TRANSPORT OF 5-METHYLTETRAHYDROFOLIC ACID ACROSS THE RAT JEJUNUM.

A THESIS

submitted for the degree of

Doctor of Philosophy

by

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SUMMARY.

The intestinal transport of 5-methyltetrahydrofolic acid was investigated using various *in vitro* preparations of rat jejunum.

The transport of 5-methyltetrahydrofolic acid using the everted sac preparation was linear with increasing incubation time at the mucosal 5-methyltetrahydrofolic acid concentration of 10^{-5}M. Tissue uptake was appreciable whether there was any concentration gradient between the mucosal and serosal medium or not.

Total transport studies carried out at mucosal concentrations of 5-methyltetrahydrofolic acid ranging from 10^{-7}M to 10^{-4}M failed to show any saturation of uptake. When the tissue uptake and serosal transfer of 5-methyltetrahydrofolic acid were considered separately at the aforesaid concentrations no saturation could be observed.

Absence of saturation kinetics, low temperature coefficient and low serosal to final mucosal 5-methyltetrahydrofolic acid concentration ratio fail to advocate, in any way a carrier mediated transport mechanism for 5-methyltetrahydrofolic acid.

Both 5-formyltetrahydrofolic acid and 2,4-dinitrophenol (DNP) were found to depress the tissue uptake and serosal transfer of 5-methyltetrahydrofolic acid if present in the mucosal medium. 5-formyltetrahydrofolic acid may compete for the brush-border binding site and thus reduce tissue uptake of 5-methyltetrahydrofolic acid but the reduced serosal transfer in presence of 5-formyltetrahydrofolic acid is difficult to explain. DNP by inhibiting proton formation may lead to a rise in the pH of the micro-

climate with a corresponding fall in the concentration of permeable zwitterion at the surface of the epithelial cell.

It was found that the radioactive mucosal efflux from the everted sacs of rat jejunum preloaded with the labelled 5-methyltetrahydrofolic acid was stimulated in the presence of mucosal unlabelled 5-methyltetrahydrofolic acid, folic acid, methotrexate and 5-formyltetrahydrofolic acid. Serosal transfer and tissue content of the labelled compounds from the sacs preloaded with the labelled 5-methyltetrahydrofolic acid were lower in presence of mucosal 5-methyltetrahydrofolic acid than in its absence.

The uptake of 5-methyltetrahydrofolic acid by preparations of isolated jejunal mucosal cells was also studied. At pH 7.0 all the uptake takes place within 3 minutes and very little or none in the following 3 minutes and 5 minutes after that. It appears that there is a rapid binding of 5-methyltetrahydrofolic acid to cell surface protein rather than transport into cell. At pH 5.0 there was again a rapid uptake within the first 3 minutes followed by a slower but significant uptake in next 3 minutes. This latter uptake represents transport into the cell. But there is no uptake after 6 minutes indicating saturation. Thus in terms of total uptake at 6 minutes or uptake between 3 and 6 minutes the uptake is greater at pH 5.0 than at pH 7.0. A similar situation may be found at 27°C.

Histochemical studies showed that these cells had lost the glycocalyx thus indicating its importance for the transporting of 5-methyltetrahydrofolic acid into the cell. At pH 5.0 about 15% of 5-methyltetrahydrofolic acid is present as neutral zwitterion and this is sufficient to allow transport into the cell to occur without the glycocalyx.

The results of this study have been discussed in

the perspective of the previous studies on 5-methyltetrahydrofolic acid and the parent compound folic acid and it has been suggested that 5-methyltetrahydrofolic acid is converted to the neutral zwitterion in an acid microclimate at the mucosal surface of the jejunum prior to transport and then transported by passive diffusion and also to some extent by solvent drag.

This work was carried out from October, 1971 to October, 1974 in the Department of Chemistry in the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.

A. Razzaque

CONTENTS

Page

Page

TABLES

Page

FIGURES

Page

PLATES

ACKNOWLEDGEMENTS

I would like to thank Dr. J. A. Blair and Professor A. J. Matty for supervision of this work, the Wellcome Trust for financial support, Mrs. P. M. Johnson for excellent typing, Dr. W. E. Jensen of the University of Chicago for the isolated cell preparation depicted in Plate IV, Mr. A. Youngs of the Department of Biological Sciences for the histological sections and my wife for her help and understanding.

SECTION 1.

INTRODUCTION AND LITERATURE SURVEY.

Part 1. Folates: a general introduction.

Part 2. Intestinal transport of folates: previous studies.

Part 3. The aims of the present study.

SECTION I. PART 1.

i) NOMENCLATURE AND ABBREVIATIONS OF PTERIDINES AND FOLATES.

The pteridines and folates are all derivatives of 'pteridine' a term coined by Wieland and Schopf (1925) for the bicyclic nitrogenous ring system as shown below, (Fig. 1.1)

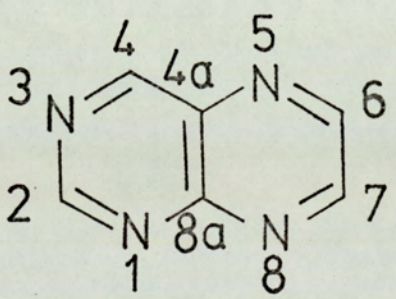


FIGURE 1.1. Pteridine.

The naturally occurring pteridines can be classified into two groups, the pterins, which are derivatives of 2-amino-4-hydroxy-pteridine (Fig. 1.2) and the lumazines which are derivatives of 2,4-dihydroxypteridine (Fig. 1.3).

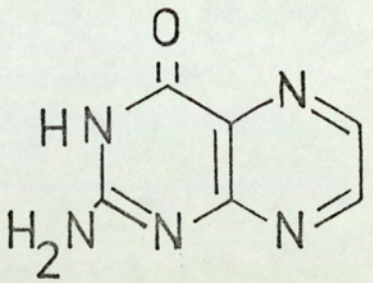


FIGURE 1.2 2-amino-4-hydroxypteridine: pterin.

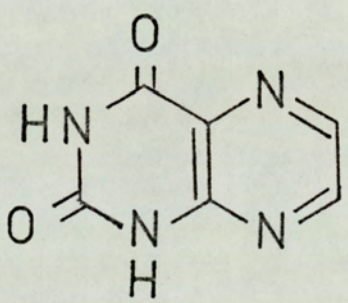


FIGURE 1.3 2,4-dihydroxypteridine: lumazine.

These compounds are often referred to as 'unconjugated' pteridines, but this usage will not be adopted.

The majority of the compounds either in solution at acid or neutral pH or in the solid state are thought to exhibit oxygen functions on the pteridine ring in the keto form, and in the formulae are drawn as such.

The abbreviations and numbering system for the

pteroates and folates are those of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry Commission on Biochemical Nomenclature. The structure of the folates are given, together with their abbreviations and common synonyms (Figures 1.6 to 1.15). Folic acid will be used in preference to the more cumbersome pteroylglutamic acid; 'folate' will be used as a generic name. Folic acid has been abbreviated to PteGlu throughout the text.

Folate polyglutamates are generally thought to be L-glutamate oligopeptides with a γ-peptide bond. The abbreviations shown refer to this type of bond, any compounds having α-bonds being written out in full. Pteroyldiglutamic acid will be indicated by the abbreviation PteGlu together with the appropriate suffix, e.g. $PteGlu_2$. Other abbreviations are used as follows:

FAD - Flavin adenine dinucleotide

NAD - Nicotinamide adenine dinucleotide.

NADP - Nicotinamide adenine dinucleotide phosphate.

ADP - Adenosine diphosphate.

ATP - Adenosine triphosphate.

RNA - Ribonucleic acid.

DNA - Deoxyribonucleic acid.

DNP - Dinitrophenol.

FIGLU - Formiminoglutamic acid.

Abbreviations and formulae for pteroic acid, folic acid and their derivatives have been shown in the next few pages. (Figures 1.4 to 1.15).

