liver removed after 10days. Folates were extracted by method (v) and the radioactivity extracted was 78%. The extract was then chromatographed on a DEAE-cellulose column (see Methods section), 5ml fractions collected and assayed for radioactivity. The recovery of radioactivity was 98%.

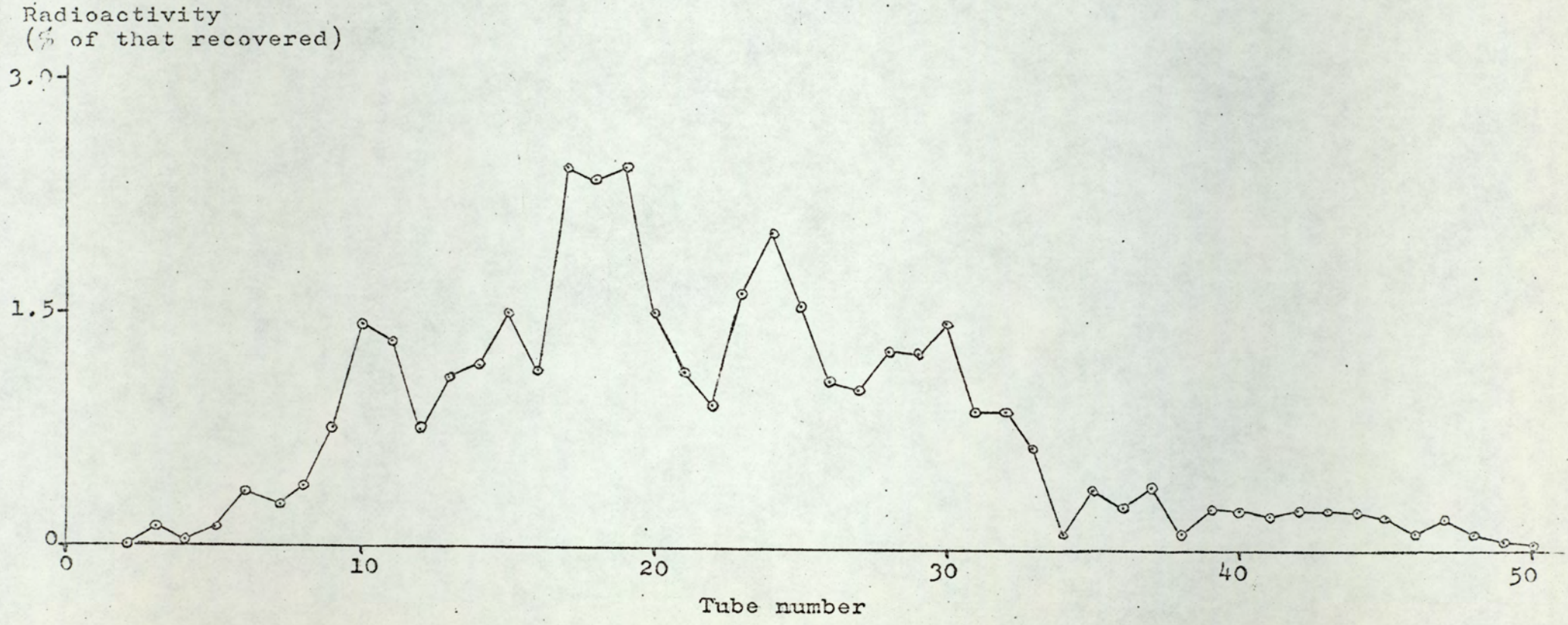


Fig.18 Hepatic radioactivity after oral administration of labelled-PteGlu.

10μCi (≡80μg) of [2^{14}C]PteGlu was administered to one rat and the liver removed after 36h. Folates were extracted by method (v) and the radioactivity extracted was 82%. The extract was then chromatographed on a DEAE-cellulose column (see Methods section), 5ml fractions collected and assayed for radioactivity. The recovery of radioactivity was 105%.

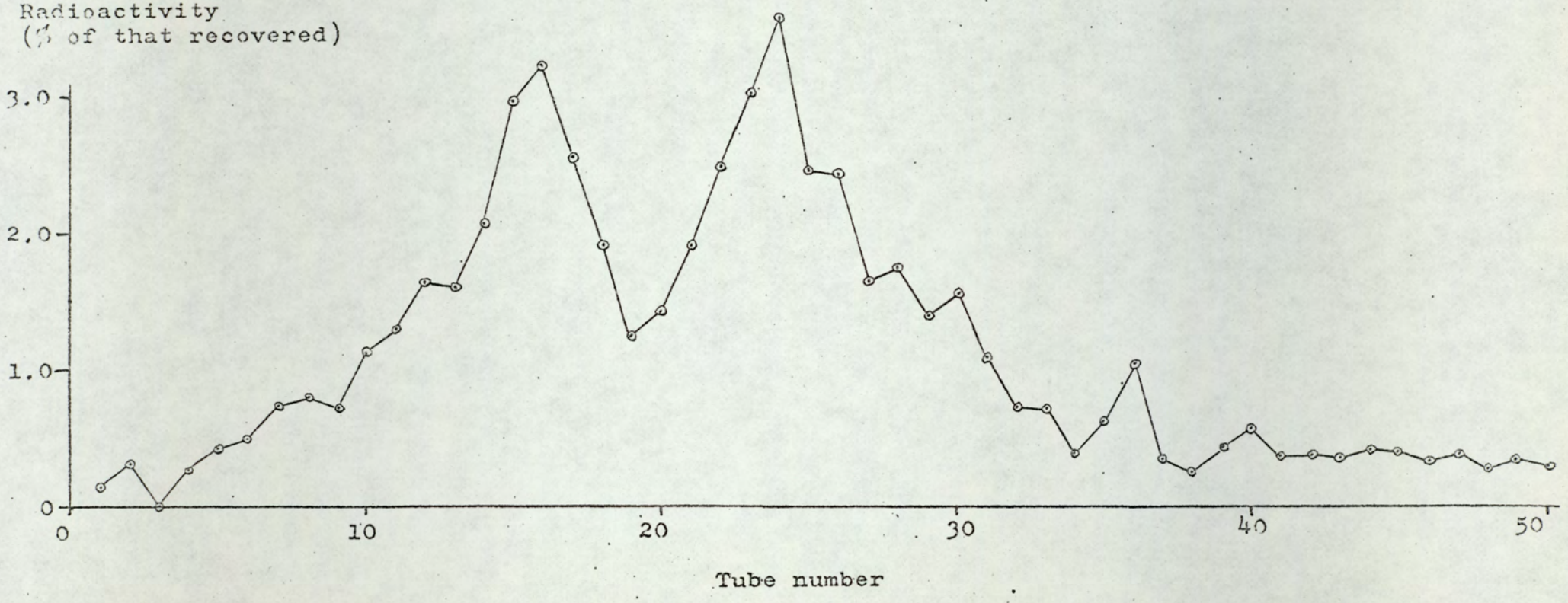


Fig.19 Hepatic radioactivity after oral administration of labelled-PteGlu.

10μCi (≡80μg) of [2-^{14}C]PteGlu were administered to one rat and the liver removed after 6h. Folates were extracted by method (v) and the radioactivity extracted was 70%. The extract was chromatographed on a DEAE-cellulose column (see Methods section), 5ml fractions collected and assayed for radioactivity. The recovery was 140%.

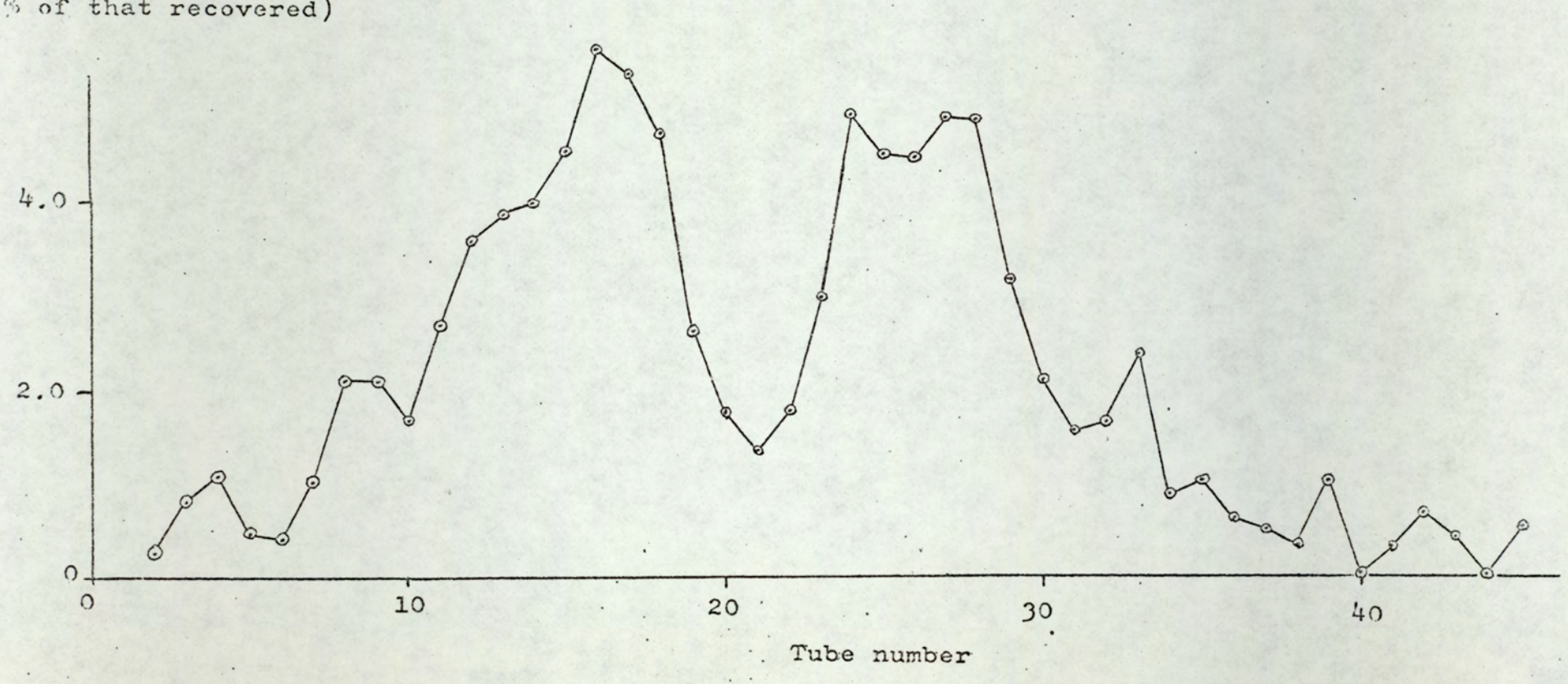


Fig.20 Hepatic radioactivity after oral administration of labelled-PteGlu and -glutamic acids.

320µg (10µCi) of [2-^{14}C]PteGlu/kg and 150µg (1mCi) of 2(n) [^{3}H]-DL-glutamic acid/kg were administered simultaneously to one rat and the liver removed after 24h. The extract was chromatographed on a Sephadex-G15 column. The recovery of carbon-14 and tritium were 57 and 63% respectively.

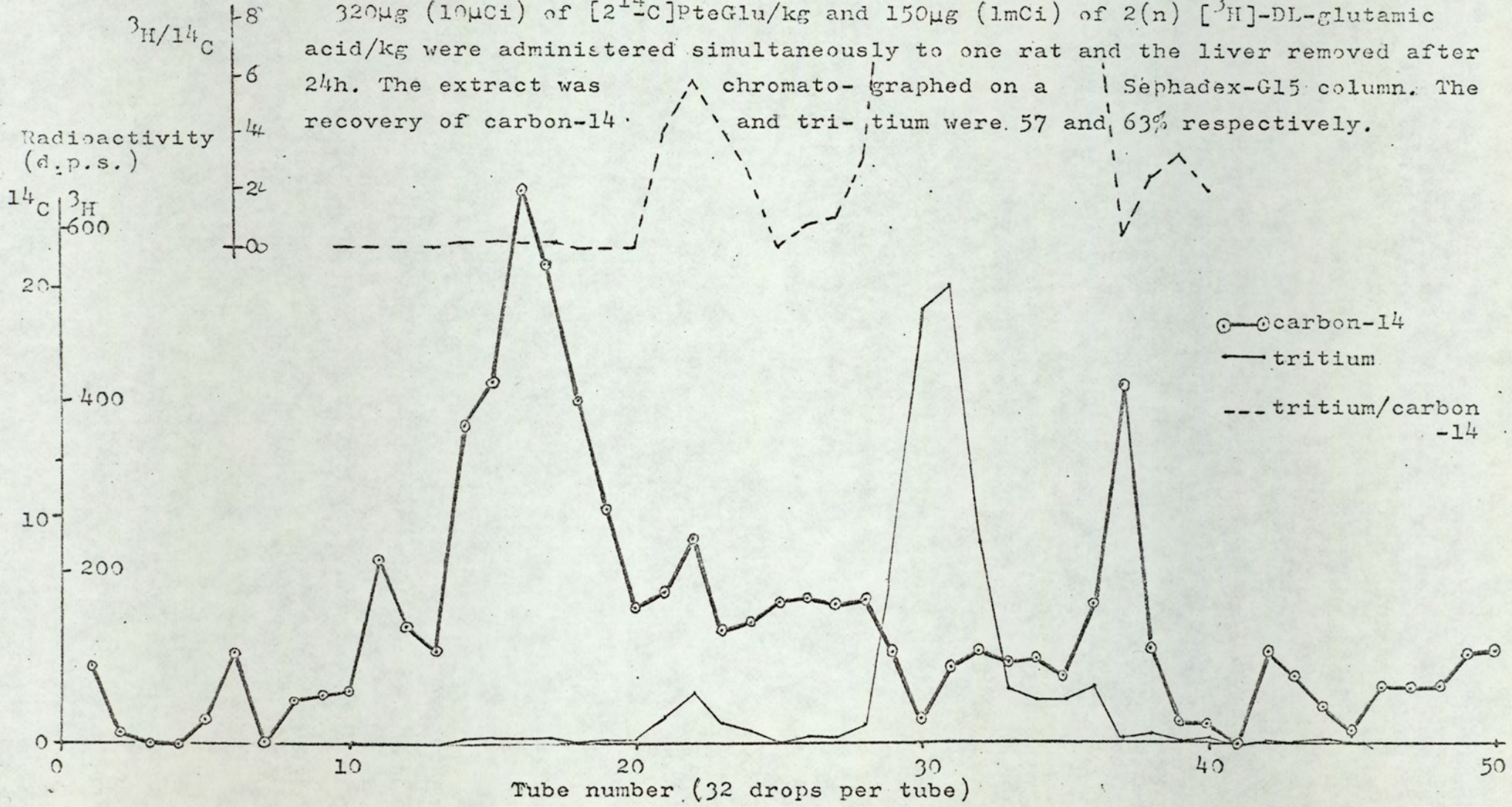


Fig.21 Hepatic radioactivity after oral administration of labelled-PteGlu and -glutamic acids.

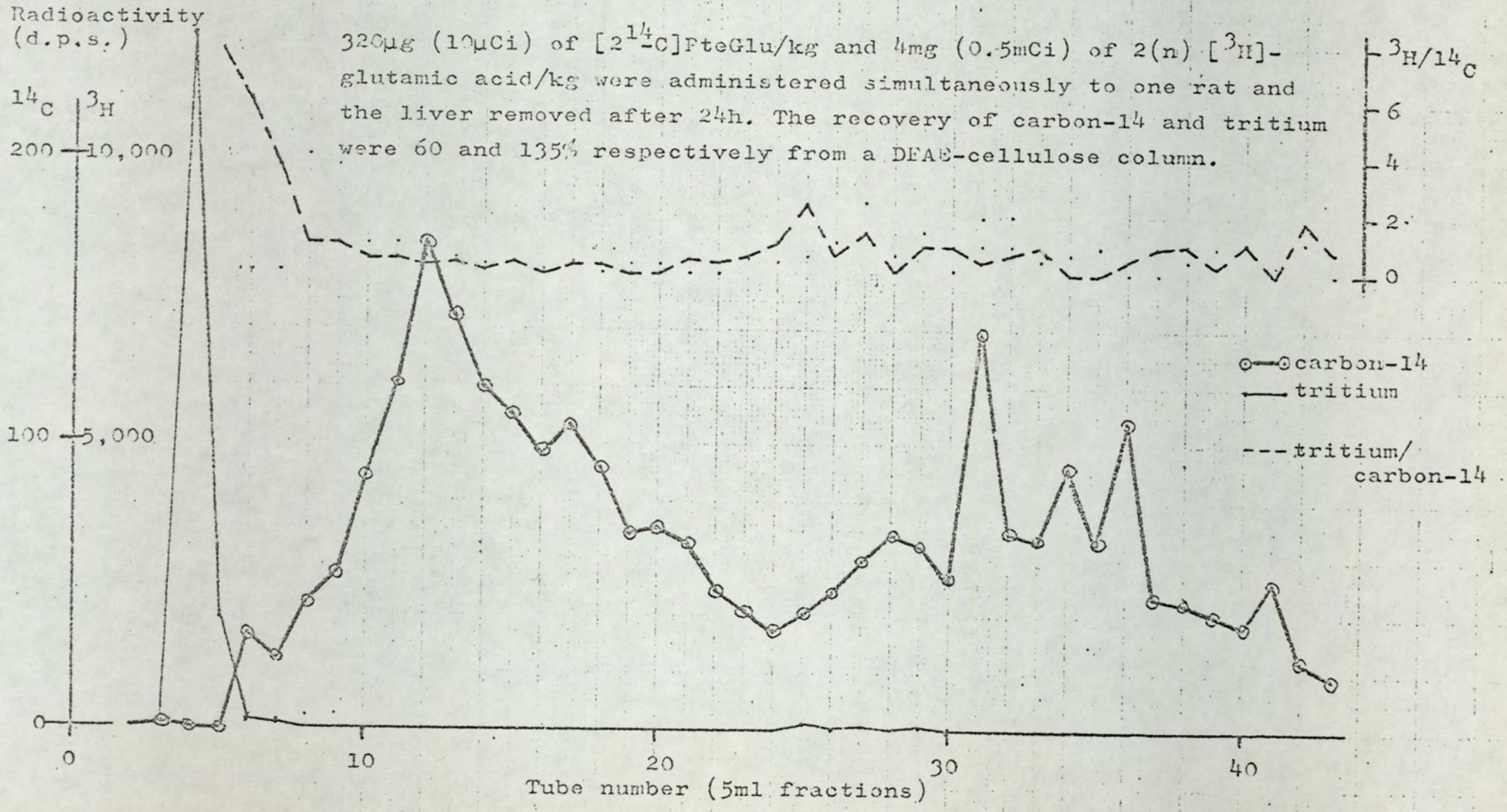


Fig.22 Hepatic radioactivity after oral administration of labelled-PteGlu and -glutamic acids.

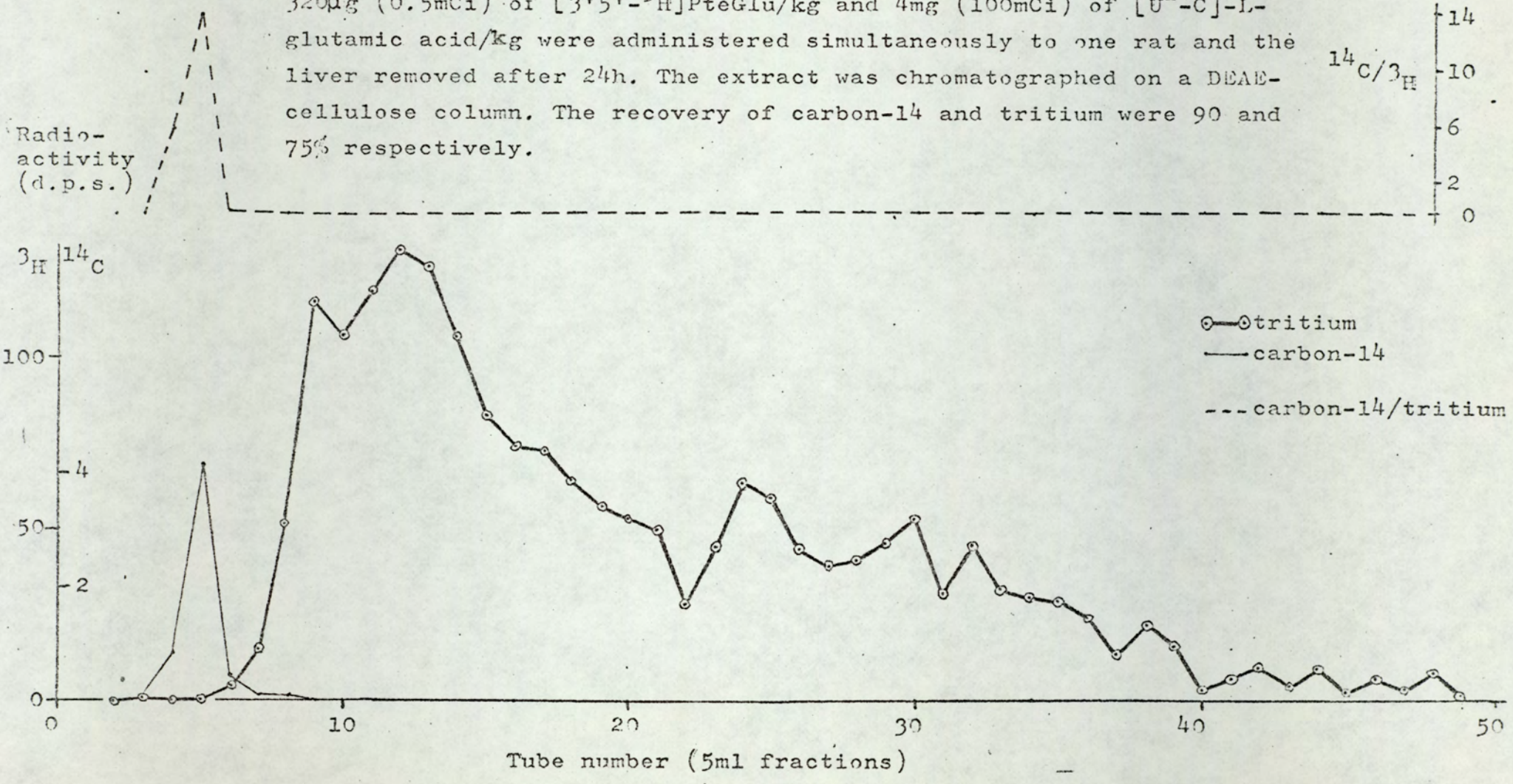


Fig.23 Hepatic radioactivity after oral administration of labelled-PteGlu and -glutamic acid.

320µg (0.5mCi) of [3',5'-^{3}H]PteGlu/kg was administered to one rat 3h before 4mg (50µCi) of [U-^{14}C]-L-glutamic acid and the liver extracted after a further 21h. The extract was chromatographed on a Sephadex-G15 column. The recovery of tritium was 82% and the carbon-14 was too low to be determined accurately.

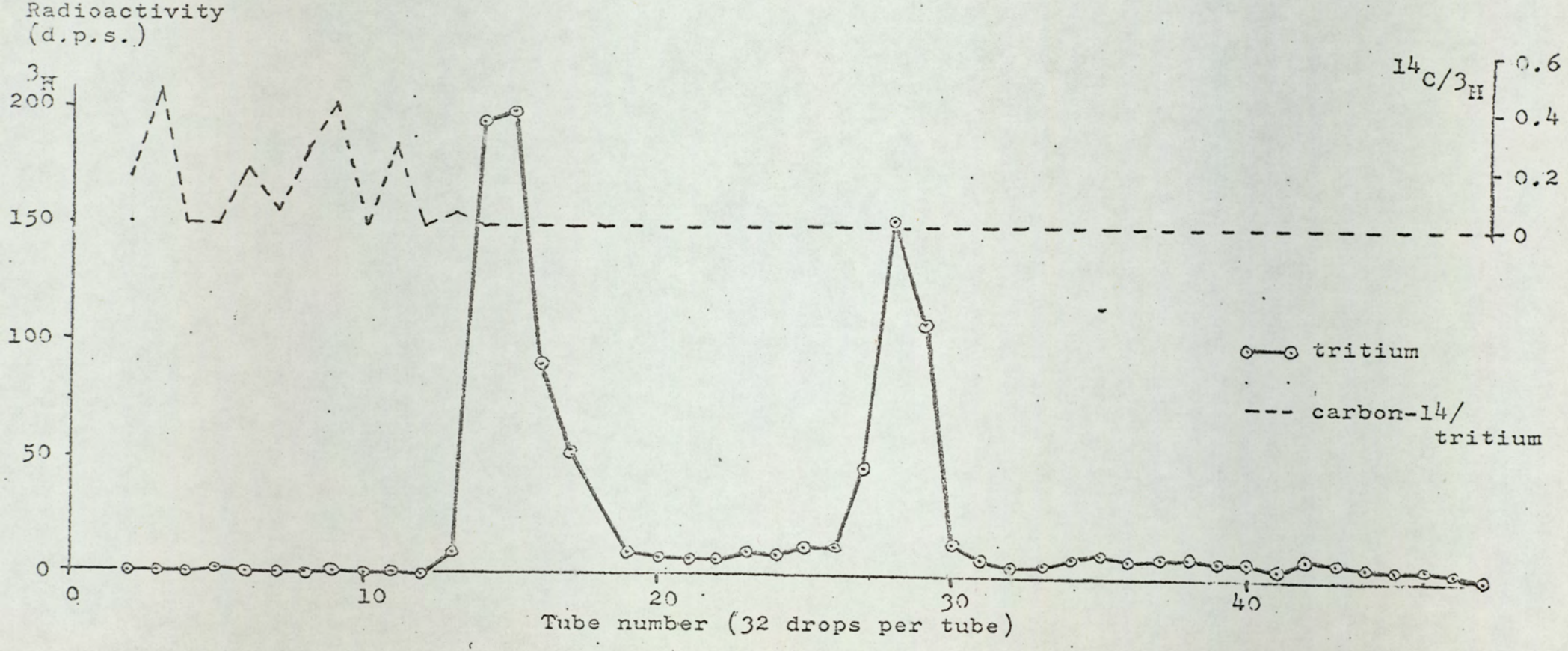


Fig.24 Urinary radioactivity after oral administration of labelled-PteGlu and -glutamic acids.

150µg (1mCi) of tritiated-glutamic acid/kg and 320µg (10µCi) of [2-^{14}C]PteGlu/kg were administered simultaneously to one rat given 1mg of glutamic acid 3 hours previously. The urine was applied directly to a column of Sephadex-G15.

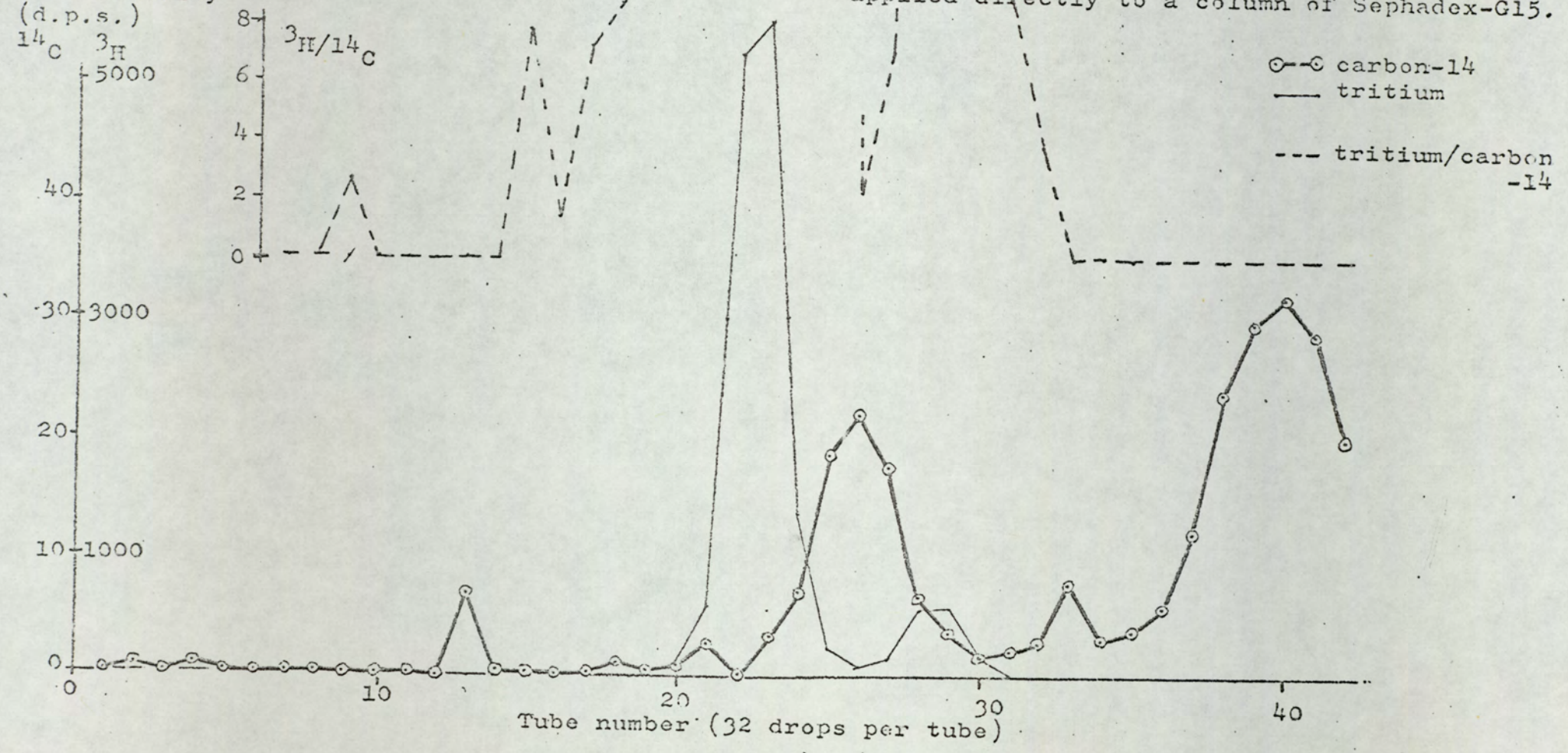


Table I. Response of L.casei, S.faecalis and P.cerevisiae to synthetic folates and pteroic acid.

Compound	L.casei	S.faecalis	P.cerevisiae
pteroic acid*	-	+	-
PteGlu*	+	+	-
10-HCO-PteGlu*	+	+	-
7,8-H_2PteGlu*	+	+	-
5-HCO-H_2PteGlu	+	+	-
10-HCO-H_2PteGlu	+	+	-
H_4PteGlu	+	+	+
5-HCO-H_4PteGlu*	+	+	+
10-HCO-H_4PteGlu	+	+	+
5-CH_3-H_4PteGlu	+	-	-
5-CH_3-H_4PteGlu*	+	-	-
$PteGlu_2$	+	+	-
$PteGlu_3$	+	-	-
$H_4PteGlu_3$	+	-?	+
$PteGlu_7$	-	-	-

Data are a combination of synthetic compounds determined (*) and literature values[8,20,30].
+ indicates a response of at least 50% of the maximum;
- indicates a response less than 5% of the maximum.

Table 2 Transfer of carbon units involving folate coenzymes

Source	Coenzyme	Acceptor	Product	Role
Formate	10-HCO-H_4PteGlu	methionine	formyl-met $_tRNA_F$	protein initiation
		5 amino- ribosyl-4 imidazole carboxamide 5'phosphate	5 formamido derivative	nucleic acid synthesis
Histidine	5-HCNH-H_4PteGlu	2 amino-N ribosyl acetamide 5'phosphate	2 formamido derivative	
		serine	glycine	amino acid synthesis
Glycine	5,10-CH_2-H_4PteGlu	CO_2	glycine	
Serine		UMP	5-CH_2OH - UMP ?	
			thymidylate	pyrimidine synthesis
		CMP	5-CH_2OH - CMP	
		UMP	5-CH_3-UMP	methylation
Methionine	5-CH_3-H_4PteGlu	homocysteine	methionine	amino acid synthesis
$(CH_3)_3S^+$			choline	transmethylation
CO_2			acetate	energy supply*
S-adenosyl-met.			methane	production of methane*
		alkene	cyclopropane derivative	lipid metabolism

* folate role not shown for vertebrates.

Table 3 Constituents of food used in all animal experiments

Food was diet 41B and the values are those published by the suppliers.

	% by weight		
Crude protein	17.069		
Crude oil	2.732	Folic acid	0.38mg/lb.
Fibre	4.352		
Digestible oil	2.114	Vitamin B_{12}	6.4µg/lb.
Digestible fibre	1.723		
Leucine	1.089		
Glycine	0.981		
Lycine	0.877		
Valine	0.837		mg/lb.
Arginine	0.801	Choline	595
Isoleucine	0.710	Inositol	≯ 100
Phenylalanine	0.674	Niacin	25.004
Threonine	0.555	Vitamin E	8.698
Tyrosine	0.362	Pantothenic acid	6.801
Histidine	0.326	Thiamine	2.809
Methionine	0.309	Pyridoxine	2.8
Cystine	0.261	Riboflavin	1.588
Tryptophan	0.192	Biotin	0.41
Calcium	1.3		I.U./lb.
Phosphorus	0.72	Vitamin A	4641
Calcium:phosphorus	1:0.6	Vitamin D_3	1160
Sodium	0.575		
Chloride	0.154		p.p.m.
		Iron	65
		Manganese	32
		Zinc	8.29
		Copper	7
		Iodine	4.18
		Cobalt	0.89

Table 4 10-HCO-PteGlu prepared by a modification of Silverman, Law and Kaufman

U.v. spectral data in 0.1M NaOH

	max (nm)	min (nm)	E_{258} ($\times 10^{-3}$)	$\frac{A_{258}}{A_{367}}$	$\frac{A_{258}}{A_{322}}$
Product	258 367	322	32	4.4	11
lit. ref. 195	258 370	320	40	4.5	13

Fluorescence spectrum in 0.1M NaOH

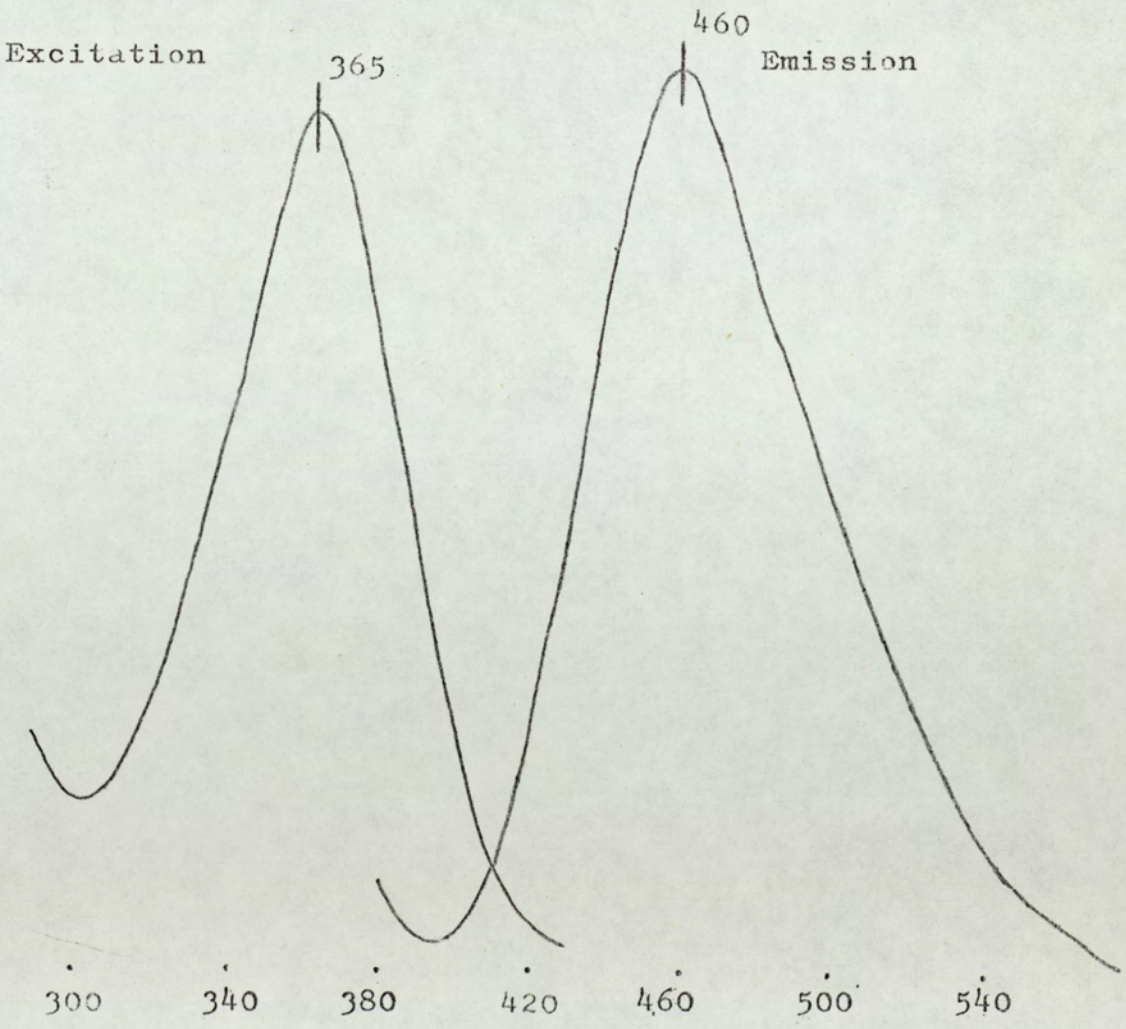


Table 5 Florisil fractionation of PteGlu and 5-CH_2-H_4PteGlu.

Compound	Solvent	Anti-oxidants	radioactivity (% of that recovered) Acid	Neutral	Ammoniacal	Recovery (% of that applied)
[2-^{14}C]-PteGlu	water	absent	16.3	4.5	79.2	97
	urine	absent	14.0	2.3	83.7	84
5[^{14}C]CH_3-H_4PteGlu	water	absent	20.2	5.9	73.9	77
5[^{14}C]CH_3-H_4PteGlu	water	present	26.7	1.2	72.1	90
	urine	present	13.0	3.0	84.0	80

Table 6 Thin-layer chromatography of compounds used in this work

Samples of fluorescing compounds (0.2 to 1μg) and of u.v.-absorbing compounds (2 to 10μg) were applied in 2μl to cellulose plates (0.2mm thick) and developed to 10cm from the origin in solvents containing 0.5% (v/v) 2-mercaptoethanol.

Compound	Characterisation under u.v. light	Rf value Solvent (a)	Solvent (b)	Solvent (c)
2-amino-4-hydroxy-pteridine (Pt)	Blue fluorescence	0.55	0.53	0.43
6 hydroxy-methyl-Pt	Light-blue fluorescence	-	0.48	-
6-formyl-Pt	Light-blue fluorescence	0.65	0.68	0.53
6-carboxylic acid-Pt	Light-blue fluorescence	0.25	0.58	0.12
Xanthopterin	Green fluorescence	0.48	0.61	0.16
Iso-xanthopterin	Light-blue fluorescence	0.38	0.40	0.17
Biopterin	Light-blue fluorescence	0.34	0.63	0.49
PteGlu	U.v.-absorbing	0.00	0.50	0.12
10-HCO-PteGlu	Light-blue fluorescence	0.65	0.92	0.30
H_2PteGlu	Light-blue fluorescence	0.55	0.39	0.30
H_4PteGlu	U.v.-absorbing	0.43	0.78	0.35
5-HCO-H_4PteGlu	U.v.-absorbing	0.69	0.87	0.32
5,10-CH=H_4-PteGlu	White fluorescence	0.38	0.65	Decom-position
5,10-CH_2-H_4-PteGlu	U.v.-absorbing	0.85 (some decom-position)	0.92	0.28
5-CH_3-H_4PteGlu	U.v.-absorbing	0.68	0.85	0.55
p-aminobenzoyl-glutamic acid	Purple fluorescence	0.77	0.94	0.47
Glutamic acid	Purple colour after ninhydrin staining	0.40	-	-

Table 7. Chromatography of pteridines and folates on Sephadex-G15.

Compounds were applied in and eluted with 0.1M ammonium acetate containing 0.2% (w/v) ascorbic acid, pH 7.0. 1.08ml fractions were collected. Flow rate was 7.9ml/h and the column height 41.5cm. Elution volumes (Ve) were the peaks determined from elution profiles.

Compound	Assay technique	Ve (ml)
Casein	u.v. absorption	14.34 (Vo)
Glutamic acid	^{3}H-l.s. counting	19.5
5-CH_3-H_4PteGlu$_3$	u.v. absorption (300nm)	15.0
PteGlu$_3$	u.v. absorption (300nm)	24.0
5-CH_3-H_4PteGlu	^{14}C-l.s. counting	25.0
10-HCO-PteGlu	^{14}C-l.s. counting	31.5
5-HCO-H_4PteGlu	^{14}C-l.s. counting	36.9
2-amino-4-hydroxy-pteridine	Fluorescence (Ext.353; Emm.450)	47.8
PteGlu	^{14}C-l.s. counting	49.2
Pteroic acid	S.faecalis	about 56.5

Fig.7. Relationship between elution from Sephadex-G15 and molecular weight.

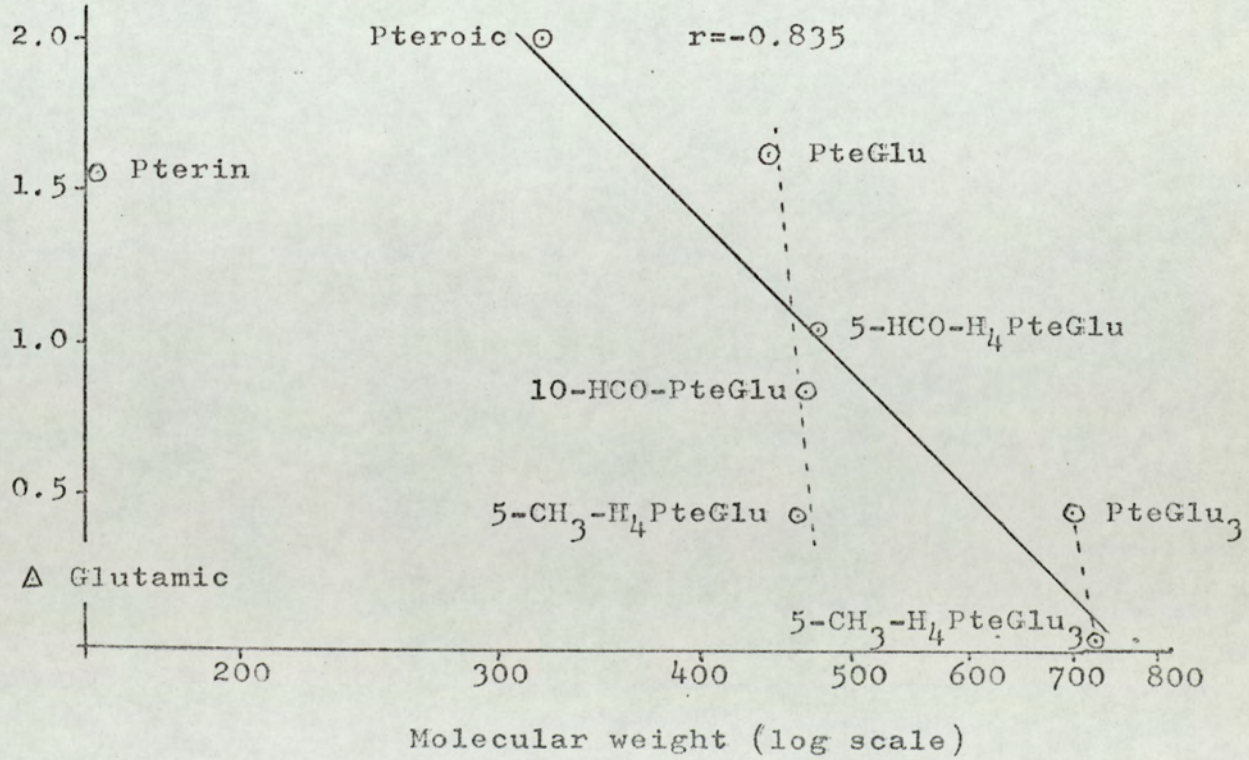


Table 8 Distribution of radioactivity after oral doses of [2-^{14}C]PteGlu.