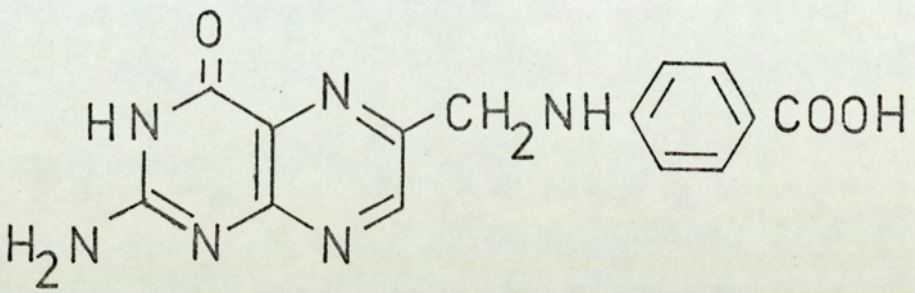


FIGURE 1.4 Pteroic acid; Pte.

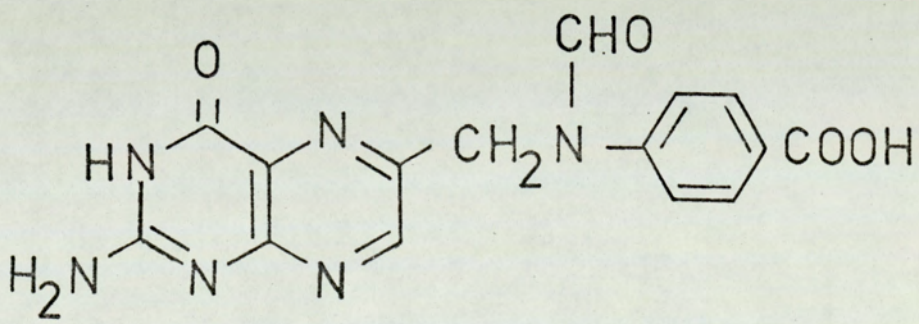


FIGURE 1.5 10-formylpteroicacid; 10CHO-Pte; Rhizopterin.

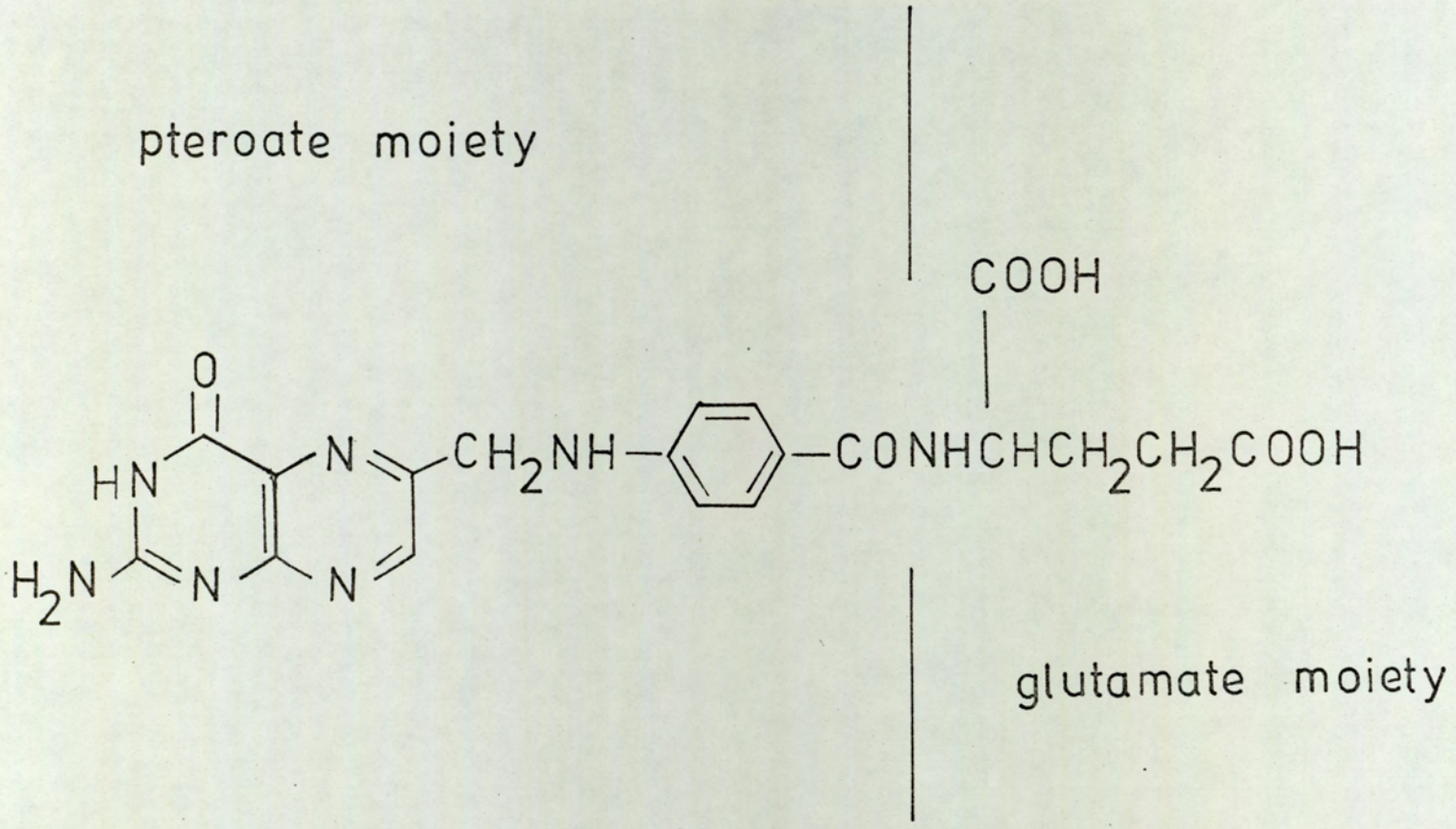
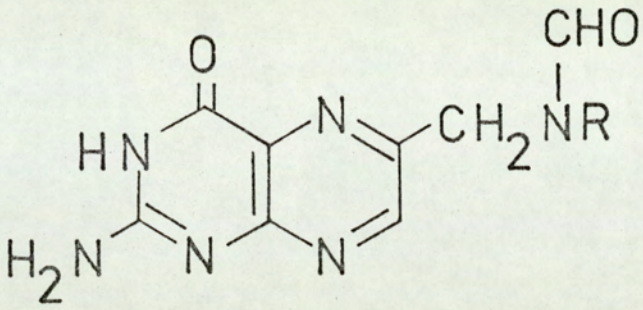


FIGURE 1.6. Pteroylmonoglutamic acid; folic acid; PteGlu.



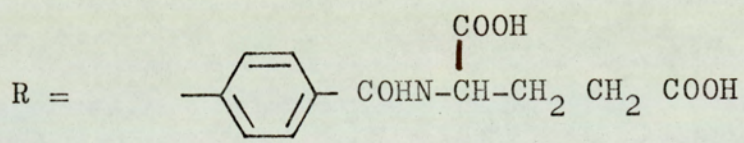


FIGURE 1.7 10-formylfolic acid; 10 CHO-PteGlu

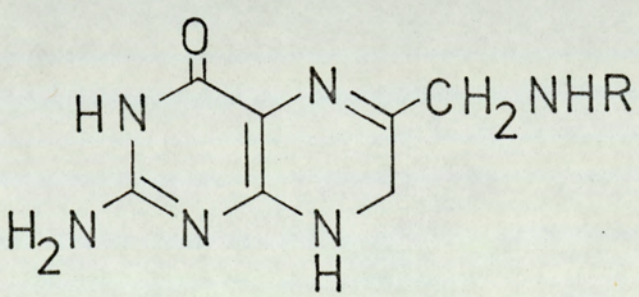


FIGURE 1.8 7,8 dihydrofolic acid; H_2 PteGlu

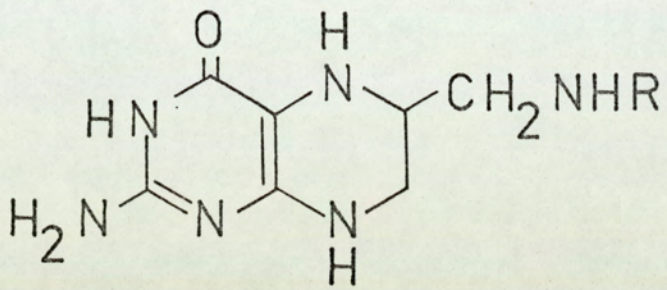


FIGURE 1.9 5,6,7,8-tetrahydrofolic acid; H_4PteGlu.