The radioactivity in the urine (collected every 24h after administration of [2-^{14}C]PteGlu) was assayed directly by liquid scintillation spectrometry. The radioactivity in the liver, kidney and faeces (collected throughout the experiment) was assayed by combustion followed by liquid scintillation counting. The specific radioactivity of the [2-^{14}C]PteGlu was 55.3mCi/mmol. Results are the mean ±S.D. for four animals.

Dose (μg/kg)	Duration of experiment (days)	Radioactivity (% of oral dose)			
		Faeces	Liver	Kidney	Urine
320	7	74.9 ± 33.1	7.3 ± 2.0	0.56 ± 0.12	32.6 ± 4.5
22	6	47.6 ± 4.7	16.1 ± 2.8	1.1 ± 0.3	12.0 ± 3.0
3.2	3	-	14.4 ± 3.7	2.2 ± 0.3	10.9 ± 3.7

Table 9 Distribution of radioactivity after oral doses of 5-CH_3-H_4[2-^{14}C]PteGlu.

The radioactivity in the urine (collected every 24h after administration of 5-CH_3-H_4[2-^{14}C]PteGlu) was assayed directly by liquid scintillation spectrometry. The radioactivity in the liver, kidney and faeces (collected throughout the experiment) was assayed by combustion followed by liquid scintillation counting. The results are expressed as mean ±S.D., no. of animals in parenthesis.

Dose (µg/kg)	Sp. radioactivity of the oral dose (mCi/mmol)	Duration of experiment (days)	Radioactivity (% of oral dose)			
			Faeces	Liver	Kidney	Urine
17.2	18.2	4	63.6 ± 22.5 (4)	7.3 ± 3.6 (4)	-	12.6 ± 3.6 (4)
18.8	14.4	6	55.2 ± 12.0 (6)	9.4 ± 3.6 (6)	2.0 ± 0.4 (5)	22.4 ± 4.3 (6)

Table 10 Urinary excretion after oral doses of 320µg [2-^{14}C]PteGlu/kg.

The results are expressed as the percentage of the administered radioactivity (mean ±S.D.; no. of animals in parenthesis) excreted in the urine. Specific radioactivity of [2-^{14}C]PteGlu was 55.3mCi/mmol.

	Radioactivity excreted in urine			
Design of experiment	1st day	2nd day	3rd day	4th day
Animals starved for two days	7.48 ± 2.19 (2)		1.38 (days 2 to 6)	
Animals starved for three days	11.17 ± 4.04 (2)			
Animals administered PteGlu at time 0 and 24h	33.61 ± 5.07 (5)	21.69 ± 9.91 (5)	1.84 ± 0.80 (5)	1.02 ± 0.20 (5)

Table 11 Urinary excretion of radioactivity after an oral dose of [2^{14}-C]PteGlu.

The results are expressed as the percentage of the administered radioactivity (mean ±S.D.; no. of animals in parenthesis) excreted in the urine after oral doses of [2^{14}-C]PteGlu, specific radioactivities were 31.4 and 50.3mCi/mmol. for doses of 40 and 56µg/kg respectively and 55.3mCi/mmol. for other doses.

Dose (µg/kg)	0-24h (1st day)	24-48h (2nd day)	48-72h (3rd day)
320	30.7 ± 6.8 (14)	1.12 ± 0.18 (4)	0.64 ± 0.29 (4)
56	3.74 ± 2.16 (15)	1.72 ± 1.14 (8)	0.34 ± 0.10 (4)
40	8.14 ± 6.03 (34)	1.86 ± 1.33 (26)	0.65 ± 8.4 (14)
32	7.95 ± 5.67 (12)	1.95 ± 0.83 (8)	1.05 ± 0.77 (8)
3.12	6.83 ± 2.91 (12)	2.66 ± 0.78 (4)	-

Table 12 Displacement of radioactivity by flushing doses.

Flushing doses of PteGlu were given by dissolving the PteGlu in a minimum volume of aq. 1% (v/v) NH_3, diluting it with water (final pH 7-8) and administering in 0.5ml by stomach intubation. Flushing dose of 5-CH_3-H_4PteGlu (Ca salt) was given in water. The results are expressed as the percentage of the administered radioactivity (mean ±S.D. of four animals) excreted in the 24h before and in the days after an oral flushing dose of unlabelled PteGlu. The specific radioactivity of the [2^{14}-C]PteGlu was 55.3mCi/mmol.

Dose of [2^{14}-C]-PteGlu (µg/kg)	Time (h) of the flushing dose after the radioactive dose	Flushing dose (µg of PteGlu/kg)	% of the administered radioactivity appearing in the urine			
				Days after flushing dose		
			24h before	1	2	3
32.0	72	200	1.72 ± 0.23	2.07 ± 0.99	1.02 ± 1.14	0.62 ± 0.09
35.2	72	400	0.39 ± 0.40	1.00 ± 0.20	0.68 ± 0.12	0.33 ± 0.14
32.0	24	400	12.1 ± 8.3	2.93 ± 0.56	1.35 ± 0.55	1.21 ± 0.51
3.95	24	400	6.87 ± 4.40	4.71 ± 1.30	-	-
2.21	24	400	7.25 ± 6.29	8.46 ± 3.00	-	-
21.8	24	1600	4.27 ± 2.23	2.44 ± 0.87	1.22 ± 0.27	-
44.3	3.5	1600	=	16.5 ± 0.8	1.12 ± 0.28	1.12 ± 0.24
43.6	Preloading dose of 1600µg of PteGlu/kg given 3.5h before the labelled dose		=	20.2 ± 4.6	0.79 ± 0.30	0.68 ± 0.42
3.2	48	524µg 5-CH_3-H_4PteGlu/kg	2.66 ± 0.78	1.87 ± 0.64	-	-

Table 13 Urinary excretion of radioactivity after an oral dose of 5-CH_3-H_4[2-^{14}C]PteGlu.

5-CH_3-H_4[2-^{14}C]PteGlu was administered in a solution of ascorbic acid (pH 6.0) and the percentage (mean ±S.D., no. of animals in parenthesis) of the oral radioactivity measured directly by liquid scintillation counting.

Dose (μg/kg)	Ascorbate solution (%)	Sp. radioactivity of oral dose (mCi/mmol)	Precentage excreted in urine		
			0-24h (1st day)	24-48h (2nd day)	48-72h (3rd day)
0.24	0.2	23.0	6.85 ± 3.65 (4)	-	-
11.5	0.2	23.0	8.69 ± 4.91 (4)	3.25 ± 1.47 (4)	2.32 ± 0.82 (4)
17.2	0.2	18.2	6.70 ± 2.48 (4)	2.71 ± 0.87 (4)	-
18.8	0.2	14.4	11.83 ± 3.86 (8)	1.76 ± 0.53 (3)	1.92 ± 1.57 (3)
13.4	1.0	10.6	18.4 (one animal)		
19.0	4.0	10.6	28.8 (one animal)		
84.0	0.2	18.2	13.3	0.40	0.61 (one animal)

Table 14 Distribution of radioactivity in compounds excreted after [2^{14}-C]PteGlu and separated on t.l.c.

After an oral dose of [2^{14}-C]PteGlu (sp. radioactivity 55.3mCi/mmol) the urine was purified by column chromatography and t.l.c. in solvent (c). The percentage of radioactivity on the chromatograms, present as the unidentified compound and as 5-CH_3-H_4[2^{14}-C]PteGlu, were estimated by densitometry of radioautographs. The specific radioactivity of the compounds was determined by a combination of liquid scintillation counting and L.casei assay on material eluted from thin-layer chromatograms run in solvents (c), (a) and then (d), and are recorded, where possible, as the mean ±S.D., no. of experiments in parenthesis using four animals in each experiment.

Dose (µg/kg)	Day	Radioactivity (%) present as		Sp. radioactivity of the unidentified compound (mCi/mmol as PteGlu)	Sp. radioactivity of 5-CH_3-H_4[2^{14}-C]PteGlu (mCi/mmol)
		unidentified compound	5-CH_3-H_4PteGlu		
2.21	1	60	22	0.38	0.097
3.95	1	24	40	0.25	0.043
21.8	1	23	14	-	0.36
32.0	1	43	29	0.86	1.06 ± 0.21 (2)
	2	83	14	0.96	0.032
	3	67	9	-	-
320	1	61	25	44.0 ± 8.92 (2)	16.45 ± 5.14 (4)

Table 15. Microbiological response to 5-CH_3-H_4PteGlu and the unidentified material excreted after oral [2-^{14}C]PteGlu.

The labelled materials were chromatographed in solvents (a), (c) and then (d) and were free from fluorescence or absorption when viewed on t.l.c. under u.v. light. Microbiological assays (see Methods section) were carried out on material isolated from at least four experiments (four rats each) and the results are the relative growth.

Compound	L.casei	S.faecalis	P.cerevisiae
5-CH_3-H_4PteGlu	100	4 to 33 (mean 12)	2.5
unidentified	100	13 to 38 (mean 25)	2.5

Table 16 Distribution of radioactivity in materials excreted in the 24 hours after oral doses of 320µg [2^{14}-C]PteGlu/kg.

After an oral dose of [2^{14}-C]PteGlu (sp. radioactivity 55.3mCi/mmol) the urine was purified by column chromatography and t.l.c. in solvent (c). The percentage of radioactivity in materials separated on chromatograms were estimated by densitometry of radioautographs. The specific radioactivity of the unidentified compound and 5-CH_3-H_4[2^{14}-C]PteGlu excreted was determined by a combination of liquid scintillation counting and L.casei assay on material eluted from thin-layer chromatograms run in solvent (c), (a) and then (d).

Design of experiment	Radioactivity (%) present at Rf			Sp. radioactivity	
	0.06	0.24	0.39	compound at Rf 0.06, (mCi/mmol of PteGlu)	5-CH_3-H_4[2^{14}-C]PteGlu Rf 0.39, (mCi/mmol)
Animals starved for two days	40	20	35	-	28.9
Animals starved for three days	60	10	20	33.3	31.2
Animals administered PteGlu at time 0 and 24h	50	8	30	-	10.6

Table 17 Distribution of radioactivity in materials excreted in the 24 hours following flushing doses and separated by t.l.c.

After a time interval following an oral dose of [2^{14}-C]PteGlu (55.3mCi/mmol) a flushing dose of PteGlu was given and the urine purified by column chromatography and t.l.c. in solvent (c). The percentage of radioactivity in materials separated on the chromatograms was estimated by densitometry of radioautographs. The specific radioactivity of the materials excreted was determined by a combination of liquid scintillation counting and L.casei assays on materials eluted from thin-layer chromatograms run in solvent (c) and then (a).

Dose (µg/kg)	Time (h) interval	Flushing dose (µg of PteGlu/kg)	Radioactivity(%) present at Rf 0.08	0.14 (PteGlu)	0.4 (5-CH_3-H_4PteGlu)	Sp. radioactivity of 5-CH_3-H_4[2^{14}-C]PteGlu (mCi/mmol)
32.0	72	200	30	none	12	-
35.2	72	400	60	none	30	0.008
32.0	24	400	59	none	26	0.013
3.95	24	400	62*	none	16	0.01
2.21	24	400	not determined			0.02
21.8	24	1600	60	-	10	0.003
44.3	3.5	1600	-**	45	40	-
43.6	1600µg of PteGlu/kg given 3.5h before the labelled dose		-**	49	30	-
3.2	48	524µg 5-CH_3-H_4PteGlu/kg	10	none	25	0.02

*Sp. radioactivity was 0.008mCi/mmol of PteGlu

**difficult to determine because of the possible overlap if bands were present at both Rf 0.08& 0.14

Table 18 Distribution of radioactivity in compounds excreted in the 24 hours following 5-CH_3-H_4[2-^{14}C]PteGlu and separated by t.l.c.

After an oral dose of 5-CH_3-H_4[2-^{14}C]PteGlu the urine was purified by column chromatography and t.l.c. in solvent (c). The percentage of radioactivity in materials separated on the chromatograms were estimated by densitometry of radioautographs. The specific radioactivity of 5-CH_3-H_4-[2-^{14}C]PteGlu excreted was determined by a combination of liquid scintillation counting and L.casei assay on material eluted from thin-layer chromatograms run in solvent (c), (a) and then (d).

Dose (µg/kg)	Ascorbate solution (%)	Radioactivity (%) present at Rf value			Sp.radioactivity of 5-CH_3-H_4[2-^{14}C]PteGlu (mCi/mmol)	
		0.08	0.25	0.4 5-CH_3-H_4-[2-^{14}C]PteGlu	administered	excreted
0.24	0.2	28*	7*	62*	23.0	-
11.5	0.2	none	38	62	23.0	9.2
17.2	0.2	7	28	58	18.2	-
18.8	0.2	none	40	45	14.4	1.8
84	0.2	5	15	80	18.2	-
13.4	1.0	15	65	20	10.6	-
19.0	4.0	52	13	28	10.6	-

* determined by elution and liquid scintillation counting (Fig.12).

Table 19 T.l.c. of aqueous extracts of livers removed after oral administration of PteGlu to rats

Rats were given 80μg of [2-^{14}C]PteGlu (55.3mCi/mmol), sacrificed at times up to 10 days after the administration and radioactivity extracted from livers. The recovery of radioactivity is the radioactivity in the aqueous extract expressed as a percentage of the total liver radioactivity (determined by combustion). Extracts were brought to pH 1.0 with conc-HCl, purified through a florisil column and the ammoniacal fraction further purified by t.l.c. on cellulose developed in solvent (c). The values in parenthesis are the percentages of the total radioactivity on the chromatogram and were estimated by densitometry of radioautographs.

Method	Time between administration and extraction	Recovery(%) of radioactivity	Rf values of labelled materials								
(i)	24h	-	.04(42)	.18(1)	.26(5)	.38(28)	.52(4)	.63(8)	.73(8)	.80(4)	
(ii)	24h	77	.08(57)		.25(4)	.29(5)	.46(19)		.67(6)	.82(10)	
(iii)	24h	51	.08(64)	.22(6)		.28(8)	.42(16)		.65(6)		
(iv)	24h	60*	.06(69)		.26(8)	.38(23)					
(v)	6h	70	.05(85)				.50(10)	.60(5)			
	24h	89	.04(80)	.22(3)			.50(16)			.85(1)	
	10 days	78	.04(74)	.20(5)		.40(6)	.50(15)				
	24h, the animal previously starved for 3 days	83	.05(84)	.20(5)		.40(11)					
	6days, the animal previously starved for 3 days	69	.04(89)			.38(8)	.55(3)				

*with the addition of 2mg of PteGlu to the solution before extracting, the recovery was 84%.

Table 20 Chromatography of synthetic folates on DEAE-cellulose column.

Data are taken from Silverman et al.[113], Wittenberg et al.[243] and Bird et al.[121] using columns made and eluted in a similar way to those used here (see Methods section).

Compound	Tube Number
10-HCO-H_4PteGlu	8
10-HCO-H_2PteGlu	9
10-HCO-PteGlu	10
5-HCO-H_4PteGlu	12
5-CH_3-H_4PteGlu	13
H_4PteGlu	15
10-HCO-H_4PteGlu$_3$	
5-HCO-H_4PteGlu$_3$	23
5-CH_3-H_4PteGlu$_3$	
H_2PteGlu	26
PteGlu	29
PteGlu$_3$	38

Table 21 L.casei response to hepatic folates separated by ion-exchange chromatography and treated with conjugase.

80µg of [2^{14}-C]PteGlu (125µCi/mg) were administered to one rat previously starved for 3 days and the liver removed after a further 24h. Radioactivity was extracted, chromatographed on a DEAE-cellulose column and fractions assayed for radioactivity (Fig.15). The amount of folate in selected tubes was then determined by the growth of L.casei before and after treatment of the fractions with chicken pancreas conjugase. The sp. radioactivities of folates were then calculated.

Tube number	Sp. radioactivity (µCi/mg of PteGlu)	
	before conjugase treatment	after conjugase treatment
4	8.4	2.9
6	>5.6	3.1
8	>8.4	5.8
11	>17.6	11.9
13	18.1	-
15	37.0	14.6
18	>76.0	6.4
22	>31.6	7.3
27	>90.0	9.4
29	>63.2	7.4
38	>20.4	8.3
43	>5.6	10.2
46	>16.0	2.8
48	>7.2	8.0

Table 22 Distribution of tritium and carbon-14 in urinary materials

Urine was collected in acid with antioxidants for 24h after administering labelled-PteGlu and -glutamic acid. The urine was purified through a florisil and the ammoniacal fraction further purified by t.l.c. in solvent (c). Radioactivity was assayed in aqueous solution by liquid scintillation counting.

	Florisil fractionation			T.l.c. purification		
	Distribution (% of that recovered*) of			labelling as glu/labelling as PteGlu at Rf		
Experiment	fraction	tritium	carbon-14	0.08	0.23	0.38
1mCi of [^{3}H]-glu (4mg/kg)	HCl	89	28			
and 10μCi of [2-^{14}C]PteGlu	H_2O	11	7			
(320μg/kg) administered together	NH_3	<1	65	0.0	0.5	0.9
[^{14}C]-glu (4mg/kg) and [^{3}H]-glu (320μg/kg) administered together	-			0.00	0.2	0.4

*the recovery of tritium was 72% and of carbon-14 was 81% of that applied.

A P P E N D I X I

DETECTION AND ASSAY OF RADIOACTIVITY

LIQUID SCINTILLATION SPECTROMETRY.

The high counting efficiency of scintillation spectrometry has led to its almost universal use in the detection of weak β emitters such as carbon-14 and tritium. The difficulties which may arise in determining these isotopes, particularly in biological systems, are the low solubility of aqueous solutions in organic scintillators and quenching effects of coloured solutions. The following is an account of the counting procedures used on such solutions isolated during work for this thesis and on the available instrumentation.

Experimental

Labelled samples. Urines collected in acid (see the Experimental section) were deep brown and contained a non-radioactive dark brown/black sediment which was removed by centrifuging the urine and decanting the supernatant. Urines collected in alkali (see the Experimental section) were pale amber. Aqueous solutions, including extracts from t.l.c. plates, varied from colourless to deep brown. The prescence of a fluorescent indicator in the cellulose had no effect on the counting rate. Tissue and faecal samples were assayed after combustion, see ref.(i). All these samples gave colourless solutions with similar counting efficiencies and will not be dealt with further.

Scintillator. Toluene scintillator, ref.(ii), and triton-X100 scintillator, ref.(iii), have been successfully used for counting of aqueous solutions, but work in this laboratory (Dransfield,E. and Beavon,J.R.G., unpublished results) has shown those scintillators unsuited for coloured samples with low count rates, either because

of low counting efficiency, prolonged chemiluminescence, or both. The scintillator used was NE220 (Nuclear Enterprises, Edinburgh, U.K.).

Method

10ml. of NE220 scintillator were placed in a quartz vial and a weighed amount (approx. 0.05ml.) of [1-^{14}C]-hexadecane was added. The vial was placed in an automatic liquid scintillation spectrometer (Nuclear Enterprises) operating at 0°C. The gain was set at 25 and the sample counted in a single channel set on integral mode with the lower discriminator set at 0.8V. The counting efficiency (c.p.m./d.p.m.) was calculated from the count rate. A pulse height spectrum was determined using a 400 channel analysor (Laben Spectroscope, model 400; Laben, Milan, Italy.) set at 20mV/channel. The counting efficiency and pulse height spectra were then similarly determined for successive additions of urine. The experiment was repeated with higher gain settings and also with scintillator containing both [1-^{14}C] hexadecane and [1,2-^{3}H] hexadecane. Sample counts were at least 5000 above background and efficiencies were determined by internal standardisation (adding the tritium first for doubly-labelled samples) using hexadecane standards (15 to 40mg), diluted if necessary, to give a count rate about 100 times higher than that of the sample.

Results and Discussion

Fig.IA-1 shows the pulse height (amplitude) spectrum of carbon-14 alone ("unquenched") and after the addition of alkaline and acidic urines to the scintillator. The area under the spectrum (up to 10v) is proportional to the counting efficiency.

The effect of adding alkaline urine was to shift the spectrum slightly towards lower amplitude, showing that the addition of alkaline urine produces only a small quenching (chemical and colour) effect. Clear homogeneous solutions in NE220 were obtained with up to 0.5ml of alkaline urine. The counting efficiency (at gain 50, lower discriminator setting 0.8V), fell from 77.4% with 0.09ml to 73.0% with 0.21ml. Further additions lowered the counting efficiency and above 0.4ml, spurious counting was observed. Alkaline urine samples were therefore routinely counted with gain 25 assaying 0.25ml. Under these conditions the counting efficiency for carbon-14 was 75% with a background of 30c.p.m., giving a "figure of merit,F" of 0.19 and hence, for a counting time of 1min., the minimum activity which could be detected with a probability of 95%, A_{min}, was 15d.p.m.