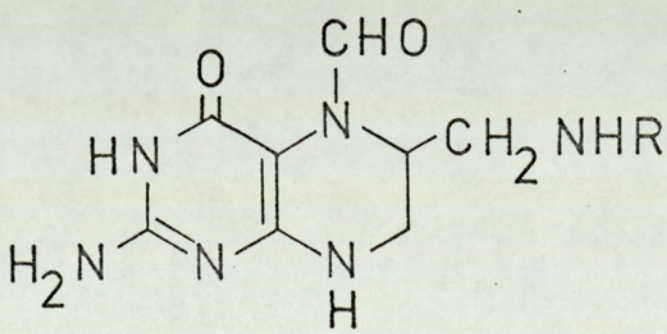


FIGURE 1.10 5-formyltetrahydrofolic acid; 5 CHO-H_4PteGlu; Citrovorum factor; folinic acid; 'Leucovorin' (trade name of Lederle Laboratories).

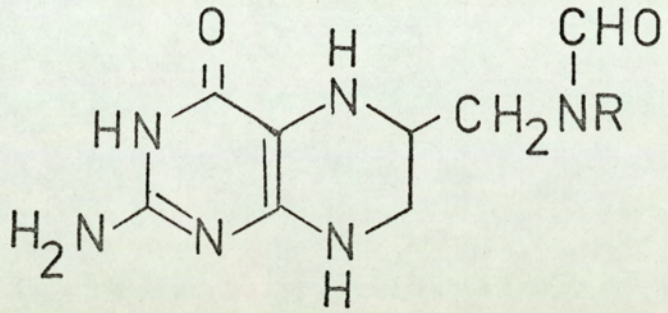


FIGURE 1.11 10-formyltetrahydrofolic acid; 10 CHO-H_4PteGlu.

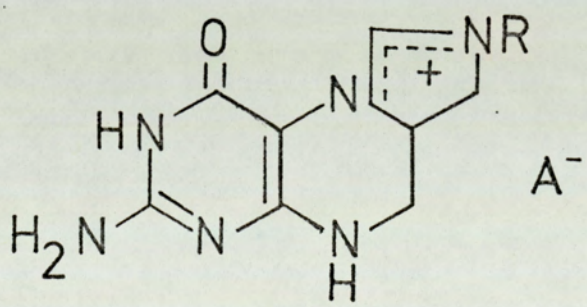


FIGURE 1.12 5,10-methenyltetrahydrofolic acid;
5,10CH = H_4PteGlu.

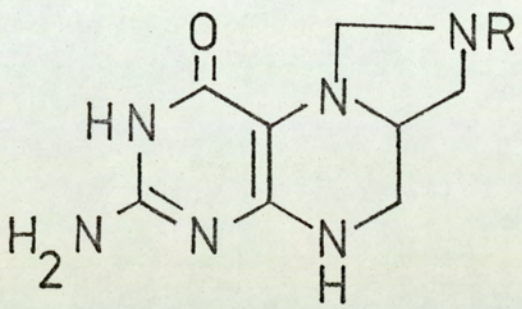


FIGURE 1.13 5,10-methylenetetrahydrofolic acid; $5,10CH_2$ - H_4PteGlu.

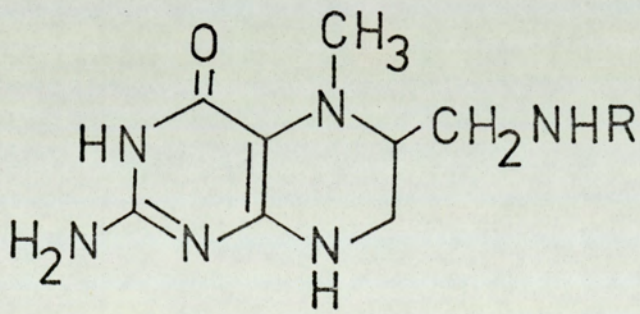


FIGURE 1.14 5-methyltetrahydrofolic acid; $5CH_3$-H_4PteGlu

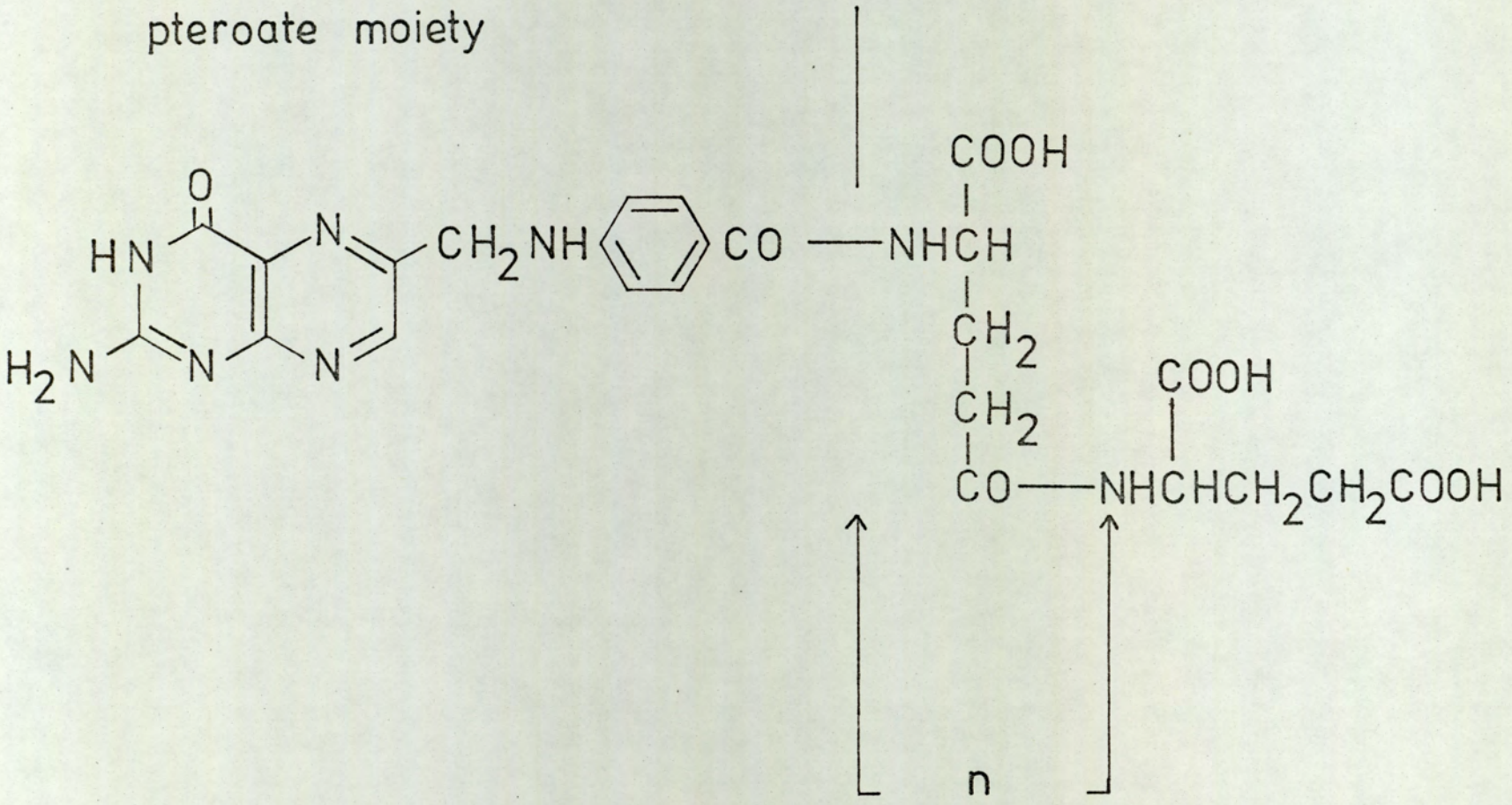


FIGURE 1.15 Folate polyglutamate; $PteGlu_n$ (n = 1 to 7); $PteGlu_2$ 'Diopterin'; $PteGlu_3$ 'Teropterin'; Derivatives are named and abbreviated similarly to the monoglutamates.

ii) HISTORICAL BACKGROUND.

In one of his earliest scientific papers, published in 1889, F. G. Hopkins described pigments which he obtained from butterfly wings (Hopkins, 1889). He encountered considerable difficulty in their analysis, for very little material was available, and the pigments were very sparingly soluble in water and not at all in organic solvents; he concluded that those were derivatives of uric acid. Later work showed them to be pteridines.