Under similar conditions acidic urine produced a much greater quenching than did alkaline urine, and this quenching was accompanied by an increased no. of pulses at low amplitude, characteristic of fluorescence, see Fig.IA-1. The pulse height spectra performed at higher gain (Fig.IA-2), showed a marked increase in quenching and fluorescence with the addition of up to 0.08ml. of urine and then little change up to 0.32ml. of urine. At gain 50, the counting efficiency for carbon-14 was 60.3% and, with successive additions (0.02ml.) of urine up to 0.08ml., increased to 83.6% due to quenching of the amplified spectrum to within the photomultiplier range. Further additions, up to 0.32ml of acidic urine, caused only a slight drop in counting efficiency. Still further urine caused extensive precipitation and eventually two heterogeneous

solutions. Acidic urines were therefore routinely counted with gain 60 assaying 0.25ml of urine. The efficiency was 85% with a background of 120c.p.m., giving F=0.006 and A_{min}=25d.p.m.

Colourless aqueous solutions produced only slight quenching (chemical) effects and were counted with the gain set at 25 assaying up to 1ml. in 10ml. scintillator.

The pulse height spectrum of tritium overlaps that of carbon-14 up to 4V. at gain 100 (Fig.IA-3). Addition of acidic urine caused quenching, fluorescence and a greater overlap of the spectra. Since the samples varied in their extent of quenching and fluorescence, radioactivity of doubly-labelled samples was determined by the simultaneous equation method, see ref.(iv), although this method gives more variable results than other methods, see ref.(v). In an attempt to obtain max. channel width with efficient counting of both isotopes in two channels, the channel widths (at gain 100) were 1.0V to 2.3V; difference mode (channel 1), and 2.3V; integral mode (channel 2). For acidic urine samples, the counting efficiencies in channels 1 and 2 for tritium were approx. 14 and 8% and for carbon-14 were approx. 16 and 69% respectively. The maximum accuracy was obtained with the $^{3}H/^{14}C$ ratio 10, see refs.(iv), (v) and (vi).

DETECTION AND LOCATION OF RADIOACTIVITY ON THIN LAYER CHROMATOGRAMS.

Several methods exist for the radioassay of t.l.c.; namely (a) external scanning, refs.(vii) to (xii);(b) radioautography, refs.(xiii) to (xvi); (c) elution followed by detection of radioactivity by G-M, ionisation or scintillation counting and (d) direct counting of absorbent refs.(xvii) to (xix).

Direct counting of absorbent requires the removal of reproducible scrapings by mechanical equipment and counting corrections must be made after evaluating self absorption losses, on absorbent particles.

Elution (with water) followed by liquid scintillation counting (in NE220) was used for extremely low radioactivity (1mμCi applied as a streak 20cm long using an SA20 streak applicator, Burkard, Rickmansworth, Herts. U.K.). The method was accurate to ±10%, but care must be taken to ensure uniformity of samples of absorbent and in extraction of radioactivity. The method relies on the quantitative elution of compounds but polar compounds can be exceedingly difficult to remove quantitatively,(ref xix).

External scanning was done with a proportional gas flow (methane/argon; 1/10) counter (Desaga t.l.c. scanner 12-2 supplied by Camlab, Cambridge, U.K.) coupled to a rate meter and chart recorder. T.l.c. plates were scanned (300mm/h) using a 'windowless window', the rate meter was set at time constant 10 and the chart drive matched to the t.l.c. scanning speed. The min. detection was 0.45mμCi (see Fig.IA-4, upper trace) scanning a solution of

2[^{14}C]PteGlu applied, in 1µl, to a t.l.c. plate (0.25mm thick, cellulose MN300uv; Macherey Nagel and Co., Düren, Germany.). The accuracy, determined by removal and weighing the area under the peaks, was ±20% and is thus less sensitive and less accurate than either direct counting of absorbent or elution.

A disadvantage common to direct counting of absorbent, elution and external scanning, however, is the inability of these methods to show the shape of the radioactivity since only an average value of radioactivity over an area of the chromatogram is recorded. This disadvantage is overcome by radioautography.

Radioautography was performed on standards of carbon-14 applied to a t.l.c. plate (see external scanning) by placing Kodak AR10 and AR50 stripping film (Kodak, Lichfield, U.K.) and X-ray film (Ilford Industrial Co; Ilford, Essex, U.K.), over the chromatogram. The films were held in position by a further glass plate and elastic bands. The films were exposed at -15°C for 24h and then developed in Kodak DX80, washed with 3% (v/v) acetic acid and fixed with Kodak FX40. Min detection with AR50 was >50mµCi; with AR10 was 0.5mµCi and with X-ray film was 0.25mµCi. The min detection with X-ray film was as recorded by Frey and Frey, ref (xiv). The blackening on AR10 stripping film was distorted due to elongation of the film, whilst X-ray film gave a reproducible record. The relative amounts amounts of radioactivity exposed to X-ray film was determined by scanning the radioautograph with a microdensitometer (Mk IIIc; Joyce, Lobel and Co. Ltd., Gateshead, Co. Durham, U.K.), and was accurate to ± 20% (Fig.IA-4, lower trace).

During drying of the spots evaporation takes place on the circumference of the spot. When the spot is completely dry a ring of radioactivity is formed around a circle of lower radioactivity. The radioautograph shows this and the densitometer scan therefore shows a double peak (Fig.IA-4, lower trace). Concurrent with the outward movement of solution during drying there is also a movement of solution towards the surface of the t.l.c. absorbent, refs.(xx) and (xxi), and thus detection on thicker plates will be the same as for thinner plates. The sensitivity of detection may be improved by spraying the chromatogram with a phosphor. However, phosphors present either as a result of spraying or direct liquid scintillation of paper chromatograms, cause difficulties in the selective elution of phosphor or labelled compounds, see ref.(xxii). Incorporation of phosphor into chromatograms may also cause contamination and reduce the recovery of labelled materials, and was therefore not used.

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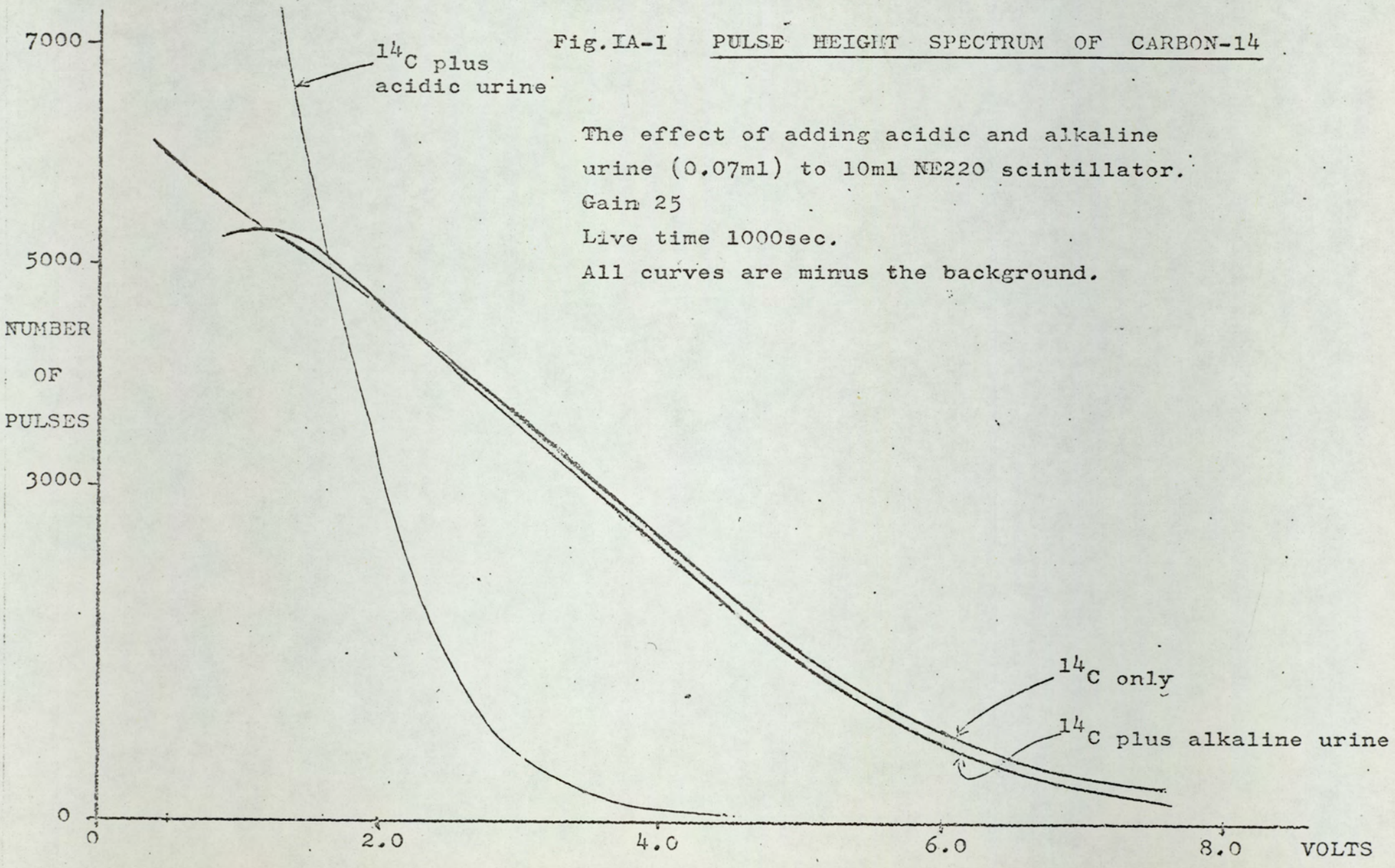
Fig.IA-1 PULSE HEIGHT SPECTRUM OF CARBON-14



The effect of adding acidic and alkaline urine (0.07ml) to 10ml NE220 scintillator.
Gain 25
Live time 1000sec.
All curves are minus the background.

Fig.IA-2 PULSE HEIGHT SPECTRUM OF CARBON-14

The effect of adding acidic urine to 10ml of NE220 scintillator.

Gain 70

Live time 1000sec.

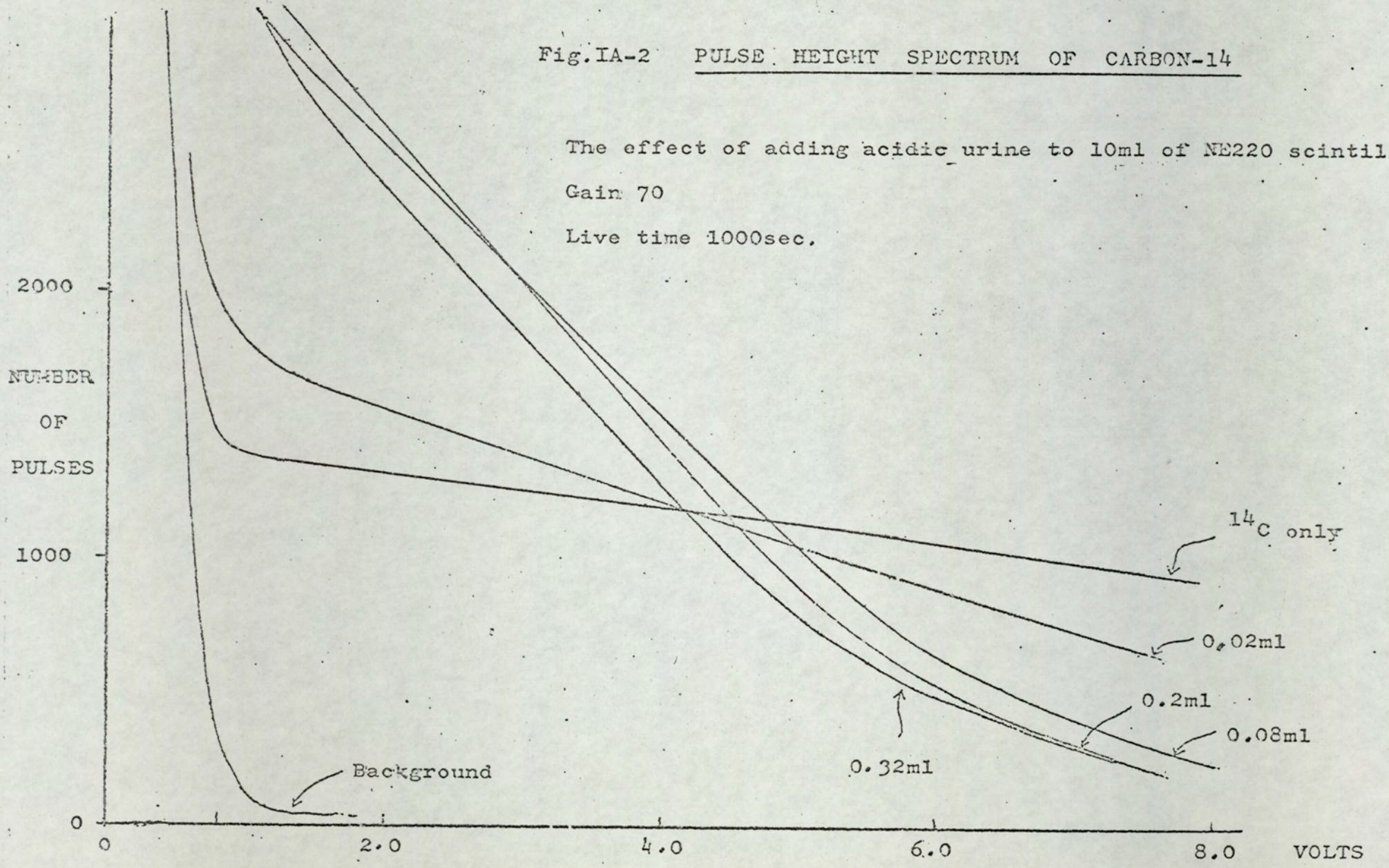


Fig.IA-3 PULSE HEIGHT SPECTRA OF TRITIUM AND CARBON-14

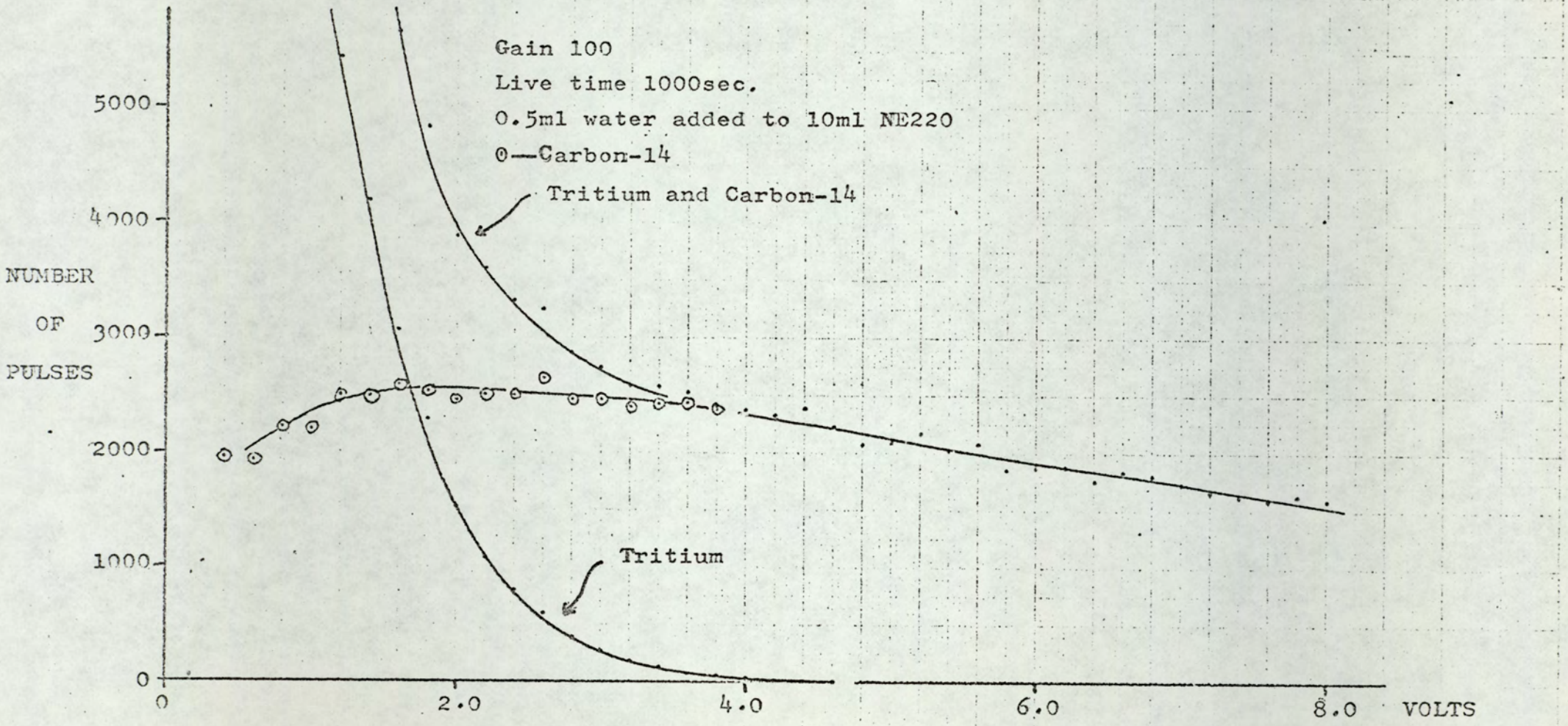
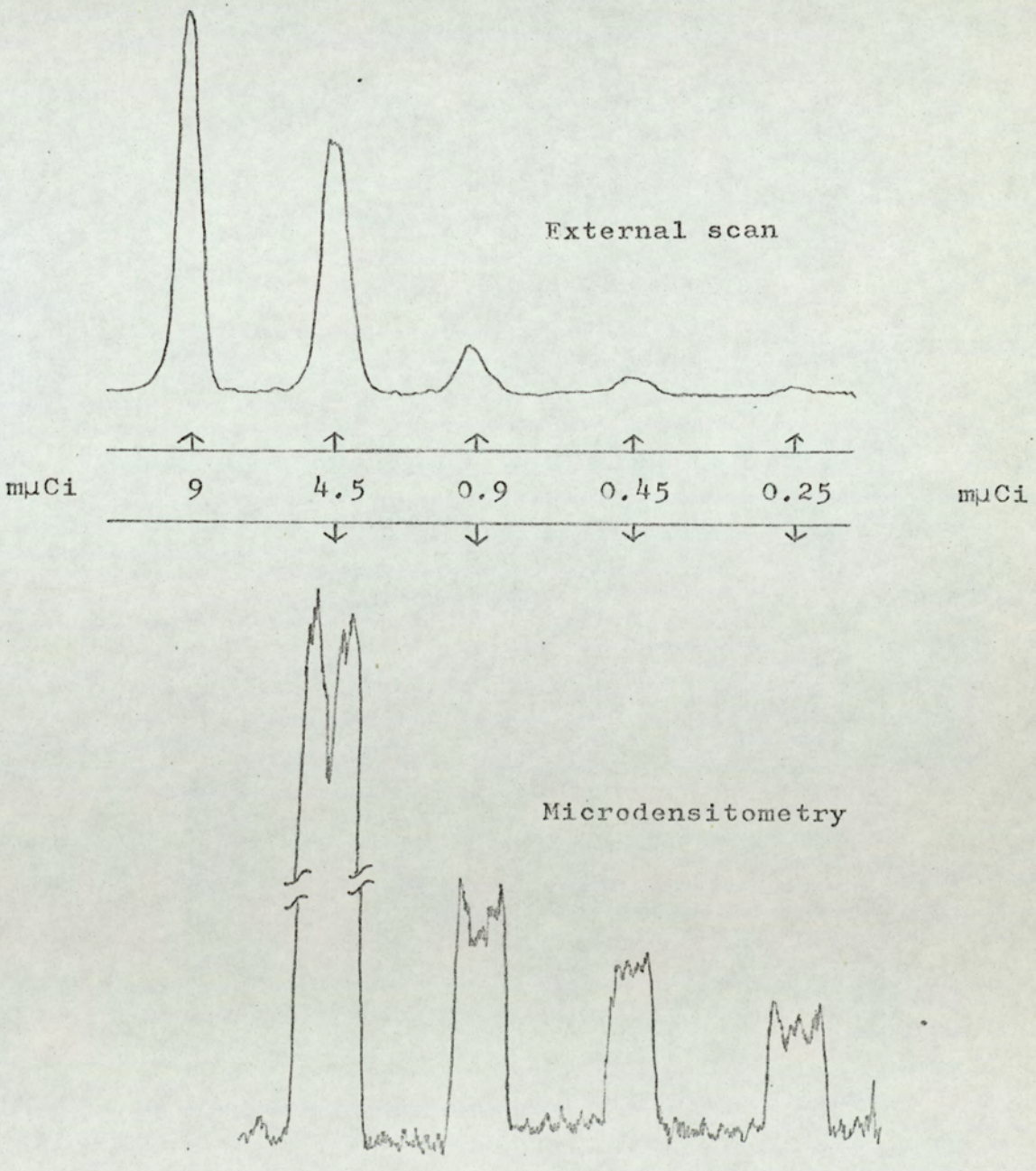


Fig.IA-4 Comparison of external scanning with radio-autography in the detection of Carbon-14 on t.l.c.

1µl spots of a solution of [2-^{14}C]PteGlu were applied to a cellulose plate (0.25mm thick).
External scanning was performed with a Desaga t.l.c. scanner.
Radioautogram was produced by 24h exposure of X-ray film, developed and then scanned with a microdensitometer.



A P P E N D I X II

THE ANOMALOUS BEHAVIOUR OF FOLIC ACID

ON THIN-LAYER CHROMATOGRAPHY

The use of t.l.c. to purify and identify trace amounts of material is now well established. Folic acid is readily detected on t.l.c. as a dark absorbing area, when viewed under u.v. light (254nm). On 0.25mm thick cellulose plates, 5µg of PteGlu were detected by this method; amounts down to 4ng were detected by radioautography (24h) or external scanning of 2[^{14}C]PteGlu. It is in this concentration range that the chromatographic behaviour of folic acid changed with its concentration.

Methods 50nCi of 2[^{14}C]PteGlu (as the dipotassium salt, sp.act. 50.3mCi/mmol. Radiochemical Centre, Amersham, U.K.) was applied to a cellulose (MN300uv) t.l.c. plate in varying amounts (0.5 to 50µg) and varying concentrations (0.5µg/µl to 25µg/µl). The plates were then run at room temp. in subdued light up to a line marked 10cm from the origin. The plates were then dried in a cold air-stream. Radioactivity was detected by external scanning and radioautography (see Appendix I).

Chromatographic solvents for t.l.c. These were as follows: (a) propan-1-ol-10%(v/v)NH_3-glycerol,(4:1:1); (b) propan-1-ol-1%(v/v)NH_3,(2:1); (c) 0.1M-sodium phosphate buffer, pH7.1; (d) 3%(w/v)NH_4Cl; (e) butan-1-ol-acetic acid -water (4:1:5, by vol. equilibrated for 20h; upper phrase); (f) 3%(w/v)NH_4Cl to pH5.5 with NH_4OH; (g) 3%(v/v) acetic acid to pH3.4 with NaOH. Chromatography solvents were also made containing a crystal of sodium sulphide or made 0.1M with respect to EDTA(sodium salt).

Results and Discussion Increasing the concentration from 0.5 to 10µg of folic acid/µl did not greatly effect the Rf values in alkaline and neutral solvent systems

(see TableIIA-1). The slight reduction in Rf value observed with higher concentrations was due to tailing. In acidic solvent systems (d) and (e), the Rf decreased with increase in concentration. The results were suggestive of decomposition at the lower concentrations. The individual spots were recovered and chromatographed together with added 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid (the likely decomposition products) and folic acid. The radioautograph showed that all the radioactivity co-chromatographed with the folic acid, and thus the behaviour of small amounts of folic acid was not due to decomposition.

The addition of Na_2S [see ref (i)] or EDTA to solvents (d) and (e) did not noticeably change the behaviour (see TableIIA-2). Small changes in Rf values are attributable to the increase in pH due to the addition of Na_2S (the pH of solvents (d) and (e) after running the chromatograms were 8.0 and 3.9 respectively) and to the increase in ionic strength with EDTA. Since heavy metals are precipitated by sodium sulphide and chelate with EDTA, the anomalous behaviour of small amounts of folic acid was not due to heavy metal salt formation nor chelation, [see ref (ii)].

The behaviour of folic acid is probably due to a concentration difference. This was established by running similar amounts of folic acid applied in different volumes of solution, see TableIIA-2. The dilution causes an increase in Rf in solvents (d) and (e). Although this behaviour depends on the concentration of folic acid it is not due to solubility since the chromatograms showed a minimum between two peaks (see FigIIA-1). An increase in Rf with

dilution caused by solubility would produce only one peak (with a forward running streak) of radioactivity.

With chromatographic solvent (g), dilute solutions (0.2-0.3µg/µl) of folic acid gave the Rf value of 0.20-0.30 and concentrated solutions (5.0-7.5µg/µl) gave the Rf value of 0.00, and this behaviour was not altered by the addition of EDTA.

Running folic acid in solvent (f) produced only one spot (Rf≈0.35), over the concentration range 0.2 to 5µg PteGlu/µl. Thus the anomalous behaviour, which disappears when the pH is altered from 4.0 [solvent (d)] to 5.5 [solvent (f)], is due to a change in the non-ionised acid.

Since this anomalous behaviour of folic acid in acidic solvent systems is not caused by decomposition, solubility or chelation and is concentration dependent, this effect is most likely resulting from the association of folic acid molecules; the species present at low concentrations and having the higher Rf value being the monomer, and the species present at higher concentrations and having the lower Rf value being the associated form.

The association of folic acid molecules may be caused by intermolecular hydrogen bonding between the non-ionised carboxyl groups, but this seems unlikely since the hydrogen bonds of non-ionised carboxyl groups in aqueous solutions are formed preferentially with the solvent molecules, [ref (iii)]. The infusibility and insolubility of folic acid in all but aqueous alkaline solutions, [ref (iv)], suggests that there is strong intermolecular interaction between the pteridine rings of folic acid molecules. This intermolecular interaction would be a reasonable explanation for the association of folic acid

in higher concentrations in acidic solvent systems. Purines have also been shown to associate in aqueous solution and have the molecular planes held in parallel to each other by the π-electrons of each ring [ref (iv) and (v)].

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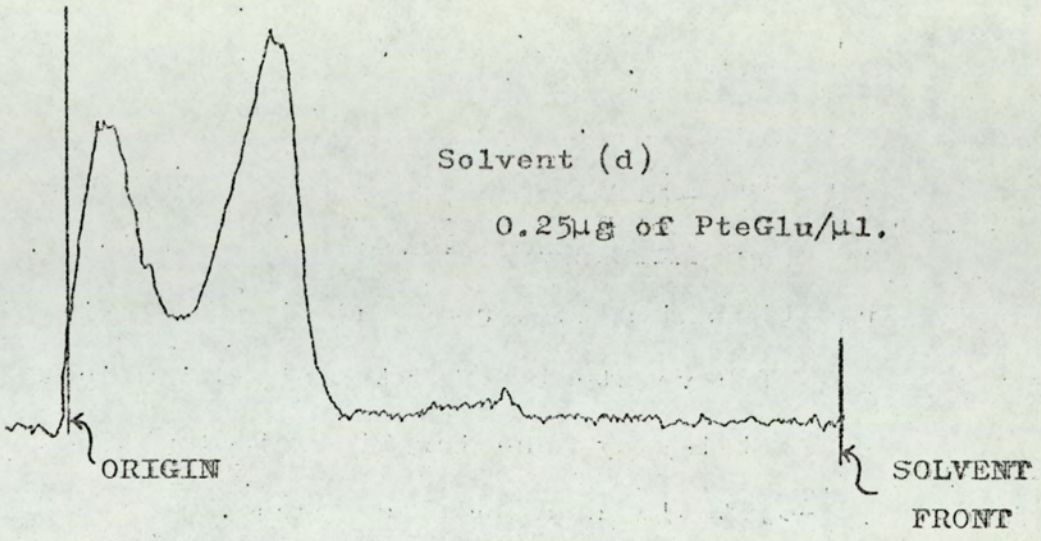
Fig IIA-1 Thin-Layer Chromatography of Folic Acid.



The effect of dilution in solvent (e).

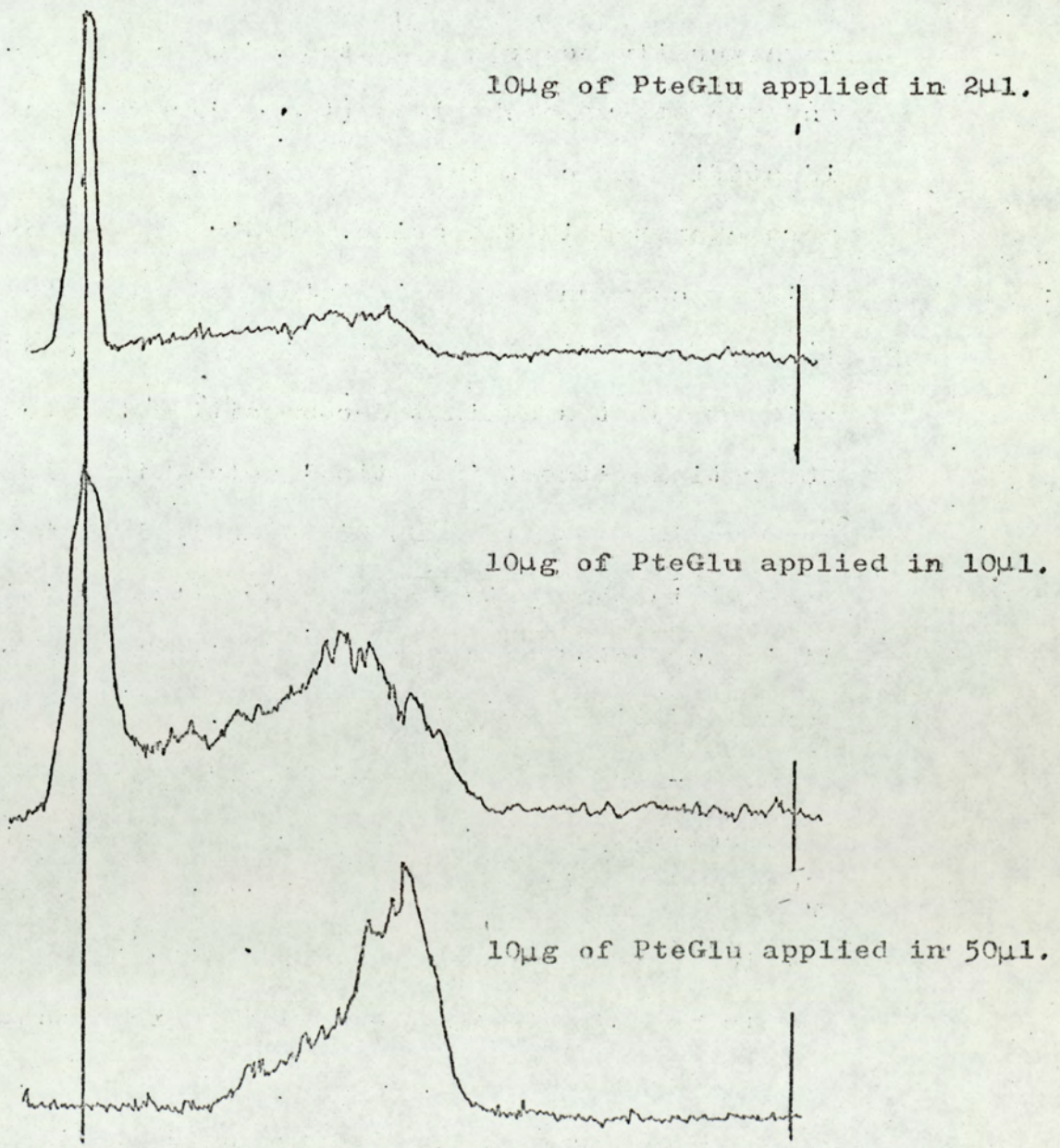


Table IIA-1 Rf values of folic acid in alkaline, neutral and acidic solvent systems.

A solution of folic acid (containing 50mμCi of Carbon-14) was applied to a chromatogram and chromatograph-ed at room temp. in subdued light (see the Methods section). The radioactivity was detected by external scanning and radioautography.

pH	Solvent	Concentration (μg applied/ μl applied)	Rf
11	(a)	0.5/1.0	0.16
		10/2.0	0.11
10.5	(b)	0.5/1.0	0.14
		10/2.0	0.11
7.1	(c)	0.5/1.0	0.57
		10/2.0	0.56
		50/5.0	0.53
4.0	(d)	0.5/1.0	0.04 0.27
		15/2.0	0.04
2.6	(e)	0.5/1.0	0.42
		8/2.0	0.00

Table IIA-2 The Effect of Dilution, Sodium sulphide and EDTA on the Rf value of Folic Acid.

0.5 and 50µg of folic acid (containing 50mµCi of Carbon-14) were applied in 2, 10 and 50µl amounts (spotted with a single application). A crystal of sodium sulphide was added to the solvent system immediately before chromatography or the solvent systems were made 0.1M with EDTA (sodium salt).

			Volume applied (µl)			plus Na_2S	plus EDTA
pH	Solvent	µg of folate	50	10	2	applied in 2µl.	applied in 2µl.
4	(d)	0.5	0.30	0.31	0.30	0.30	0.26
		50	0.28	0.28	0.04	0.04	0.02
2.6	(e)	0.5	0.50	0.50	0.45	0.51	0.49
		50	0.50	0.00	0.00	0.03	0.00

A P P E N D I X III

FORMYLATION OF TETRAHYDROFOLATES

10-HCO-H_4PteGlu and 5,10-CH=H_4PteGlu are known to act as co-factors in one-carbon (at the oxidation level of formate) transfer reactions in polypeptide initiation and purine biosynthesis. These two folate coenzymes are biosynthesised by tetrahydrofolate interconversions and have been chemically synthesised from 10-HCO-PteGlu, 5-HCO-, 5-HCNH-H_4PteGlu, see ref.(vii), and H_4PteGlu (Fig.IIIA-1).

H_4PteGlu reacts with formic acid to produce 10-HCO-H_4PteGlu which rearranges in acidic solutions to produce the more stable 5,10-CH=H_4PteGlu, see ref (i). A similar overall reaction has been carried out using 5,10-CH_2-5-CH_3-, and 5-C_2H_5-H_4PteGlu derivatives, as follows.

MATERIALS

5-CH_3- and 5-C_2H_5-H_4PteGlu were synthesised as in ref. (ii) and 5,10-CH_2-H_4PteGlu by a modification of that method. H_4PteGlu was synthesised by catalytic hydrogenation of PteGlu, ref (iii). 5-HCO-H_4PteGlu (calcium leucovorin) was a gift of Lederle Laboratories, Pearl River, N.Y., U.S.A. Purity (determined by uv spectroscopy; Pye Unicam SP.700) of H_4PteGlu and the 5,10-CH_2-, 5-CH_3-, and 5-C_2H_5- derivatives was 85,90,82 and 67% respectively.

METHODS

Solvents for t.l.c. These were as follows: (a) 1.0M aq. formic acid; (b) propan-1-ol—butan-1-ol—0.1M-HCl (2:1:1, by vol.); (c) aq. -HCl, pH 2.0; (d) butan-1-ol—acetic acid—water (4:1:5, by vol. equilibrated for 20h; upper phase); (e) 5%(v/v) acetic acid in water-saturated butan-1-ol; (f) 0.2M-sodium acetate buffer pH 4.0.

General method carried out with H_4PteGlu and 5-CH_3-H_4PteGlu. These folates (6mg) were dissolved in 98% (w/v) formic acid (2ml) containing 1% (w/v) ascorbic acid and

covered with a layer of toluene. The solution was left at room temp. for 3h and then freeze-dried. The freeze-dried solid was dissolved in the min. vol. of water and purified by t.l.c. on cellulose (MN 300u.v., 1mm thick) developed in solvent (d). Compounds visible under u.v. light (365nm) were extracted with 0.1M-HCl and u.v. spectra and fluorescence spectra (Aminco Bowman, American Instrument Co. Inc., Maryland, U.S.A.) determined. The reaction was also carried out at 75°C.

Kinetic Studies performed with H_4PteGlu and the 5-CH_2-, 5-CH_3-, and 5-C_2H_5-H_4PteGlu derivatives. Folates (approx. 15mg) were dissolved in 5ml of a solution of formic acid (either 98% (w/v), 10% (v/v) aq., or 10% (v/v) in conc. -HCl) containing 1% (w/v) ascorbic acid and covered with a layer of toluene. Samples (0.1ml) were withdrawn at time intervals, made up to 5ml with 0.1M-HCl and the fluorescence at 470nm (excitation 355nm) was measured. The amount of 5,10-CH=H_4PteGlu present was calculated from a standard curve produced with authentic 5,10-CH=H_4PteGlu (5-HCO-H_4PteGlu dissolved in 0.1M-HCl). After 3h, 5µl of the solution was spotted on t.l.c. plates and developed in solvents (a), (b), (d) and (e).

RESULTS

The product of the reaction of formic acid with H_4PteGlu and 5-CH_3-H_4PteGlu gave a major white fluorescing band, Rf 0.25 in solvent (d). This compound had a fluorescence excitation max. at 335nm and emission max. at 470nm (see Fig.IIIA-2); a u.v. spectral max. at 350nm in 0.1M-HCl which disappeared when the solution was made alkaline and reappeared on acidification, and had Rf values 0.36 in solvent (a), 0.22 in solvent (b), 0.34 in solvent (c),

0.25 in solvent (d), 0.06 in solvent (e) and 0.38 in solvent (f). Authentic 5,10-CH=H_4PteGlu had a similar fluorescence spectrum (see Fig.IIIA-2), similar u.v. spectrum and had Rf values 0.38, 0.21, 0.34, 0.22, 0.05 and 0.39 in solvents (a), (b), (c), (d), (e) and (f) respectively.

Similar results were obtained when the formylation reaction was carried out at 75°C.

Results of kinetic studies on H_4PteGlu and the 5,10-CH_2-, 5-CH_3-, 5-C_2H_5-H_4PteGlu derivatives are shown in Figs.IIIA-3,-4 and -5. After 3h all these solutions, chromatographed on t.l.c., showed a white fluorescence with Rf values 0.37, 0.23, 0.25 and 0.07 in solvents (a), (b), (d) and (e) respectively.

5-CH_3-H_4PteGlu dissolved in 1% or 50% (v/v) aq.HCl containing 1% (w/v) ascorbic acid and covered with a layer of toluene, did not produce a white fluorescent compound with the Rf value of 5,10-CH=H_4PteGlu when the mixture was chromatographed in solvents (b), (c), (d) nor (e).

DISCUSSION

H_4PteGlu and the 5,10-CH_2-, 5-CH_3- and 5-C_2H_5-H_4PteGlu derivatives, in the prescence of formic acid, produced a white fluorescing compound. This fluorescent compound was characterised as 5,10-CH=H_4PteGlu by its Rf value in 6 solvent systems, u.v. spectra and fluorescence spectra (although the prescence of cellulose and/or its fluorescent additive shifted the excitation spectrum to lower wavelength).

Under similar conditions in the absence of formic acid, 5,10-CH=H_4PteGlu was not produced. The reaction was therefore dependent on the presence of formic acid and,

under these conditions, 5,10-CH=H_4PteGlu was not produced by oxidation of the N-5-alkyl group.

The kinetics of the formylation were studied by fluorescence spectroscopy because the emission max. at 470nm is characteristic of 5,10-CH=H_4PteGlu, see ref (iv); whereas its u.v. absorption, max. at 355nm at pH 1.0, is also given by pteridines, see ref (v) page 92, which may be formed in side reactions.

At the three pHs (<3.0) studied the rearrangement of 10-HCO-H_4PteGlu is fast (half time <8min), see refs (i) and (vi).

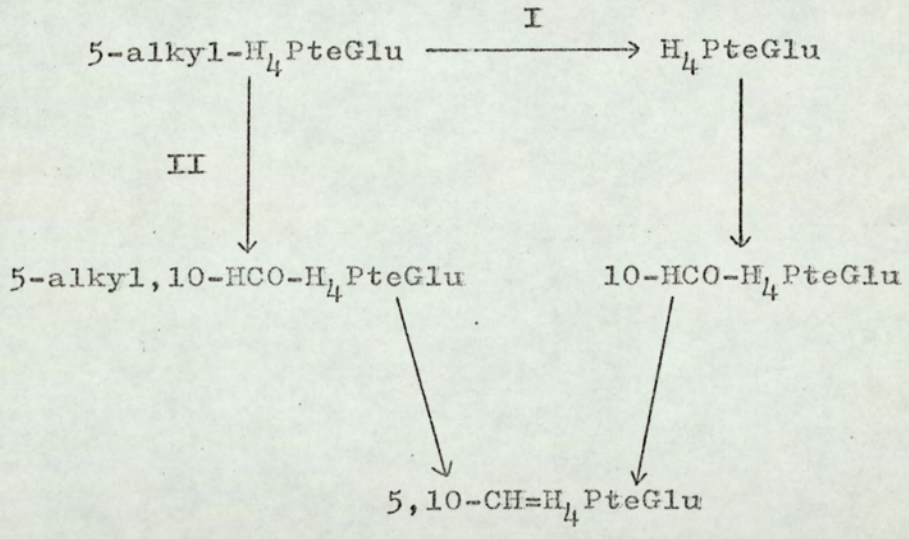
The rate of formation of 5,10-CH=H_4PteGlu (over the first 100min) from H_4PteGlu fell (to $\frac{1}{5}$) when the formic acid concentration is reduced from 98% to 10% aq. formic acid. The rate curves of these two reactions are clearly sigmoidal showing that the rate of formylation is of the same order as the rearrangement step. Substituting conc. HCl for water with 10% formic acid caused a drop in the rate and also the total amount of 5,10-CH=H_4PteGlu formed. This fall in rate and the amount 5,10-CH=H_4PteGlu produced is probably due to decomposition (oxidation?) of the H_4PteGlu, caused by the HCl.

The rate of formation of 5,10-CH=H_4PteGlu (over the first 100min) from 5,10-CH_2-H_4PteGlu was greatest with 10% aq. formic acid and least with 10% formic acid in conc. HCl, and the amounts of 5,10-CH=H_4PteGlu formed were less than with H_4PteGlu under comparable conditions. The reaction therefore proceeded via the acid-catalysed hydrolysis of 5,10-CH_2-H_4PteGlu to H_4PteGlu, see ref (viii), followed by formylation and rearrangement similar to H_4PteGlu. The reduced rate with 10% formic acid in conc. HCl is caused

by decomposition of the H_4PteGlu formed.

The conversion of 5-CH_3-H_4PteGlu to 5,10-CH=H_4PteGlu was greatest with 10% formic acid in conc. HCl and the reaction rate with 98% formic acid was similar to that with 10% aq. formic acid. The conversion therefore depended to a greater extent on the acidity rather than on the concentration of formic acid. The opposite was found for 5-C_2H_5-H_4PteGlu, when the rate was reduced by a reduction in formic acid concentration and was least with 10% formic acid in conc. HCl.

The conversion of 5-alkyl-H_4PteGlu analogues to 5,10-CH=H_4PteGlu could proceed as follows:



Loss of the alkyl group followed by formylation (reaction I) has H_4PteGlu as an intermediate, whereas formylation before loss of the alkyl group (reaction II) does not.

Although it was not possible to confirm which of these mechanisms was operating, the conversions of 5-CH_3-H_4PteGlu by a pathway similar to reaction II and 5-C_2H_5-H_4PteGlu by reaction I are consistent with the observed

effect of HCl which reduces the rate of conversion of H_4PteGlu (or compounds producing H_4PteGlu as an intermediate) to 5,10-CH=H_4PteGlu.