Weiland and Schopf (1925) isolated a pure yellow pigment from the wings of the brimstone butterfly and the same workers (1926) prepared a white pigment from the wings of the cabbage butterfly. These pigments were named by them xanthopterin and leucopterin respectively, as an indication of the colour and source of these compounds (Gk. xanthos, yellow; leukos, white; pteron, wing).

Difficulties were encountered in determining the structures of these compounds and it was not until 1940 that Purrmann showed that xanthopterin and leucopterin have the structures (Figure 1.16) and (Figure 1.17) respectively (Purrmann,1940, 1941a), Purrmann(1941b)also determined the structure of the insect pigment isoxanthopterin (Figure 1.18).

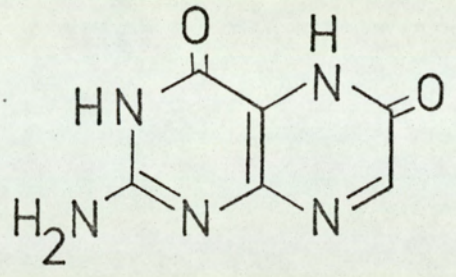


FIGURE 1.16 Xanthopterin.

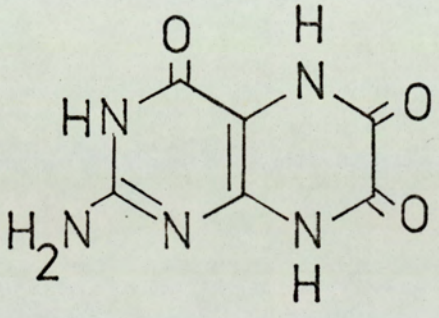


FIGURE 1.17 Leucopterin.

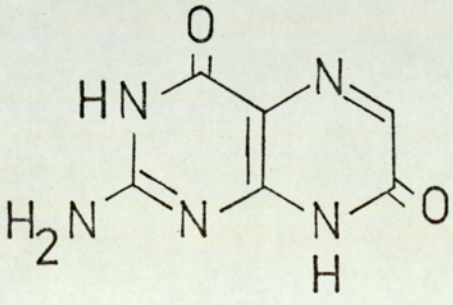


FIGURE 1.18 Isoxanthopterin.

A very large volume of work has dealt with the distribution of pterins in almost all phyla of the animal and plant kingdoms.

The classical studies of Dr. Lucy Wills (1931) in Bombay, India, drew attention to the importance of nutritional megaloblastic anaemia in pregnant women. She reported that extracts of autolyzed yeast or liver are effective in the treatment of tropical macrocytic anaemia. She also reproduced this anaemia in monkeys by means of a diet composed chiefly of polished rice and white bread, similar to that eaten by her patients. This anaemia did not respond to any vitamin known at that time, nor to purified liver extract (presumably containing cyanocobalamin). Yet good clinical responses were obtained with an autolysed yeast or liver extracts (Wills, 1933; Wills, Clutterbuck & Evans, 1937a,b) which was generally ineffective in Addisonian pernicious anaemia. It was evident, therefore, that yeast contained some unknown anti-anaemic principle (the 'Wills' factor) that was different from the factor present in purified liver extract. It was also termed as Vitamin M.

It has also been found that chicks fed a purified diet had their growth retarded, but the growth could be stimulated by a dietary factor from yeast, alfalfa, wheat bran and liver, variously called factor U (Stokstad & Manning, 1938), Factors R & S (Schumacher, Heuser & Norris, 1940), Vitamin B_c (Hogan & Parrott, 1940), and Vitamin M, this latter from studies on nutritional cytopenia in monkeys (Wills & Stewart, 1935; Day, Langston & Darby, 1938; Langston, Darby, Shukers & Day, 1938).

A critical step leading towards the isolation and

identification of these factors was the discovery that a factor in charcoal eluate of yeast served as a growth factor for Lactobacillus casei (Snell & Peterson, 1940; Hutchings, Bohonos & Peterson, 1941) and it was called the Norite eluate factor.

Some yeast extracts which were curative in the macrocytic anaemia of chicks and monkeys would not support the growth of L. casei (Binkley, Bird, Bloom, et al, 1944). It thus appeared that the chick anaemia assay and the L. casei assay were not measuring the same principle.

This apparent paradox was clarified by Bird and his co-workers (Bird, Bressler & Brown et al, 1945) who showed that the anti-anaemia principle of yeast would support the growth of L. casei if the yeast extract was first digested with crude tissue homogenates obtained from animal sources. They gave the name "vitamin B_c conjugate" to the complex form of the anti-anaemia principle already known as vitamin B_c, and they named the enzymes which release vitamin B_c from that complex "conjugases". Exploiting the microbiological and chick assays, Stokstad (1943) and Pfiffner and his coworkers (1943) independently isolated the free vitamin from yeast and liver. They also utilized conjugases as an adjunct to microbiological methods and accomplished the isolation and identification of the conjugated vitamin from yeast (Pfiffner, Calkins, O'Dell et al, 1945).

A factor active for Streptococcus faecalis was also isolated from spinach leaves (Mitchell, Snell & Williams, 1941, 1944) and was named folic acid.

The synthesis of pteroylglutamic acid (Angier, Boothe, Hutchings, et al, 1946) at Lederle and American

Cyanamid and confirmation of its structural identity (Figure 1.6) with "folic acid" or "Lactobacillus caseifactor" represented the culmination of more than a decade of investigation into the nature of a variety of seemingly unrelated growth principles or anti-anaemia factors.

Derivatives of folic acid have been isolated, which exhibited that the naturally occurring folates were reduced, substituted derivatives; $5CHO\text{-}H_4PteGlu$ was isolated from liver (Sauberlich & Baumann, 1948; Bond, Bardos, Sibley & Shive, 1949; Keresztesy & Silverman, 1951; Sauberlich, 1952) and the existence of its isomer, $10CHO\text{-}H_4\text{-}PteGlu$ and cyclised product $5,10CH{=}H_4PteGlu$ recognised. Later came the discovery of $5CH_3\text{-}H_4PteGlu$ in horse liver (Donaldson & Keresztesy, 1959, a,b; 1961 a,b).

As this thesis solely documents the studies on the uptake of 5-methyltetrahydrofolic acid by rat jejunum along with the possible metabolism during the transport, attempts have been made to elaborate the points associated with 5-methyltetrahydrofolic acid in comparison with other folates. Hence a detailed description of the isolation and identification of the compound has been furnished here.

When a hot aqueous extract of horse liver was incubated with a hog liver homogenate there was a significant increase in Citrovorum Factor activity. The form of folic acid which was converted to Citrovorum Factor was tentatively designated as prefolic acid. There were several forms of prefolic acid in liver. One of these prefolic acids, prefolic A, was partially purified and its enzymatic conversion to Citrovorum Factor was found to proceed via two stages. In the first stage the prefolic acid A was

converted to a product with the properties of H_4PteGlu and required FAD as a cofactor. In the second stage the intermediate was formylated to Citrovorum Factor (Donaldson & Keresztesy, 1959 a,b).

Prefolic A did not support the growth of S. faecalis or L. citrovorum and required in addition to FAD-linked enzyme system for its conversion to H_4PteGlu, an electron acceptor, such as O_2, indigo disulphonate or triphenyltetrazoleum. The other requirement was ascorbic acid, which appeared to act as a protective agent (reducing agent) for H_4PteGlu and could be replaced by cysteine, glutathione or mercaptoethanol (Donaldson & Keresztesy, 1959a).

Larrabee and Buchanan (1961) found an intermediate formed during the synthesis of methionine from N^5,N^{10} methylene tetrahydrofolic acid and homocysteine in presence of NAD, FAD, ATP, Mg^{++} and two enzymes, this was one carbon substituent folate derivative incapable of replacing folic acid or tetrahydrofolic acid and yielded approximately one molecule of $-CH_3$. per molecule of the compound. Prefolic A, exhibited special characteristics similar to H_2PteGlu and H_4PteGlu. Mild oxidation of the compound resulted in an acid labile form which could be reconverted to Prefolic A by ascorbic acid and mercaptoethanol (Donaldson & Keresztesy, 1961b). The above observations suggested the possibility that prefolic A might be a CH_3H_4PteGlu.

An enzymatic system isolated from a mutant (Davis & Mingioli, 1950) of Escherichia coli was found to be capable of carrying out the reaction N^5,N^{10}-methylene H_4PteGlu + homocysteine $\longrightarrow$ methionine + H_4PteGlu. This enzymatic system was comprised of two enzymes. One

enzyme was designated as the "205 - 2" enzyme. The other enzyme contained vitamin B_{12} or a derivative as a prosthetic group and was designated as the "B_{12}-enzyme" (Hatch et al, 1961).

When the enzyme "205 - 2" was incubated with NAD and N^5, N^{10} methylene H_4PteGlu labelled in mythylene carbon with ^{14}C, a radioactive product was formed which upon hydrolysis yielded one molecule of glutamic acid/molecule of C-labelled with ^{14}C. The product exhibited maximum absorption at 290 mμ, did not react with aldehyde binding reagent, yielded radioactive CH_3I when treated with HI. This was designated as Compound I. When this compound was incubated with homocysteine, NAD, FAD, ATP, Mg^{++} and "B_{12} enzyme" methionine and H_4PteGlu were formed. The latter was identified by its ability to support growth of L. citrovorum. $N^{10}CH_3H_4$PteGlu could not serve as the substrate of the above reaction. From the above findings N^5 CH_3H_4PteGlu was proposed as the tentative structure of compound I (Larrabee, Rosenthal, Cathou & Buchanan, 1961).

Prefolic A. when enzymatically oxidised to H_4PteGlu one molecule of HCHO was formed. The reversal of the reaction, i.e. the biosynthesis of prefolic A by reduction of methylene tetrahydrofolic acid by NAD in presence of HCHO (Donaldson & Keresztesy, 1961a), and the chemical synthesis of prefolic A by $NaBH_4$ reduction of methylene tetrahydrofolic acid (Keresztesy & Donaldson, 1961) further confirmed that prefolic A was a methyl substituted derivative of H_4PteGlu. The location of the $-CH_3$ on prefolic A was not established. However, N^5 position was the most likely one. In support of this was the greater stability of prefolic A

when compared with N^{10} substituted reduced folic acid derivatives. Blakley (1959, 1960) reported that N^{10} substituted hydropteridines bound significant amounts of HCHO, whereas N^5 substituted derivatives bound little or no HCHO. These observations supported the view that prefolic A was $5\text{-}CH_3\text{-}H_4PteGlu$. Sakami and Ukstins (1961) has postulated that the major KBH_4 reduction production of a mixture of $H_4PteGlu$ and HCHO was $5CH_3H_4PteGlu$ and showed that it was the intermediate in methionine synthesis. On the basis of all the observations, findings and arguments the prefolic A was characterised as a $N^5CH_3H_4PteGlu$ (Donaldson and Keresztesy, 1962).

The compound isolated from yeast (Pfiffner, Calkins, O'Dell, Bloom, Brown, Campbell & Bird, 1945), which was active as an anti-anaemia factor in the chick, but inactive for microbiological assay until digested with proteolytic enzyme, 'conjugase' was given the structure (Figure 1.15, $n = 5$) and was the first member of the folate polyglutamates: it remains the best characterized naturally extracted polyglutamate. Much of the dietary folate contains polypeptide, generally considered to be polyglutamate, and they also seem to occur in other tissues.

iii) OCCURRENCE AND METABOLIC FUNCTIONS OF PTERIDINES.

Pteridines are widely distributed in nature, e.g. in insects, amphibia, reptiles, fish, crustacea, micro-organisms and plants. In many cases the functions are not known. The occurrence of pterins in phyla mentioned is well reviewed by Blakley (1969), and will not be considered further.

The occurrence of pterins in mammals has not been well documented. Xanthopterin has been isolated from human urine (Koschara, 1936). Isoxanthopterin has been found in human urine (Blair, 1958; Foxall, 1967) and un-specified pterins active for Crithidia fasciculata have been found in blood and serum of man, cattle, sheep, horse, rabbit and chicken (Frank, Baker & Sobotka, 1963). Biopterin (Figure 1.19) is found in

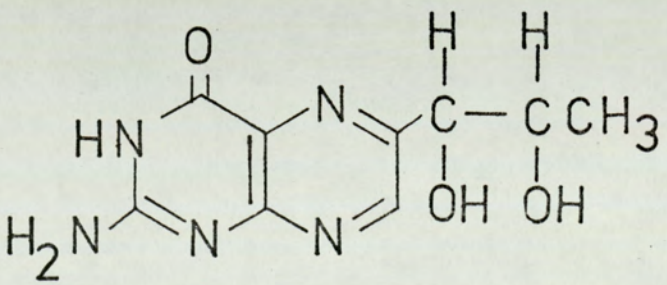


FIGURE 1.19 Biopterin.

human urine (Broquist & Albrecht, 1955; Patterson et al, 1956; Fukushima & Shiota, 1972), and is of interest since its origins within the animal are not known, and its

reduced derivative tetrahydrobiopterin (Figure 1.20).

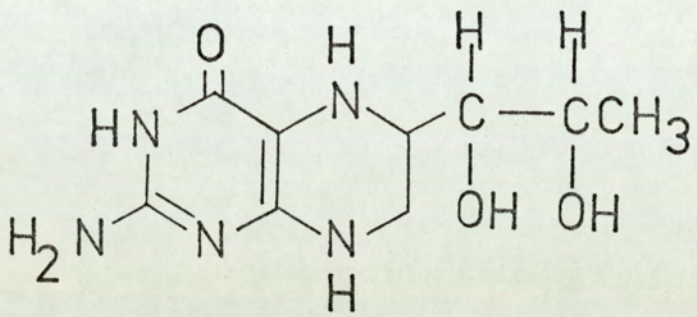


FIGURE 1.20 Tetrahydrobiopterin.

is a cofactor for the oxidation of phenylalanine to tyrosine (Figure 1.21).

This latter reaction has been extensively studied. The enzyme system, phenylalanine hydroxylase occurs in all mammalian liver examined. Cofactor activity was shown to be necessary (Kaufman, 1958a) and the naturally occurring cofactor was isolated as dihydrobiopterin (Kaufman, 1963) although its functional form is tetrahydrobiopterin (Rembold & Metzger, 1967). H_4PteGlu was thought to be active in the phenylalanine hydroxylating system (Kaufman, 1958b) although later work has shown that an impurity, 2-amino-4-hydroxy-6-methyltetrahydropteridine, present in the H_4PteGlu is the active material (Lloyd, Mori & Kaufman, 1971). The reaction scheme in Figure 1.21 has been taken from the review (Kaufman, 1967).

The origin of biopterin in mammals is unknown. Intraperitoneal injection of (2-^{14}C) tetrahydrobiopterin into rats gave biopterin as 15% of total urinary metabolites;