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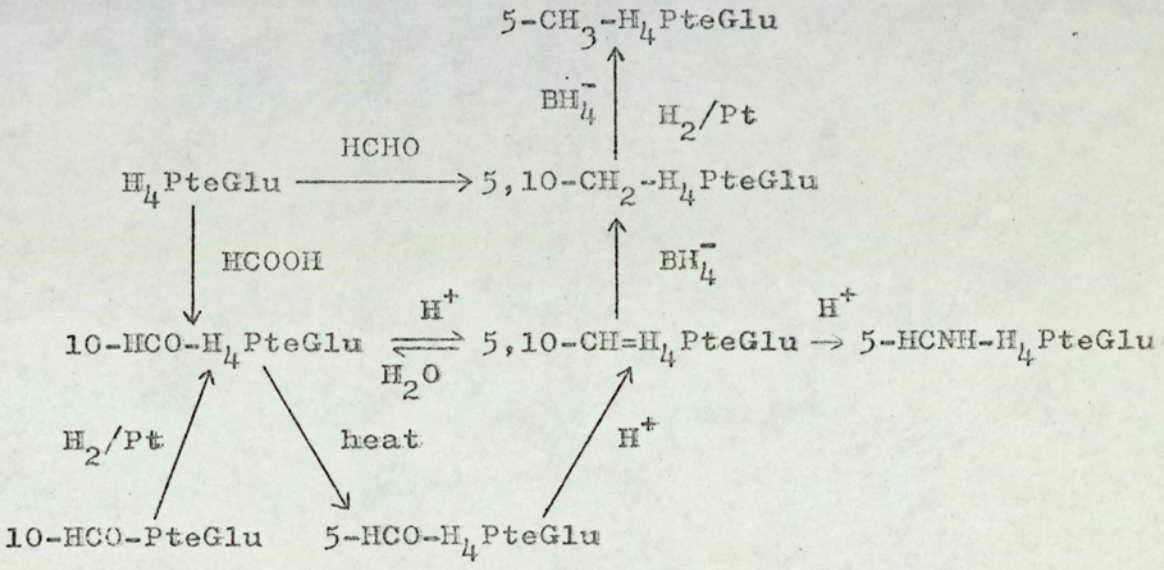
Fig.IIIA-1. Synthesis of 10-HCO-H_4PteGlu and 5,10-CH=H_4PteGlu

(a) Chemical synthesis



(b) Biosynthesis

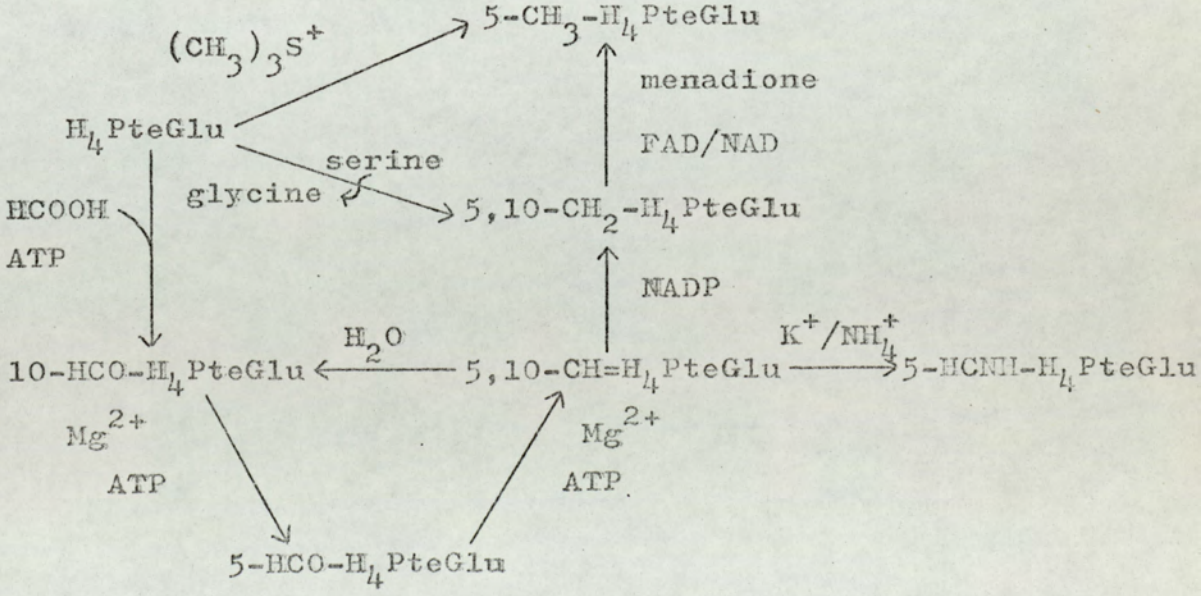


Fig. IIIA-2. Formylation of 5-CH_3-H_4PteGlu

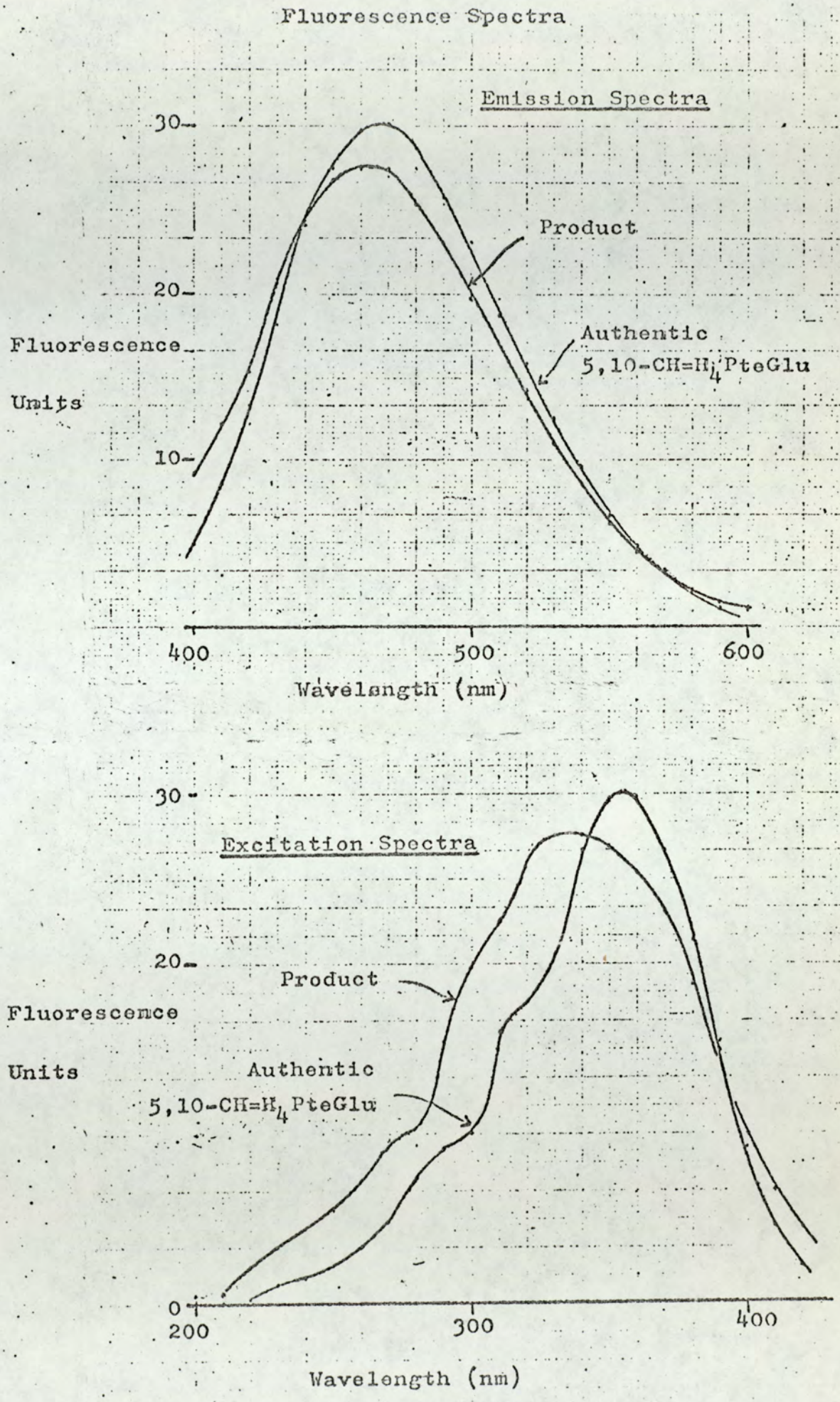
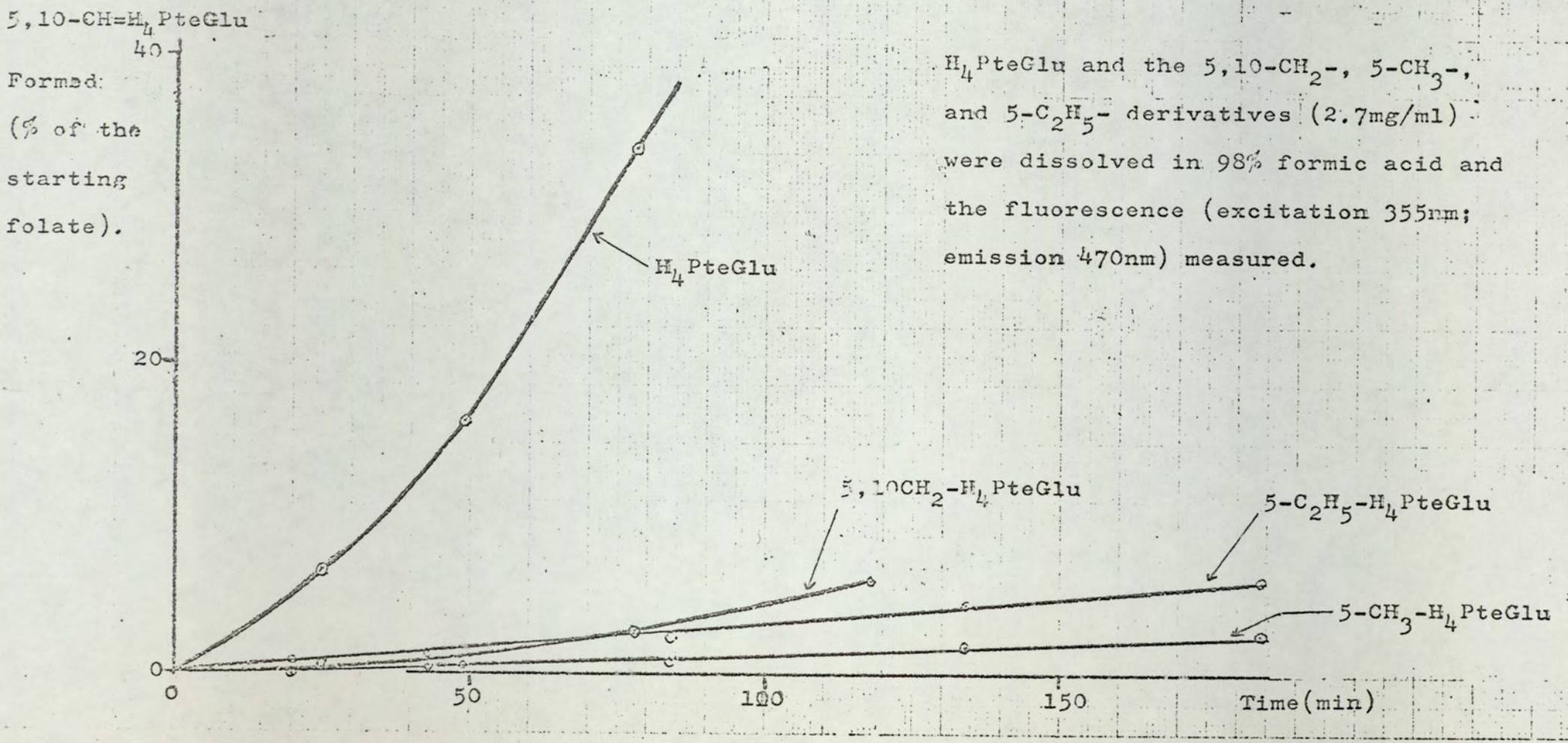


Fig.IIIA-3. Formylation of Tetrahydrofolates

98% formic acid



H_4PteGlu and the 5,10-CH_2-, 5-CH_3-, and 5-C_2H_5- derivatives (2.7mg/ml) were dissolved in 98% formic acid and the fluorescence (excitation 355nm; emission 470nm) measured.

Fig.IIIA-4. Formylation of Tetrahydrofolates

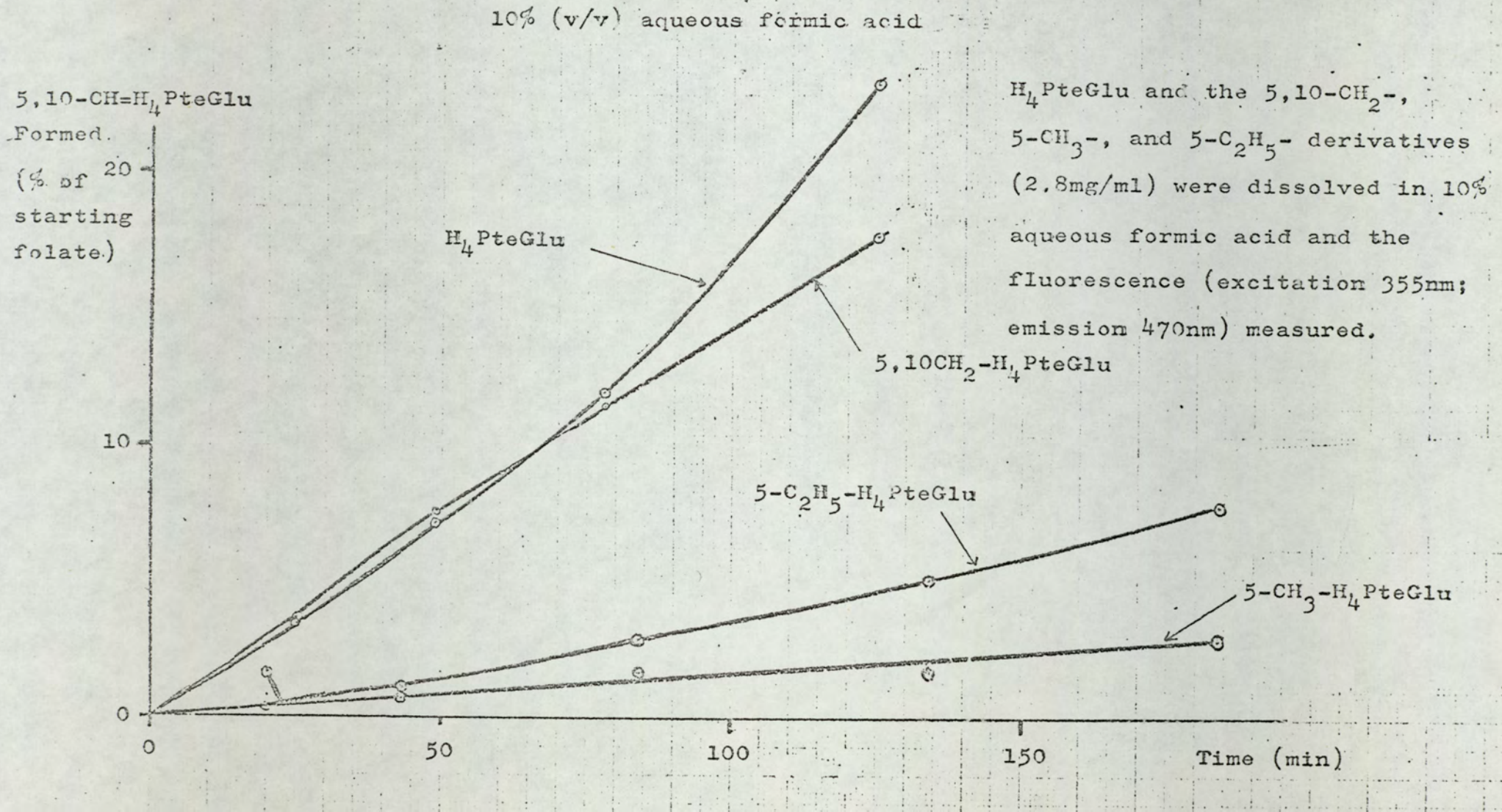
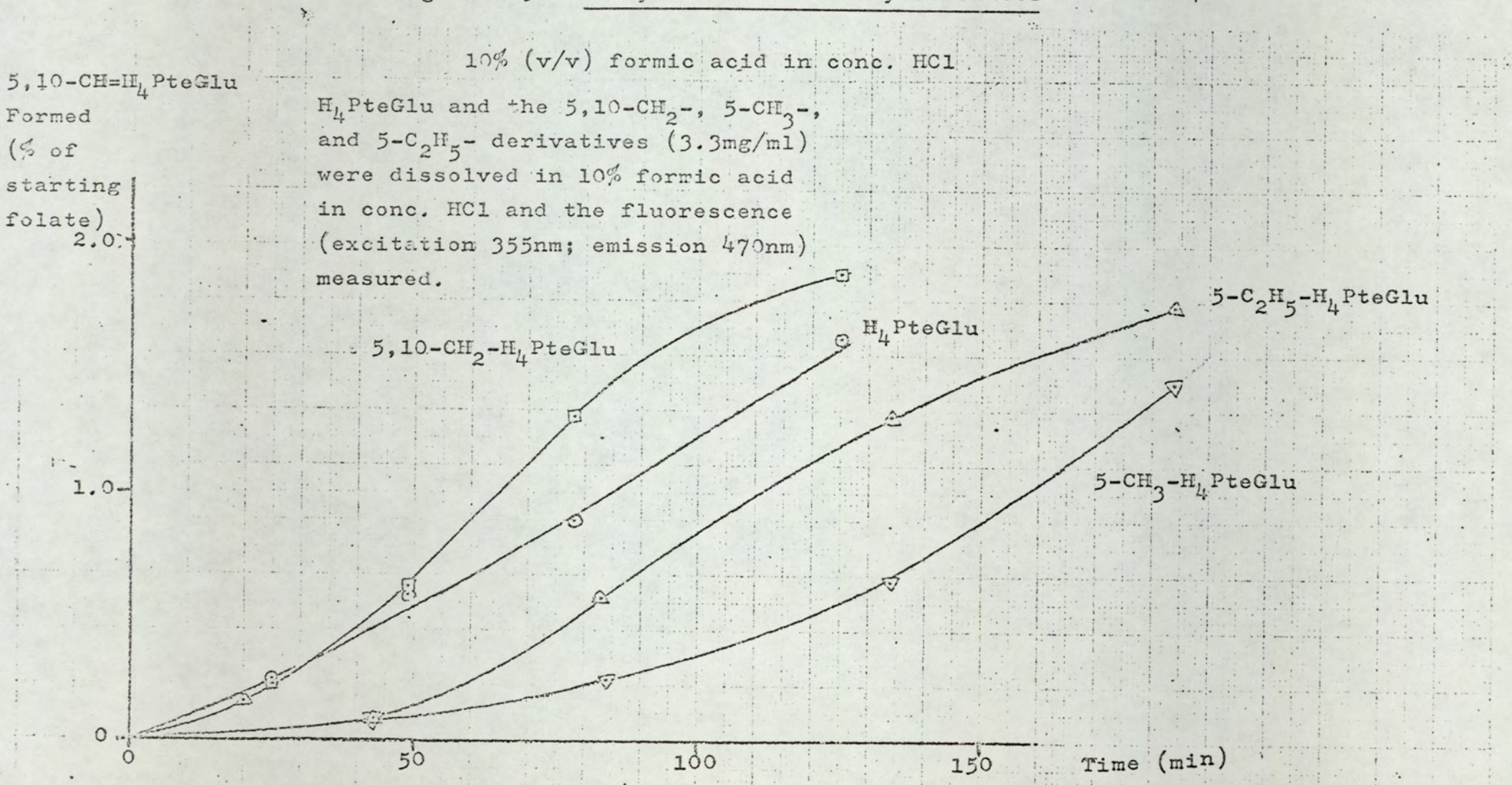


Fig.IIIA-5. Formylation of Tetrahydrofolates



A P P E N D I X IV

THE FATE OF ORAL ADMINISTRATION OF THE MAJOR URINARY METABOLITE FORMED FROM [2-^{14}C] PTEGLU

Despite intensive efforts to characterise the major urinary metabolite formed after administration of [2^{14}-C]-PteGlu to rats, the compound was not identified. However, a preliminary metabolic study was carried out both to provide evidence for the structure of the compound and to determine its metabolism for future analysis. The importance of this compound as a naturally occurring folate in the rat was discussed in sections II and III.

Materials and Methods

The compound was isolated from 24h urines collected in acid with ascorbate after the administration of [2^{14}-C]-PteGlu (320µg/Kg, sp. radioactivity 55.3mCi/mmol) to each of 16 rats. The compound was isolated by purification of urine through a florisil column followed by t.l.c. in solvents (c), (a) and then (d), using antioxidants throughout. The specific radioactivity was determined by radiochemical and L.casei assays and was 33.3mCi/mmol of PteGlu. Assays and chromatography were as previously described.

Results and Discussion

The distribution and excretion of radioactivity after a dose of 25µg/Kg, (Table IVA-1) was similar to that following PteGlu or 5-CH_3-H_4PteGlu administration although the radioactivity excreted in the urine, collected on the first day after administration (Table IVA-2), was slightly higher (Student's *t* test, $p<5\%$). The higher excretion was to be expected since this compound was the major urinary compound but was only a minor compound of liver folates (Section II).

Purification of the first day urine showed that the radioactivity was present mainly as the compound administered and had Rf values of 0.15 and 0.40 in solvents (c)

and (a) respectively. A small amount of a compound at Rf 0.43 in solvent (c), Table IVA-3, co-chromatographed with 5-CH_3-H_4PteGlu in solvent (a), Rf 0.50.