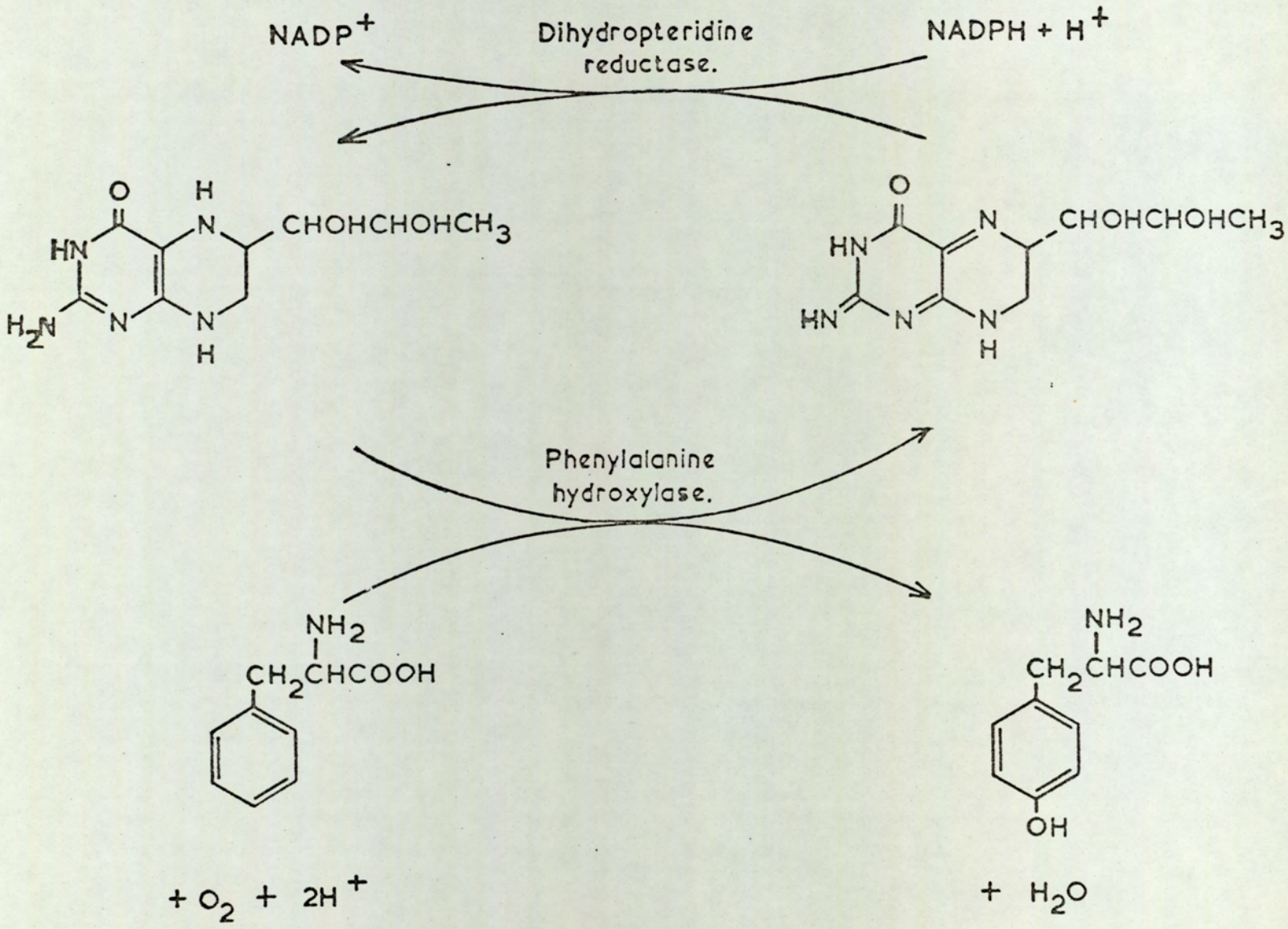


FIGURE 1.21 The hydroxylation of phenylalanine to tyrosine.

(2-^{14}C)-tetrahydroneopterin, (6,7,1',2',3',^{14}C)-tetrahydroneopterin, (6,7,1'-^{14}C) 7,8-dihydro-6-hydroxymethylpterin gave no excreted biopterin (Rembold, Chandrashekar & Sudershan, 1971). On a "biopterin free" diet, rats excrete 20 μg of biopterin in urine in 24 hours (Pabst & Rembold, 1966). Reports of the conversion of PteGlu to biopterin in Crithidia fasciculata (Cowperthwaite et al, 1953; Kidder & Dutta, 1958; and Kidder et al, 1967), and to pterin: 2 amino-4-hydroxypteridine and isoxanthopterin in man (Blair, 1958) have appeared. Assay of human urine with Crithidia fasciculata (Leeming & Blair, 1974) shows 7,8 dihydrobiopterin as major component with tetrahydrobiopterin probably the minor. High folic acid intake does not affect the level of biopterin and neopterins in urine, hence its role as precursor of pterins is not established (Fukushima & Shiota, 1972). Beavon (1973) reported that 10-CHO PteGlu when given orally to rats was not found to be metabolized, but gave large amount of excreted pteridines a distinguishing feature as compared with other compounds. The nature of the pteridines was not identified.

iv) OCCURRENCE AND BIOLOGICAL SIGNIFICANCE OF FOLATES AND 5-METHYLTETRAHYDROFOLIC ACID.

Folate is found in a wide variety of foodstuffs. Most food and other folates have been determined by micro-biological assay. The micro-organisms used were *Lacto-bacillus casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*. The response of these micro-organisms to the principal folates is shown in Table 1.1. The normal adult Western diet contains about 600-700 µg of folate daily as determined by *L. casei* assay after conjugase treatment and three quarters of this is believed to be in the polyglutamate form (Butterworth *et al*, 1963; Chanarin *et al*, 1968; Chanarin, 1969). Hurdle *et al* (1968), on the other hand do not contribute to the view that three quarters of dietary folates are polyglutamates, they rather, report that heat labile simple folates are often present in greater amount than complex polyglutamates. Liver and yeast which are the richest sources of dietary folate, contain respectively, 5.10 µg and 3.05 µg of folate per gram of wet weight (Hurdle *et al*, 1968). Literature values differ widely as to the folate content of liver, e.g. the value in the rat being 20 µg/g of wet tissue (Bird, McGlohon & Vaitkus, 1965), 9 - 15 µg/g of wet tissue (Grossowicz, Rachmilewitz & Izak, 1963; Grossowicz, Izak & Rachmilewitz, 1964) or 7 - 15 µg/g of wet tissue (Bennett, Berry, Chanarin & Ardeman, 1964). Chicken liver contains 10 µg/g of wet tissue (Noronha & Silverman, 1962). Some workers have found 5-CH_3H_4PteGlu and 5CHO-H_4PteGlu along with considerable quantities of polyglutamates of these compounds as the major folates

TABLE 1.1

Microbiological response to Folates.*

	L. casei	S. faecalis	P. cerevisiae
Pteroate	-	+	-
PteGlu	+	+	-
H_2PteGlu	+	+	-
H_4PteGlu	+	+	+
5CHO-H_4PteGlu	+	+	+
10CHO-PteGlu	+	+	-
10CHO-H_2PteGlu	+	+	-
10CHO-H_4PteGlu	+	+	+
5CH_3-H_4PteGlu	+	-	-
$PteGlu_2$	+	+	-
$PteGlu_3$	+	-	-
$PteGlu_7$	-	-	-

+ indicates a response of at least 50% of the maximum.

- indicates a response less than 5% of the maximum.

*Adapted from Blakley (1969) and Chanarin (1969)

(Bird et al, 1965) in the rat liver, whereas others have found a predominance of pentaglutamates, e.g. 5-$CH_3H_4PteGlu_5$, 10CHO $PteGlu_5$, $H_4PteGlu_5$ in the rat liver (Shin et al, 1972; Houlihan & Scott, 1972; Thenen et al, 1973). Brown et al (1974) found 5-$CH_3H_4PteGlu_{5,6}$ as the predominant folate in monkey liver after 3 days following administration of tritium labelled PteGlu. About 85% of the guinea pig liver folates were reduced pteroyl polyglutamates and 53% comprise the methylated derivatives (Corrocher et al, 1972). The predominant forms of folates in sheep liver were identified to be hexaglutamates of H_4-Pte-Glu, $5CH_3$-$H_4PteGlu$, 5CHO-$H_4PteGlu$ and 10CHO $H_4PteGlu$ (Osborne-White & Smith, 1973).

Some 75 - 90% of folate in a mixed diet is in the form of a polyglutamate, that is, the pteroylglutamic acid moiety has a chain of generally six or more glutamic acid residues (Butterworth, Santini & Frommeyer, 1963; Chanarin, Rothman, Perry & Stratfull, 1968). Analysis of pea seedlings, yeast, and milk indicates that significant amounts of reduced folate, primarily $5CH_3H_4PteGlu$ either in the free form and/or in the conjugated form are found (Bird et al, 1965; Ramasastri, 1965; Roos et al, 1968). Other major compounds in the diet are 10-formyl-pteroyl-polyglutamate, and 5-formyl-tetrahydropteroylpolyglutamate (Butterworth et al, 1963).

Folates occur in blood, serum and plasma. Red cells have relatively large amounts, 105 - 407 ng/ml (Chanarin, 1969), 210 $\pm$ 57 ng/ml (Rothenberg et al, 1974) and 307 ng/ml (Khalil et al, 1973) in man. Serum folate in normal subjects varies widely, results of several studies

ranging from 5.1 to 16.0 ng/ml being given by Chanarin (1969). 5-CH_3H_4PteGlu is the principal form in serum (Herbert, Larrabee & Buchanan, 1962; Noronha & Silverman, 1962; Bird et al, 1965; and Baker et al, 1965).