The compound was therefore excreted largely without metabolism and suggests that it was absorbed without change. Since this compound was probably only a minor compound of liver, the small dilution (5.6 times compared to 60 times after PteGlu administration) must have occurred by the de novo synthesis of the compound during that 24h period. The compound is therefore a naturally occurring folate. Metabolism of the compound would have produced a further dilution of radioactivity by the body folates and this was reflected in the dilution (about 50 times) of the excreted 5-CH_3-H_4PteGlu. The compound was therefore metabolised into naturally occurring folates of which 5-CH_3-H_4PteGlu was excreted.

Further discussion on the fate of this compound would be premature.

Table IVA-1 Distribution of radioactivity following oral doses of the unidentified metabolite.

The labelled material was administered as a solution in 0.5ml of 0.2% (w/v) ascorbate solution, pH 6.0. The radioactivity in the urine was assayed directly by liquid scintillation spectrometry. The radioactivity in the liver, kidney and faeces (collected throughout the six day experiment) was assayed by combustion followed by liquid scintillation counting (see Methods section).

The specific radioactivity of the administered materials was 33.3mCi/mmol of PteGlu. The results are expressed as the percentage mean ±S.D. for 5 animals.

	Radioactivity (% of oral dose)			
Dosage (µg as PteGlu/kg)	Faeces	Liver	Kidney	Urine
24.5	55.26 ± 17.48	4.11 ± 2.47	1.41 ± 0.60	22.08 ± 12.75

Table IVA-2 Urinary excretion of radioactivity after an oral dose of the unidentified metabolite.

The labelled material was administered as a solution in 0.5ml of 0.2% (w/v) ascorbate solution, pH 6.0. The radioactivity was determined directly by liquid scintillation counting. The results are expressed as the percentage of the administered radioactivity (mean ±SD; no. of rats in parenthesis) excreted in the urine after oral administration of the unidentified material. The specific radioactivity of the administered materials was 33.3mCi/mmol of PteGlu.

Days after administration	Radioactivity (%) excreted after doses of the unidentified material (24.5µg as PteGlu/kg)
1	14.09 ± 8.89 (8)
2	1.92 ± 0.79 (3)
3	1.18 ± 0.58 (3)
4-6	1.10 ± 1.07 (3)

Table IVA-3 Distribution of radioactivity in materials excreted after oral administration of the unidentified metabolite.

After an oral dose of the labelled metabolite (33.3mCi/mmol of PteGlu) the urine collected for 24h, purified on a florisil column and the ammoniacal fraction by t.l.c. in solvent (c). the percentage of radioactivity in materials separated on the chromatogram was estimated by densitometry of an autoradiograph. The specific radioactivity of materials was determined by a combination of liquid scintillation counting and L.casei assay on materials eluted from the chromatogram.

Dose (µg as PteGlu/kg)	Rf value	Radioactivity (%)	Sp. radioactivity (mCi/mmol of PteGlu)
24.5	0.15	92	5.75
	0.43	8	0.62

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Anomalous behaviour of radioactive folic acid on thin-layer chromatography

The use of thin-layer chromatography to determine the purity and identity of trace amounts of organic compounds is well established. We now report an example in which the chromatographic behaviour changed with concentration of the substance under investigation.

Folic acid (pteroyl-L-monoglutamic acid) may be readily detected by TLC on cellulose powder in amounts greater than 5 μg by its appearance as a dark absorbent spot when viewed in 254 mμ light. With butanol–acetic acid–water (4:1:5, upper phase pH 2.6) as developing solvent, folic acid has an R_F value of 0.0 and this conveniently distinguishes it from its fluorescent decomposition products which move away from the origin.

When [2-^{14}C]folic acid (0.45 μg/μl; specific activity 50.3 mCi/mmole; Radiochemical Centre, Amersham) was assayed for purity in this system a major spot, as determined by autoradiography, of an R_F value of 0.42 was obtained with nothing at the origin. This led to the initial conclusion that the sample of folic acid had extensively decomposed although this radioactive material was not identical with the anticipated decomposition products. When TLC of the radioactive compound (0.45 μg/μl) was carried out in 0.1 *M* phosphate buffer (pH 7.0) and in propanol–1% aq. ammonia (2:1) the major radioactive spot had the same chromatographic behaviour as cold folic acid (5 μg/μl). When folic acid was added to the radioactive folic acid so as to produce a wide range of concentrations and TLC was carried out in butanol–acetic acid–water (4:1:5, upper phase) the R_F values of the major radioactive species varied as shown in Tables I and II.

TABLE I

R_F VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
[2-^{14}C]folic acid 0.45 μg/μl.

Nonradioactive folic acid added	R_F
0.0	0.42
8 μg/2 μl	0.00
20 μg/3 μl	0.00
40 μg/5 μl	0.00

TABLE II

R_F VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
Mixture of 0.5 μg [2-^{14}C]folic acid and 10–15 μg nonradioactive folic acid.

Volume of solution applied (μl)	R_F
2	0.0
10	0.0
50	0.50

These data show that folic acid in butanol–acetic acid–water has an R_F value of 0.0 when applied in concentrated solutions (more than 1 $\mu g/\mu l$) and an R_F value of 0.42 when applied in dilute solution (0.5 $\mu g/\mu l$).

As these differences in chromatographic behaviour could be attributed to chelation of the folic acid with metals, chromatograms were run in butanol–acetic acid–water saturated with ethylene diamine tetracetic acid and butanol–acetic acid–water containing a crystal of sodium sulphide[1]. Again dilute solutions of folic acid had an R_F value of 0.49 and concentrated solutions an R_F value of 0.00.

With 3% aqueous acetic acid (adjusted to pH 3.4 with NaOH) dilute solutions (0.2–0.3 $\mu g/\mu l$) of folic acid gave an R_F value of 0.20–0.30 and concentrated solutions (5–7.5 $\mu g/\mu l$) an R_F value of 0.00; the behaviour was not altered by the addition of ethylene diaminetetracetic acid.

With 3% aqueous ammonium chloride at pH 4.0 dilute solutions of folic acid (0.45 $\mu g/\mu l$) gave two spots (R_F 0.04 and R_F 0.27) coalescing into one R_F (0.04) at higher concentrations (7.5 $\mu g/\mu l$) but at pH 5.5 only one spot (R_F 0.30–0.40) was obtained at all concentrations.

The absence of any effect of adding sodium sulphide or ethylene diamine-tetra-acetic acid establishes that this anomalous effect is not due to chelation of the folic acid. As the variation in R_F values with concentration is found only in acidic solvent systems and disappears when the pH of the 3% aqueous ammonium chloride system is changed from 4.0 to 5.5 this behaviour is due to the non-ionised acid. The variation in chromatographic behaviour with concentration of folic acid in the acidic solvents used is caused by the association of the acid, the species present at low concentrations and having the higher R_F value being a monomer and the species present at higher concentrations and having the lower R_F value being the associated form. The association of the folic acid molecules could be caused by intermolecular hydrogen bonding between the non-ionised carboxyl groups but this seems unlikely as the hydrogen bonds of non-ionised carboxyl groups in aqueous solutions are made preferentially with the solvent molecules and not with each other[2]. There is strong intermolecular interaction between the pteridine rings of folic acid as evidenced by its infusibility and insolubility in all solvents but aqueous alkaline solutions[1]. This intermolecular interaction would be a reasonable explanation for the association of folic acid in higher concentrations in acidic solvent systems. A similar association in aqueous solutions has been established for purines where it has been shown that purines associate with the molecular planes parallel to each other held by interactions between the π-electrons of each ring[3,4].

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The Urinary Excretion of Orally Administered Pteroyl-L-glutamic Acid by the Rat

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1. The urinary excretion of folates after oral administration of [2-^{14}C]pteroyl-L-glutamic acid was studied by assaying the radioactivity in the urine and in materials purified and characterized by t.l.c. 2. Radioactivity excreted was 6.8, 5.9 and 30.7% of the oral dose in the first 24h after doses of 3.1, 32 and 320μg/kg respectively. 3. Extensive decomposition of urinary folates to pteroyl-L-glutamic acid was prevented by antioxidants or collection of urine frozen. 4. At the three dosages, two major and one minor radioactive compounds were isolated. One of the major metabolites was 5-methyltetrahydropteroylglutamic acid. The others were unidentified but were not pteroylglutamic acid, 7,8-dihydro-, 5,6,7,8-tetrahydro-, 5- or 10-formyl-tetrahydro-, 5,10-methylidyne-tetrahydro-, 5-formimidoyl-tetrahydro-, 5,10-methylene-tetrahydro-, 5-methyltetrahydro-pteroylglutamic acid, nor any decomposition products of these compounds formed during isolation. Labelled unconjugated pteridines were absent. 5. Labelled pteroyl-L-glutamic acid was displaced by oral administration of unlabelled pteroyl-L-glutamic acid (1.6mg/kg) when given 3.5h after, but not when given 24h after the labelled dose. 6. The results show that orally administered [2-^{14}C]pteroyl-L-glutamic acid is absorbed without metabolism and is then metabolized into naturally occurring tetrahydrofolates. 7. These findings are discussed with reference to previous work.

Since 1935 (Decastello, 1935) a vast amount of literature has been published about the urinary excretion of folates and pteridines, not only for their interest *per se*, but also to provide possible insight into the folate relationships in normal and diseased metabolism.

Of these reports only a few are concerned with the non-microbiological identification of pteridines and folates. These reports include the isolation of uropterin (later identified as xanthopterin) (Koschara & Hrubesch, 1939), isoxanthopterin (Blair, 1958), biopterin (Broquist & Albrecht, 1955; Patterson, Broquist, Albrecht, Saltza & Stokstad, 1955; Patterson, Saltza & Stokstad, 1956), neopterin (Sakurai & Goto, 1967), PteGlu* (Baker *et al.* 1965; Anderson, Belcher, Chanarin & Mollin, 1960) and 5,10-CH=H_4PteGlu (Silverman, Ebaugh & Gardiner, 1956; Albrecht & Broquist, 1956).

By far the largest amount of literature, however, deals with the microbiological assay of urinary folates. Microbiological assays suffer from several disadvantages, chiefly in their lack of specificity (Blakley, 1969), and have been much criticized (Wieland, Hutchings & Williams, 1952; Johns, Sperti & Burgen, 1961; Kinnear, Johns, MacIntosh, Burgen & Cameron, 1963). This lack of specificity is particularly important when the compound is not characterized by other methods or when impurities are present. It is therefore desirable to develop a simple non-microbiological assay technique for urinary folates. The use of radioactive labelling is advantageous in the study of urinary folates because of the presence of only trace amounts. Tritiated folic acid, first used in 1960 (Anderson *et al.* 1960; Burgen, 1961), although having the main advantages of high specific radioactivity and that it may be used for folate study in man, suffers from the disadvantage of non-specific labelling (Anderson *et al.* 1960; Johns *et al.* 1961; Burgen, 1961; Zakrzewski, Evans & Phillips, 1970) and has been criticized on the grounds of possible impurities (Blakley, 1969).

We now report the results of our studies on the urinary excretion of orally administered

* Abbreviations: PteGlu, pteroyl-L-glutamic acid; H_2PteGlu and H_4PteGlu, 7,8-dihydro- and 5,6,7,8-tetrahydro-pteroyl-L-glutamic acid; 5(and 10)-HCO-, 5(and 10)-formyl-; 5,10-CH=, 5,10-methylidyne-; 5,10-CH_2-, 5,10-methylene-; 5-CH_3-, 5-methyl-; 5-HCNH-, 5-formimidoyl- derivatives.

[2-^{14}C]PteGlu in the rat, by using the labelling to provide accurate determination of the amounts excreted and t.l.c. to purify and identify the compounds.

MATERIALS

Animals and chemicals. The animals used were adult male Wistar rats, weighing 200–350g, purchased from Scientific Products Farm, Ash, Canterbury, Kent, U.K. Food was diet 41B, supplied by Pilsbury, Edgbaston, Birmingham, U.K. Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivity of [2-^{14}C]pteroyl-L-glutamic acid, as the dipotassium salt, was 55.3mCi/mmol and that of [1-^{14}C]hexadecane was 1.06μCi/g. Pteridines and pteroyl-L-glutamic acid were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. H_2PteGlu and H_4PteGlu were freshly prepared by the methods of Futterman (1957) and Hatefi, Talbert, Osborn & Huennekens (1960) respectively. Calcium leucovorin was a gift of Lederle Laboratories, Pearl River, N.Y., U.S.A. 5-CH_3-H_4PteGlu was prepared by the method of Blair & Saunders (1970) and 5,10-CH_2-H_4PteGlu by a modification of that method. Characterization and purity of the compounds were periodically determined by t.l.c. and/or by u.v. spectroscopy (Pye Unicam SP.700).

The liquid scintillators used were NE220 (Nuclear Enterprises, Edinburgh, U.K.) and a toluene-based scintillator containing 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (Koch–Light) made up to 1 litre with A.R. toluene.

Antioxidants used were ascorbic acid (British Drug Houses Ltd., Poole, Dorset, U.K.) and 2-mercaptoethanol (Koch–Light). Florisil (60–100 mesh) was purchased from Koch–Light and cellulose (MN300uv) from Macherey Nagel and Co., Düren, Germany.

Chromatography solvents for t.l.c. These were as follows: (*a*) butan-1-ol–acetic acid–water (4:1:5, by vol., equilibrated for 20h; upper phase); (*b*) 0.1M-sodium phosphate buffer, pH7.0; (*c*) propan-1-ol–aq. 1% (v/v) NH_3 (2:1, v/v); (*d*) butan-1-ol–acetic acid–water (20:3:7, by vol.); (*e*) M-formic acid; (*f*) 0.01M-HCl, pH2.0; (*g*) 0.2M-sodium acetate buffer, pH4.0; (*h*) propan-1-ol–butan-1-ol–0.1M-HCl (2:1:1, by vol.); (*i*) 5% (v/v) acetic acid in water-saturated butan-1-ol; (*j*) 2% (w/v) ammonium acetate–pyridine (19:1, v/v).

Antioxidant, when used, was 0.5% (v/v) 2-mercaptoethanol for all chromatography solvents and extractions from the cellulose plates (performed with water).

Table 1 shows the characteristic R_F values of the pteridines and folates used during this work. Detection levels were of the order of 25μg/cm^2 for light-absorbing compounds and 2μg/cm^2 for fluorescent compounds. The R_F values of the compounds when added to urine were distorted and greater attention was paid to the relative R_F values, characteristic absorption or fluorescence and co-chromatography.

METHODS

[2-^{14}C]PteGlu was administered to the rats, the urine was collected, assayed for radioactivity and then purified initially through a Florisil column followed by t.l.c. on cellulose.

Administration of [2-^{14}C]*pteroylglutamic acid.* This was administered in 0.5ml of water by stomach intubation to adult male rats. A control rat was given 0.5ml of water only. The animals were housed separately in wire-

Table 1. *Thin-layer chromatography of pteridines and folates*

Samples of fluorescing compounds (1μg) and of u.v.-absorbing compounds (10μg) were applied in 2μl to cellulose plates (250μm thick) and developed to 10cm from the origin in solvents containing 0.5% (v/v) 2-mercaptoethanol.

Compound	Characterization under u.v. light (nm)	R_F value		
		Solvent (*a*)	Solvent (*b*)	Solvent (*c*)
2-Amino-4-hydroxypteridine	Blue fluorescence (365)	0.55	0.53	0.43
2-Amino-4-hydroxy-6-formylpteridine	Light-blue fluorescence (365)	0.65	0.68	0.53
2-Amino-4-hydroxypteridine-6-carboxylic acid	Light-blue fluorescence (365)	0.25	0.58	0.12
Xanthopterin	Green fluorescence (365)	0.48	0.61	0.16
Isoxanthopterin	Light-blue fluorescence (365)	0.38	0.40	0.17
Biopterin	Light-blue fluorescence (365)	0.34	0.63	0.49
PteGlu	U.v.-absorbing (254)	0.00	0.50	0.12
10-HCO-PteGlu	Light-blue fluorescence (365)	0.65	0.92	0.30
7,8-H_2PteGlu	Light-blue fluorescence (365)	0.55	0.39	0.30
H_4PteGlu	U.v.-absorbing (254)	0.43	0.78	0.35
5-HCO-H_4PteGlu	U.v.-absorbing (254)	0.69	0.87	0.32
5,10-CH=H_4PteGlu	White fluorescence (365)	0.38	0.65 (tailing)	Decomposition
5,10-CH_2-H_4PteGlu	U.v.-absorbing (254)	0.85 (some decomposition)	0.92	0.28
5-CH_3-H_4PteGlu	U.v.-absorbing (254)	0.68	0.85	0.55

bottomed cages fitted with a urine–faeces separator (Metabowl; Jencons Ltd., Hemel Hempstead, Herts., U.K.). Food and tap water were given *ad libitum*.

Urine collection. This was performed in subdued light every 24h for up to 7 days after administration of labelled PteGlu. Urines were collected in acid (final pH 1–2), alkali (final concentration 1–2M-KOH) or frozen (acetone–solid CO_2, −78°C). Antioxidants, when used, were ascorbic acid (final concentration 1–2%, w/v) for urine collected in acid, and 2-mercaptoethanol (final concentration about 0.5%, w/v) for urine collected in alkali. Urines with antioxidant were collected under toluene (5ml).

Determination of radioactivity. This was done for urines and other aqueous solutions directly by using NE220 liquid scintillator in an automatic scintillation spectrometer (Nuclear Enterprises) operating at 0°C. The background count was approx. 2 c.p.s. Assay of radioactivity in tissues and faeces was performed by combustion (Kalberer & Rutschmann, 1961). The freeze-dried material was ground in a pestle and mortar and a sample (about 50mg) was burned in 2 litres of oxygen. Carbon dioxide was absorbed in 25ml of a solution of monoethanolamine (120ml made up to 1litre with A.R. methanol) and the radioactivity of a 5ml sample was counted in 5ml of toluene-based scintillator. The background count was about 1 c.p.s. The recovery of radioactivity was 96%. [1-^{14}C]Hexadecane was used as internal standard for each sample. Total sample counts were at least 5000 above background.

Purification by chromatography on Florisil. Florisil (8g) was washed with 100ml of aq. 1% (v/v) HCl, then to neutrality with water, followed by 100ml of aq. 14% (v/v) NH_3, then to neutrality with water and finally with 50ml of aq. 1% (v/v) HCl. This suspension was packed into a glass column (11.5cm×1.3cm) and shielded from the light. Urine samples were freeze-dried and applied in 25ml of solution at about pH1.0. Elution was carried out with 30ml of aq. 1% (v/v) HCl (acid fraction), followed by 30ml of water (neutral fraction) and finally 25ml of aq. 14% (v/v) NH_3 (ammoniacal fraction). Antioxidants, when used, were 2% (w/v) ascorbic acid in acidic solutions and 0.5% (v/v) 2-mercaptoethanol in neutral and alkaline solutions.

There was no significant difference between either the distribution or the recovery of radioactivity from the Florisil column, from urines collected with and without antioxidant, or with and without added folate. The percentage distribution of the radioactivity expressed as the mean ±S.D. for 23 experiments, was 23.9±11.1, 6.8±4.4 and 69.3±13.0 in the acid, neutral and ammoniacal fractions respectively. Recovery of radioactivity was 83.3±14.0%.

Both [2-^{14}C]PteGlu and 5CH_3-H_4[^{14}C]PteGlu when applied in water or urine to a Florisil column were similarly distributed between the three fractions. T.l.c. of the three fractions from labelled urine showed the same labelled materials in each fraction. For further purification only the ammoniacal fraction was used. This fraction contained most of the radioactivity and was not grossly contaminated with inorganic material.

Purification by t.l.c. The ammoniacal fraction from the Florisil column was freeze-dried, dissolved in approx. 2ml of water and centrifuged. Any solid material was washed twice with a small volume of water, the washings were combined with the original supernatant and further purified by t.l.c. as follows.

The solution was applied as a streak (with a SA20 streak applicator; Burkard, Rickmansworth, Herts., U.K.) to cellulose t.l.c. plates (200mm×200mm×1mm). Further purification was on plates 1mm or 250μm thick. All chromatograms were developed to 10cm from the origin. Recovery of label ([2-^{14}C]PteGlu) from cellulose plates was 70%.

Because of the anomalous behaviour of small amounts of folic acid (Blair & Dransfield, 1969) in solvent (*a*), 2mg of non-radioactive PteGlu (R_F0.00) was added as a routine to the Florisil ammoniacal fraction before chromatography. It was also found that this acidic solvent system caused retention of radioactive material at the origin; subsequent chromatography in systems (*b*) and (*c*) showed that this material was not PteGlu. This retention may be due to washings from the Florisil column. To avoid erroneous conclusions due to this retention, chromatograms were also run in the alkaline solvent (*c*), which did not cause retention at the origin.

Radioautography. This was used for the detection and location of labelled compounds on chromatograms. An X-ray film (Ilford Industrial G, Ilford, Essex, U.K.) was placed over the chromatogram and held in position by a further glass plate and elastic bands. X-ray films were exposed at −15°C for up to 21 days, developed in Kodak DX 80, washed in 3% (v/v) acetic acid and fixed in Kodak FX 40. Minimum detection of radioactivity by this technique was approx. 2.5nCi/cm^2 in 24h. The relative amounts of labelled compounds were estimated by scanning the radioautograph with a microdensitometer (MK IIIc; Joyce, Lobel and Co. Ltd., Gateshead, Co. Durham, U.K.).

Microbiological assays. Samples were dissolved in 0.1M-sodium phosphate buffer, pH7.0, containing 1% (w/v) ascorbic acid. The organisms used were *Lactobacillus casei* N.C.I.B. 8010 grown in Q-Ess medium (gift of BBL, Division of Bioquest, U.S.A.), *Streptococcus faecalis* N.C.I.B. 8043, grown in Difco 0319-15 medium and *Pediococcus cerevisiae* N.C.I.B. 7837 grown in Difco 0456-15 medium (Difco Laboratories, Detroit, Mich., U.S.A.). Incubation was for 18h at 37°C and the extent of growth was determined turbidimetrically. Assays were performed in duplicate at least. Chicken pancreas conjugase was prepared by the method of Perry & Chanarin (1968).

Reduction of labelled compounds. This was done with excess of $NaBH_4$ in 2% (w/v) ammonium acetate, pH7.0, containing 0.5% (v/v) 2-mercaptoethanol, for 20min at room temperature, or by catalytic hydrogenation in acetic acid, H_2/Pt, for 20min at room temperature.

Formylation of 5-methyltetrahydropteroyl-L-glutamic acid. Formic acid (98%, w/v) reacts with H_4PteGlu to produce 10-CHO-H_4PteGlu (May *et al.* 1951), which rearranges in acidic solutions to the more stable 5,10-CH=H_4PteGlu (May *et al.* 1951). A similar overall reaction has been carried out in this laboratory by using 5-CH_3-H_4PteGlu. The 5-CH_3-H_4PteGlu (6mg) was dissolved in 98% formic acid (2ml) containing 1% (w/v) ascorbic acid and covered with a layer of toluene. After 3h the mixture was purified by t.l.c. on cellulose. The major fluorescent material (white; under 365nm u.v. light), R_F 0.35 in solvent (*a*), was identified as 5,10-CH=H_4PteGlu by fluorescence

spectrum (excitation maximum 335nm; emission maximum 470nm), u.v. spectrum (peak at 350nm in 0.1M-HCl disappears when the solution is made alkaline and reappears on acidification) and t.l.c. on cellulose [R_F 0.36 in solvent (*e*), 0.34 in solvent (*f*), 0.38 in solvent (*g*), 0.22 in solvent (*h*) and 0.06 in solvent (*i*)]. Authentic 5,10-CH=H_4PteGlu (calcium leucovorin dissolved in 0.1M-HCl) had R_F values 0.35, 0.34, 0.39, 0.21 and 0.05 in these solvents respectively and co-chromatographed with the material in solvent systems (*a*) and (*h*).

The reaction with 5-CH_3-H_4PteGlu was slower than with H_4PteGlu. The reaction was also carried out at 75°C and with aq. 10% formic acid at room temperature.

RESULTS

The urinary excretion of radioactivity after an oral dose of [2-^{14}C]PteGlu is shown in Table 2.

Identification of the labelled urinary components was attempted by purification of urine collected under various conditions as follows.

Collection of urine without antioxidants. When urine was collected in either acid or alkali without antioxidants present, extensive and variable decomposition of radioactive material to PteGlu occurred, during both collection and purification procedures; consequently all subsequent experiments were performed with added antioxidants (see the Methods section).

Collection of urine in acid with use of antioxidants throughout. Typical chromatograms of the ammoniacal Florisil fraction showed radioactivity at R_F values (0.00, due to anomalous retention), 0.28, 0.42 and 0.52 in solvent (*a*) and corresponding R_F values 0.25, 0.10 and 0.40 in solvent (*c*). Labelled PteGlu was absent. The compound with R_F 0.52 in solvent (*a*) and 0.40 in solvent (*c*) was identified as 5-CH_3-H_4PteGlu by co-chromatography in solvent systems (*a*), (*b*), (*c*) and (*j*).

Radioactive compounds were purified free from any fluorescence or u.v. absorption by chromatography in solvent (*a*), extraction, freeze-drying and repetition of this process in solvents (*c*) and (*d*). The amounts of radioactive compounds were too small to be detected under u.v. light by using this method.

Collection of the urine frozen followed by freeze-drying. Antioxidants were used during purification. Chromatography in solvent (*a*) showed the presence of five labelled compounds with R_F values (0.00), 0.22, 0.31, 0.43 (major radioactivity) and 0.52 (identified as 5-CH_3-H_4PteGlu). Labelled PteGlu was absent. The major radioactivity was further purified in solvent (*c*) and had R_F 0.10, and then in solvent (*d*), in which it had R_F 0.47, and was then free from all fluorescence or u.v. absorption.

Labelled 5-CH_3-H_4PteGlu was identified (by its R_F value in two solvents) by a similar purification in all but one of 12 subsequent experiments. Characterization by co-chromatography in three solvents was carried out in eight of these experiments.

The relative microbiological activity of the major unidentified compound, R_F 0.42 in system (*a*) and 0.10 in system (*c*), was 100 for *L. casei*, 13–38 (mean 25) for *S. faecalis* and <2.5 for *P. cerevisiae*. The corresponding values for the isolated 5-CH_3-H_4[2-^{14}C]PteGlu were 100, 4–33 (mean 12) and <2.5. *L. casei* assays on the unidentified material before and after conjugase treatment were exactly the same indicating that this compound is not a heptaglutamate. The response of *S. faecalis* and *P. cerevisiae* to conjugase-treated material was not determined.

Collection of urines with added folates. Because of the instability of the unidentified compound and its decomposition to PteGlu, even when antioxidant was present, we decided to investigate the identity of this compound by the addition of folates to the urine collection flask, when, even if decomposition did occur, the radioactive material would correspond to a fluorescent pteridine formed from the decomposed folate.

When urine was collected in the presence of added PteGlu (0.5mg), H_2PteGlu (1mg), H_4PteGlu (5mg), 5-HCO-H_4PteGlu (0.5mg dissolved in 1M-HCl), 5,10-CH_2-H_4PteGlu (2mg), in separate experiments, the radioactive compounds did not correspond to the fluorescence or u.v. absorption of the added folate or, in the case of H_4-PteGlu and

Table 2. *Urinary excretion of radioactivity after an oral dose of* [2-^{14}C]*pteroyl-*L*-glutamic acid*

The results are expressed as the percentage of the administered radioactivity (mean ±S.D.; no. of determinations in parentheses) excreted in the urine after oral doses of [2-^{14}C]PteGlu, specific radioactivity 55.3mCi/mmol.

Dose (μg/kg)	Percentage excreted in urine		
	0–24h (1st day)	24–48h (2nd day)	48–72h (3rd day)
3.12	6.83±2.91 (12)	2.66±0.78 (4)	—
32.0	5.89±2.56 (8)	1.95±0.83 (8)	1.05±0.77 (8)
320	30.7±6.8 (14)	1.22±0.18 (4)	0.64±0.29 (4)

5,10-CH_2-H_4PteGlu, which were not themselves isolated, of their decomposition products. Added 5-CH_3-H_4PteGlu did not alter the radioactive materials isolated.

Reduction of the unidentified compound. The attempted reduction of the labelled material (purified through Florisil and by t.l.c. in two solvent systems) with sodium borohydride and by catalytic hydrogenation, did not change the pattern of radioactive spots when compared with control experiments.

Purification of the second-day urines. This was performed in a manner exactly similar to that used for the first-day urines, antioxidants being used throughout. The radioautographs were essentially similar to those of the chromatograms produced by the first-day urines. Radioactive PteGlu was absent. The major radioactive component, R_F 0.48 in solvent (*a*) and 0.11 in solvent (*c*), was not identified. The compound with R_F 0.55 in solvent (*a*) was identified as 5-CH_3-H_4PteGlu by co-chromatography in solvent (*b*), R_F 0.83, and solvent (*c*), R_F 0.41.

Purification of the third-day urines. This was performed with use of antioxidants throughout. The radioautograph showed the presence of similar radioactive compounds and labelled 5-CH_3-H_4-PteGlu was identified by its R_F value in solvent systems (*a*) and (*c*).

Studies on the two major metabolites in the urine. The ratio of the major unidentified material to the 5-CH_3-H_4[2-^{14}C]PteGlu, isolated on the first day after a dose of 32 μg/kg, varied from 0.5–7.7 (mean 2.7) to 1.0 in twelve experiments. The percentage of the total radioactivity on the chromatograms in the unidentified material and 5-CH_3-H_4PteGlu, and the specific radioactivity of the 5-CH_3-H_4-PteGlu, are shown in Table 3.

The specific radioactivity of the unidentified material, assuming full activity for *L. casei*, isolated on the first day after an oral dose of 32 μg/kg, was 2.15 mCi/g of PteGlu, giving a dilution of the starting material of 59.1. This was similar to the dilution (52.2) of the 5-CH_3-H_4PteGlu.

The results of a series of orally administered 'flushing' doses of unlabelled PteGlu are shown in Table 4. Flushing doses of up to 1.6 mg/kg body wt., given at 24 h or later after oral doses of 3.95 or 32 μg of labelled PteGlu/kg, did not produce a significant increase in radioactivity in the urine, and did not give labelled PteGlu in the urine. The percentage of the radioactivity in the urine present as 5-CH_3-H_4PteGlu was 9.5 after a flushing dose of 200 μg/kg given 72 h later, 26 (specific radioactivity 0.013 mCi/mmol) after a flushing dose of 400 μg/kg given 24 h later, and 30 (specific radioactivity 0.008 mCi/mmol) after a flushing dose of 400 μg/kg given 72 h later.

A significant increase (Student's *t* test, $P < 0.001$) in radioactivity occurred when doses of 1.6 mg of unlabelled PteGlu/kg were given 3.5 h after or 3.5 h before the labelled dose (44 μg/kg). Labelled PteGlu was excreted and represented 45% and 49% respectively of the radioactivity detected on the chromatograms.

Tissue radioactivity. The radioactivity present in the faeces, urine, liver and kidney after oral doses of [2-^{14}C]PteGlu is shown in Table 5.

DISCUSSION

The experimental results show that of a dose of 80 μg (320 μg/kg) only 20–40% of the PteGlu was absorbed, and of this 81% was excreted in the urine in 7 days. At the PteGlu dose (5.5 μg) equivalent to the physiological range of folate (approx. 5 μg per day), 50% was absorbed and of that 31% was excreted in 6 days. Although the faecal excretion of radioactivity was not determined for a dose of 0.8 μg, it seems likely that about 50% was absorbed, by comparison of the tissue contents with those after a dose of 5.5 μg. A similar amount of excretion

Table 3. *Studies on the isolated* 5-CH_3-[2-^{14}C]H_4PteGlu

After an oral dose of [2-^{14}C]PteGlu (specific radioactivity 55.3 mCi/mmol) the urine was purified by column chromatography and t.l.c. The percentages of the radioactivity on the chromatograms present as the unidentified compound and as 5-CH_3-H_4PteGlu were estimated by densitometry of radioautographs and the specific radioactivity of 5-CH_3-H_4PteGlu by a combination of liquid-scintillation counting and *L. casei* assay, on material eluted from thin-layer chromatograms.

Dose (μg/kg)	Day	Radioactivity (%) present as: Unidentified compound	Radioactivity (%) present as: 5-CH_3-H_4PteGlu	Sp. radioactivity of 5-CH_3-H_4[2-^{14}C]-PteGlu (mCi/mmol)
2.2	1	60	22	0.097
32.0	1	43.3	29	1.06
	2	82.6	14	0.032
	3	67.0	8.6	—
320	1	52	27	17.1

Table 4. *Displacement of radioactivity by flushing doses*

Flushing doses of PteGlu were given by dissolving the PteGlu in a minimum volume of aq. 1% (v/v) NH_3, diluting it with water (final pH 7–8) and administering in 0.5 ml by stomach intubation. The results are expressed as the percentage of the administered radioactivity (mean ±S.D. of four animals) excreted in the 24 h before and in the days after an oral flushing dose of unlabelled PteGlu. The specific radioactivity of the [2-^{14}C]PteGlu was 55.3 mCi/mmol.

Dose of [2-^{14}C]-PteGlu (μg/kg)	Time (h) of the flushing dose after the radioactive dose	Flushing dose (μg of PteGlu/kg)	% of the administered radioactivity appearing in the urine			
				Days after flushing dose		
			24 h before	1	2	3
32.0	72	200	1.72±0.23	2.07±0.99	1.02±1.14	0.62±0.09
35.2	72	400	0.39±0.40	1.00±0.20*	0.68±0.12	0.33±0.14
32.0	24	400	12.1 ±8.3	2.93±0.56†	1.35±0.55	1.21±0.51
3.95	24	400	6.87±4.40	4.71±1.30	—	—
21.8	24	1600	4.27±2.23	2.44±0.87	1.22±0.27	—
44.3	3.5	1600	=	16.5 ±0.8	1.12±0.28	1.12±0.24
43.6	Preloading dose of 1600 μg of PteGlu/kg given 3.5 h before the labelled dose		=	20.2 ±4.6	0.79±0.30	0.68±0.42

* Specific radioactivity of the 5-CH_3-H_4PteGlu was 0.008 mCi/mmol.
† Specific radioactivity of the 5-CH_3-H_4PteGlu was 0.013 mCi/mmol.

Table 5. *Distribution of radioactivity after oral doses of* [2-^{14}C]PteGlu

The radioactivity in the urine (collected every 24 h after administration of [2-^{14}C]PteGlu) was assayed directly by liquid-scintillation spectrometry. The radioactivity in the liver, kidney and faeces (collected throughout the experiment) was assayed by combustion followed by liquid-scintillation counting (see the Methods section). The specific radioactivity of the [2-^{14}C]PteGlu was 55.3 mCi/mmol. Results are the mean ±S.D. for four animals.

Dose (μg/kg)	Duration of experiment (days)	Radioactivity (% of oral dose)			
		Faeces	Urine	Liver	Kidney
320	7	74.9±33.1	32.6±4.5	7.3±2.0	0.56±0.12
22	6	47.6±4.7	12.0±3.0	16.1±2.8	1.1 ±0.3
3.2	3	—	10.9±3.7	14.4±3.7	2.2 ±0.3

was observed in the rat by using *S. faecalis* assays (Swendseid, Bird, Brown & Bethell, 1947). During studies on humans, Retief (1969), using *L. casei* assays, obtained similar results, whereas higher values were recorded by Jukes, Franklin, Stokstad & Boehne (1947) using *L. casei*, by Girdwood & Delamore (1961) using *S. faecalis* assays and by Anderson *et al.* (1960) using tritiated PteGlu. Wide variations of excretion have been recorded and may reflect differences in folate absorption and metabolism, in the microbiological activity of isolated materials and in the specific radioactivity when labelled PteGlu was used. The amounts of label remaining in the liver were approximately an order of magnitude greater than those in the kidney at all three doses and are within the ratios reported by using *L. casei* assays (Grossowicz, Rachmilewitz & Izak, 1963; Grossowicz, Izak & Rachmilewitz, 1964).

There was no significant difference in the percentage of the administered radioactivity (approx. 6%) appearing in the urine in the 24 h period after oral doses of 3.2 and 32 μg/kg of [2-^{14}C]PteGlu, but a marked increase (to 30%) when the dose was raised to 320 μg/kg. On the second and third days, the urinary excretion of radioactivity after the largest dose fell sharply, whereas that after the smaller doses fell more gradually. This pattern of urinary excretion is in agreement with those found by Steinkamp, Shukers, Totter & Day (1946), Jukes *et al.* (1947), Denko (1951) and Girdwood & Delamore (1961), in that the largest amount of the excreted material from an oral dose is excreted within 24 h.

After an oral dose of 32 μg/kg collection in acid and alkali and purification of the first-day urine without antioxidants led to decomposition and only PteGlu was identified. A similar purification procedure with antioxidants present led to the isolation

of tetrahydropteroylglutamates, and PteGlu was absent. Similar results were obtained by collection in acid, alkali and with frozen urine. PteGlu, isolated by several workers (Kinnear *et al.* 1963; Anderson *et al.* 1960; Johns *et al.* 1961, using [^{3}H]PteGlu and DEAE-cellulose purification; and Baker *et al.* 1965, using bioautography), probably arose by oxidation due to the absence of antioxidants as in our work. McLean & Chanarin (1966), using antioxidants, also obtained PteGlu. Specific radioactivities, which would indicate whether or not decomposition had taken place, were not recorded by the authors who used labelled material.

During the present work two major (representing some 85% of the radioactivity) and one minor labelled metabolites were isolated. One of the major metabolites was identified as 5-CH_3-H_4PteGlu. The other was not identified but was not PteGlu, 7,8-H_2PteGlu, H_4PteGlu, 5-HCO-H_4PteGlu, 5,10-CH=H_4PteGlu, 10-HCO-H_4PteGlu, 5-HCNH-H_4-PteGlu, 5,10-CH_2-H_4PteGlu or 5-CH_3-H_4PteGlu, nor any artifact formed from these during the isolation. Labelled unconjugated pteridines were not isolated, although they were not extensively studied because their R_F values differed from the radioactive materials and the radioactive compounds decomposed to PteGlu when antioxidants were absent. 2-Amino-4-hydroxypteridine and 10-formylpteroylglutamic acid appeared as extremely small amounts of radioactivity on only two occasions and they were derived from other labelled materials.

The microbiological activity of the 5-CH_3-H_4-PteGlu showed maximum activity for *L. casei* and little activity for *S. faecalis*, which was nevertheless higher than expected and may have been caused by decomposition during the assay. The unidentified material showed similar specific radioactivity to the 5-CH_3-H_4PteGlu and therefore was a naturally occurring metabolite of PteGlu. Its activity with *L. casei* was identical before and after conjugase treatment; the molecule therefore is unlikely to contain more than three glutamic acid residues. The compound was not reducible. From this evidence and the R_F values, it is tentatively identified as the di- or tri-glutamate of 5-methyltetrahydropteroate. Because of the similarity in microbiological activity these methods cannot distinguish between the unidentified compound and 5-CH_3-H_4PteGlu.

Early work suggested an increase in *P. cerevisiae* activity (citrovorum factor) in the urine after oral administration of PteGlu but isolation media often contained no antioxidants and compounds with citrovorum factor activity were accompanied by unidentified *S. faecalis*-active material. To account for the citrovorum factor activity, first reported by Sauberlich (1949), two groups of workers (Albrecht & Broquist, 1956; Silverman *et al.* 1956) proposed that it was 5,10-CH=H_4PteGlu, which was isolated by Silverman *et al.* (1956). These results must be viewed with caution, since both groups used very large doses (up to 175 mg for humans) and Silverman *et al.* (1956) used formic acid, which, we have shown, reacts with 5-CH_3-H_4PteGlu to give 5,10-CH=H_4-PteGlu. Low conversions into urinary citrovorum factor activity have been reported for PteGlu when given orally (Anderson *et al.* 1960; Broquist, Stokstad & Jukes, 1951; Baker *et al.* 1965; Oji, Wada & Yoshida, 1951) or subcutaneously (Guggenheim, Halevy, Neumann & Usiel, 1956). More recently McLean & Chanarin (1966) have shown no activity for *P. cerevisiae*. We did not obtain 5,10-CH=H_4PteGlu and the compounds isolated showed no activity for *P. cerevisiae*. Comparable observations have been reported for blood; Usdin (1959), using no antioxidants, obtained 5-HCO-H_4PteGlu (active for *P. cerevisiae*) although 5-CH_3-H_4PteGlu was the major folate present.

Labelled unconjugated pteridines were not present in the urine. Neopterin (Sakurai & Goto, 1967) and biopterin (Broquist & Albrecht, 1955; Patterson *et al.* 1955, 1956) may have been derived from compounds in the diet or by synthesis from purines (reviewed by Kaufman, 1967), although this has not been shown for mammals. Isoxanthopterin (Blair, 1958) may have been derived by folate degradation. Orally, subcutaneously or intravenously administered PteGlu did not give rise to increased xanthopterin in the urine (Rauen & Haller, 1950); the origin of xanthopterin (Koschara & Hrubesch, 1939) is uncertain.

The 5-CH_3-H_4PteGlu and the unidentified material were also obtained on the second and third days of excretion, and also at oral doses of 3.2 and 320 μg/kg. The specific radioactivity of the 5-CH_3-H_4PteGlu isolated from day 1 samples at the three dosages showed that this metabolite had been diluted by 100–200 μg of folate, which is about half the total hepatic folate found by using *L. casei* (Bird, McGlohon & Vaitkus, 1965).

A large dose (1.6 mg/kg) of non-labelled PteGlu given orally 3.5 h before or 3.5 h after [2-^{14}C]PteGlu produced an increase in radioactivity and labelled PteGlu in the urine. This is in agreement with absorption of PteGlu without metabolism (Whitehead & Cooper, 1967; Butterworth, Baugh & Krumdieck, 1969; Smith, Matty & Blair, 1970), but in disagreement with Cohen (1967) who obtained metabolites on the serosal side of everted-sac preparations, although the possibility that these arose from endogenous folate was not ruled out. Chanarin & Perry (1969) obtained an increase in growth-promoting activity for *L. casei* but no increase in activity for *S. faecalis* (which grows with H_2PteGlu and H_4PteGlu) in the systemic blood

after oral doses of tritiated H_2PteGlu and H_4-PteGlu, and concluded that metabolism to 5-CH_3-H_4PteGlu occurred during absorption. However, the peak of radioactivity occurred significantly earlier than the peak of *L. casei* activity and the inability of these investigators to measure portal-blood folate also does not warrant their conclusion (Bernstein, Gutstein, Weiner & Efron, 1970).

Flushing doses of unlabelled PteGlu given 24h or more after the [2-^{14}C]PteGlu produced no significant increase in radioactivity excreted. Similar results were obtained for humans (Butterworth *et al.* 1969). The small amounts of radioactivity were accompanied by low specific radioactivity of the metabolites, showing extensive dilution owing to metabolism of the flushing dose. Labelled PteGlu was not present in the urine, which suggests that the [2-^{14}C]PteGlu had been completely metabolized within 24h. This was substantiated in that labelled PteGlu was not present in the liver 24h after an oral dose of 320μg/kg (J. A. Blair & E. Dransfield, unpublished work). These results are in disagreement with those of Johns *et al.* (1961) who obtained displacement of tritiated PteGlu by intravenous flushing doses. They did not use antioxidants and their results are subject to criticism on the grounds of decomposition of metabolites and the displacement of radioactivity found by them, but not by ourselves nor by Butterworth *et al.* (1969), may reflect the high specific radioactivity of the tritiated PteGlu.

Metabolic studies can now be undertaken by using the biologically active diastereoisomers isolated during this work.

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