Apparently between 60 and 90 µg of folate enters the bile each day. Biliary folate is mainly in the form of 5-CH_3H_4PteGlu and formylfolates (Bernstein et al, 1970a; Pratt & Cooper, 1971; Lavoie & Cooper, 1974). An unidentified radioactive folate appeared in bile upon intravenous infusion of [^{3}H] folic acid into patients with biliary fistulae (Lavoie & Cooper, 1974). It contained both the pteridine and para-aminobenzoate portions of folate but appeared not to support the growth of the test micro-organisms and not be bound to a protein or a chain of γ-glutamates. Of the few other tissues that have been studied, rat kidney contains 1.6 µg/g of folate, spleen and intestine 0.6 µg/g (Grossowicz, Rachmilewitz & Izak, 1963). Human marrow cells contain high levels of S. faecalis active folate about 95 - 726 ng/ml (Cooke et al, 1959). Folates in human milk, cow's milk and goat's milk show mean levels of 24, 38 and 6 ng/ml respectively (Matoth, Pincus and Sroka, 1965; Ramasastri, 1965 and Ford et al, 1972). A mean value of 23.6 ng/ml was reported for human cerebro-spinal fluid (Wells & Casey, 1967). 5CH_3H_4PteGlu is again the predominant form in milk and cerebro-spinal fluid (Ford et al, 1972; and Chanarin et al, 1974).

Milk has been reported to contain folate binding proteins (Metz & Herbert, 1967; Ghitis, Mandelbaum-Shavit & Grossowicz, 1969; Ford et al, 1972) and there is also good evidence that a large proportion of serum folates are

protein bound (Markkanen, 1968; Retief & Huskisson, 1970; Markkanen & Peltola, 1971; Markkanen et al, 1972a), but contradictory reports that human blood serum does not contain a strong folic acid-binder have also appeared (Metz, Zalusky & Herbert, 1968; Mantzos, Alevizou-Terzaki & Gyftaki, 1974). Mantzos et al (1974) also found that with the exception of pig plasma none of the other animal plasma, e.g. sheep, goat, cattle, horse, rabbit, dog, rat, guinea pig and chicken contained any significant folic acid-binders.

The normal adult daily requirement of folate is approximately 100 μg (Herbert, 1968) and the total body stores of folate which are situated mainly in the liver are about 10 mg. A normal human daily folate intake has been estimated to vary from about 700 μg (Butterworth et al, 1963; and Chanarin et al, 1968) to 1000 to 1500 μg (Jandl & Lear, 1956) per day.

The major metabolic pathways of folic acid and its reduced derivatives have been substantially established in a variety of prokaryotic and eukaryotic organisms (Stokstad & Koch, 1967; Blakley, 1969).

In the mammal the major reactions (Figure 1.22) are seen to constitute a series of interlocking pathways in which, (i) folic acid is reduced to dihydrofolate and then to tetrahydrofolate in the presence of reduced pyridine nucleotides by an enzyme known as dihydrofolate reductase. Reduction to tetrahydrofolate is a basic requirement for coenzyme function. (ii) Tetrahydrofolic acid is the precursor of coenzymes which act in the transfer of 1-carbon units (Table 1.2). (iii) Folate coenzymes participate in certain aspects of purine and pyrimidine synthesis, formation of methionine from homocysteine, metabolism of other amino acids including

the catabolism of histidine and interconversion of serine and glycine, oxidation-reduction reactions of some folic acid derivatives and formylation reactions. The overall pathway is confined to the cellular cytoplasm although certain enzymes are known to be present within different organelles (Brown et al, 1965; Blakley, 1969; Rowe & Lewis, 1973). Enzymes involved in the various folate coenzyme participated reactions are dihydrofolate reductase, 10-formyltetrahydrofolate synthetase, serine transhydroxymethylase, methylene tetrahydrofolatedehydrogenase, methenyltetrahydrofolate cyclohydrolase, 5 methyltetrahydrofolate: L-homocysteine cobalamin S-methyltransferase and thymidylate synthetase.

The interconversion of serine and glycine mediated by serine hydroxymethyl transferase is the principal source of 1-carbon units in animal cells and in many organisms. Mansouri et al (1972) had shown that the biosynthesis of this enzyme in E. coli was controlled by the level of methionine in the growth medium but dihydrofolate reductase, thymidylate synthetase, and N^5,N^{10} methylenetetrahydrofolate dehydrogenase were unaffected. In experiments with lymphocytes from patients with folate deficiency, ^{14}C-formate incorporation into serine, methionine, R.N.A. and protein is found to be decreased (Ellegaard & Esmann, 1973).

According to the current concepts $5CH_3$-H_4PteGlu the predominant folate form in serum, can be converted to other active folate forms only after its conversion to tetrahydrofolate during the biosynthesis of methionine from homocysteine requiring cobalamin coenzyme (Blakley, 1969; Chello & Bertino, 1973). The reaction as furnished

FIGURE 1.22

METABOLIC FUNCTIONS OF FOLATE DERIVATIVES.

(modified from Blakley, 1969)

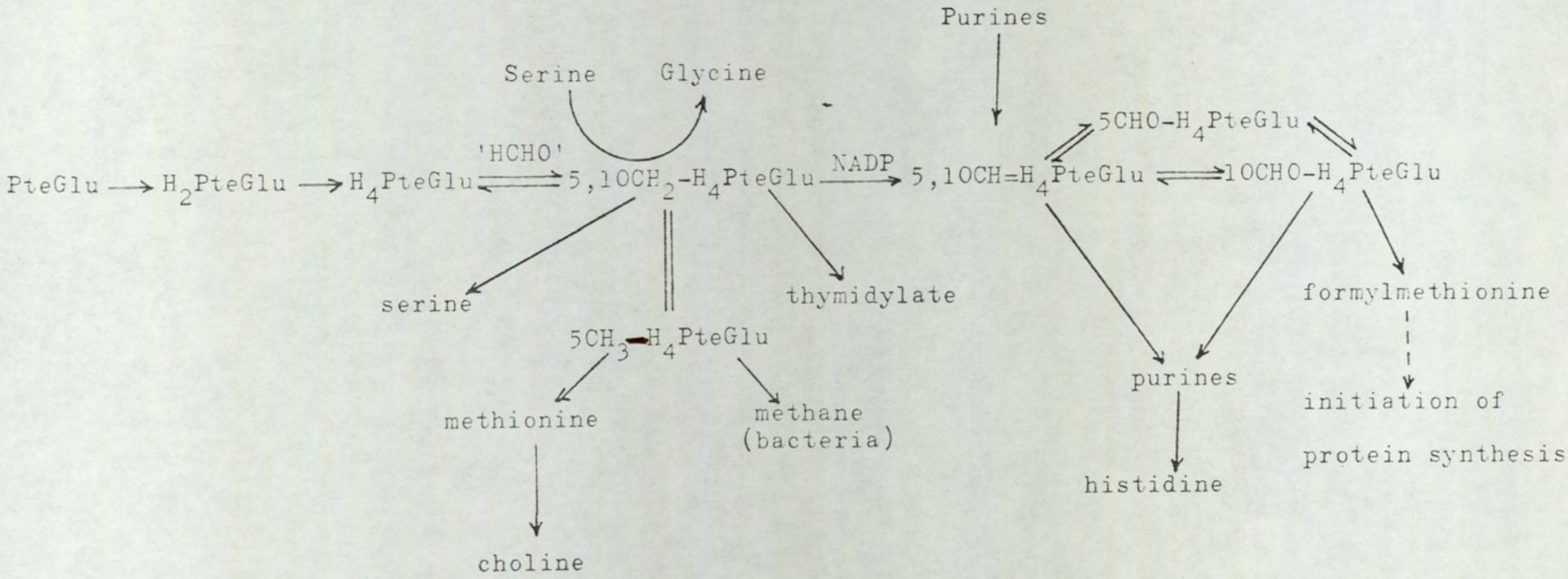


TABLE 1.2

METABOLICALLY ACTIVE FORMS OF FOLIC ACID

(Adapted from Stokstad & Koch, 1967)

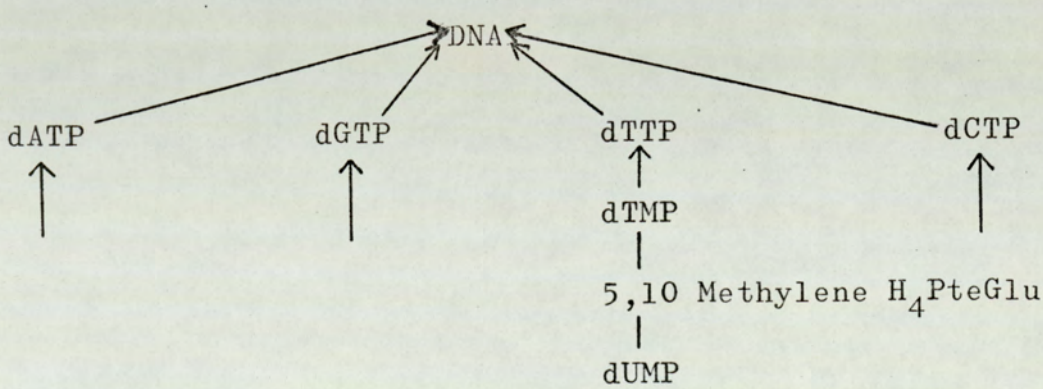
Form	1-Carbon Unit	Oxidation State
10-CHO-H_4 PteGlu	- CHO	Formate
5-CHO-H_4 PteGlu	- CHO	Formate
5,10-CH = PteGlu	= CH	Formate
5,CHNH H_4PteGlu	- CH = NH	Formate
5,10-CH_2=H_4PteGlu	- CH_2	Formaldehyde
5CH_3H_4PteGlu	- CH_3	Methanol

below:

$$5\ CH_3H_4PteGlu + Homocysteine \xrightarrow[Enzyme]{B_{12}} Methionine + H_4PteGlu$$

plays an important role in methyl group metabolism by supplying the methionine for S-adenosylmethionine synthesis, which subsequently participates in forming methylated polynucleotides, epinephrine, melatonin, creatine, phosphatidylcholine and other important metabolites.
So the folate coenzymes occupy important positions in the metabolic pathways leading to DNA and protein synthesis. Figure 1.23 illustrates the position of 5,10 methylenetetrahydrofolate in the production of thymidylate, one of the nucleotides precursors of DNA.

FIGURE 1.23



d - deoxy
A - Adenosine
G - Guanosine
U - Uracil
T - Thymidine
C - Cytidine
TP - Triphosphate
MP - Monophosphate

(Adapted from Hoffbrand & Tripp, 1972).

In man, megaloblastic anaemia is the almost invariable accompaniment of folate deficiency. It is thought to be caused by a disturbance of DNA synthesis in the bone marrow. Hoffbrand and Tripp (1972) have shown that acute folate deficiency caused by the anti-folate drug, methotrexate, does indeed cause a fall in intracellular concentration of dTTP, and increases levels of dATP, and they suggest that the latter effect leads to the inhibition of enzymes concerned in DNA synthesis and repair, in addition to the inhibitory effect of diminished dTTP supply. More recently, however, Hoffbrand et al (1974) have shown that bone marrow cells cultured from patients with untreated megaloblastic anaemia appear to have normal intracellular levels of both these nucleotides. The physiology of megaloblastic anaemia thus remains a matter for continued research.

The role of folate deficiency in the pathogenesis of megaloblastosis in protein-calorie malnutrition was claimed to be confirmed (Halsted et al, 1969; Khalil et al, 1973) by determining the serum and erythrocyte folate concentrations of folate.

Folic acid deficiency may be a significant factor in the pathogenesis of growth retardation and hypogonadism in children with sickle cell anaemia (Watson-Williams, 1962; Lopez et al, 1973), but the role of folic acid in growth retardation has been contradicted (Liu & Augusta, 1974).

Urinary folate loss may be a significant factor in the aetiology of folate deficiency of chronic liver disease (Retief & Huskisson, 1969).

Folic acid appears to play a major role in the metabolism of the developing foetus, and in animal experiments

folate deficiency is capable of causing a wide variety of congenital malformations, including abortion. Hibbard and Hibbard (1968) suggest that malformations were twice as common in the babies of mothers with megaloblastic anaemia of pregnancy compared with the general population, but Scott and coworkers (1970) failed to find any association between foetal malformations and maternal folate deficiency. On present evidence it seems likely that the spontaneous folate deficiency of human pregnancy is not associated with foetal malformations (Speidel, 1973).

A number of studies have established that red cell and plasma folate levels fall significantly during the neonatal period (Strelling et al, 1966; Roberts et al, 1969), high renal loss of folate has been suggested to be responsible for the observed fall (Landon & Hey, 1974). Blair (1970) and Hunter et al (1970) recorded toxic reactions in healthy volunteers given daily doses of 15 mg. of folic acid. Katz (1973) considered that large doses might cause neurologic relapse. Folic acid has been reported to induce acute renal failure apparently by increase in blood urea and cessation of urine flow (Schmidt et al, 1969; Searle & Blair, 1973; Schmidt et al, 1973).

In man folate deficiency may occur as a result of dietary deficiency, malabsorption, increased loss from the body and increased catabolism.

Folic acid in its different metabolic forms are inter-related to many other nutrients in biochemical and metabolic functions. Vitamin B_{12} is essential for the enzymatic conversion of $5\text{-}CH_3\text{-}H_4PteGlu$ to $H_4PteGlu$ during the biosynthesis of methionine from homocysteine (Blakley,

1969; Chello & Bertino, 1973; Tisman & Herbert, 1973). Vitamin B_{12} deficiency thereby leads to decreased DNA synthesis and consequent megaloblastosis. This theory is known as "methyltetrahydrofolate trap" or "folate trap" (Noronha & Silverman, 1961; Herbert & Zalusky, 1962) hypothesis. Further evidence in favour of the "folate trap" hypothesis has been provided (Nixon & Bertino, 1972a; Gutstein et al, 1973). Halsted et al (1969) reported an increase in the serum vitamin B_{12} level with a corresponding decrease in serum folate level in kwashiorkor, but Khalil et al (1973) on the other hand could find no change in the vitamin B_{12} level in serum of protein calorie malnutrition patients; although erythrocyte and serum folate were deficient.

It has been reported that the bone marrow folate and vitamin B_{12} contents are almost 8 times larger than the total pool of circulating vitamins and a size relationship existed between folate and vitamin B_{12} in blood, marrow, and liver with the folate pool 7 to 10 times larger than vitamin B_{12} pool (Trubowitz et al, 1974). In anaemia of infancy and childhood high serum folate was found to associate with vitamin B_{12} deficiency but it was low in case of iron deficiency. Red cell folate, on the other hand, was high in iron deficiency with or without concurrent lack of vitamin B_{12} was high, and upon iron repletion, it significantly fell. However, the red cell folate rose when vitamin B_{12} therapy was given, on both occasions the blood folate rose (Saraya et al, 1973). Oxidation of $3^{14}C$ serine to $^{14}CO_2$ has been found to be decreased in both folate and vitamin B_{12} deficiency (DeGrazia et al, 1972).

Folate deficiency has been reported to cause malabsorption of thiamine in alcoholics (Thomson et al, 1971) and induce a thiamine deficit in blood and liver of rats (Thomson et al, 1972). Pyridoxine treatment on homocystinurea causes lowering of folate level but when supplemented with folate improves the biochemical and clinical status (Wilcken & Turner, 1973). Clarke (1973) observed that mean corpuscular riboflavin concentration values of mother and prenates of the group which received iron, folic acid and riboflavin and those of the group which took no iron or vitamins were similar, normal and significantly higher than those of the groups which were treated with iron and folic acid without riboflavin. Biotin influences folic acid metabolism and particularly its utilization for the biosynthesis of coenzymatic derivatives (Noronha & Sreenivasan, 1959; Marchetti et al, 1965; Pasquali et al, 1968). Ascorbic acid has been claimed to have a protective effect on folic acid metabolism and to enhance the conversion of folic acid to tetrahydrofolic acid (Nichol & Welch, 1950; Jandl & Gabuzda, 1953; Terroine, 1960 and King, 1968). Loh and Dempsey (1974) have shown that both the dietary ascorbic acid intake and plasma ascorbic acid concentrations are inversely related to the serum folic acid in normal human subjects. They suggest that the increase in the metabolic availability of ascorbic acid (plasma ascorbic acid) probably increases the catabolic use of folic acid in haemopoiesis and thus a low serum folic acid. They did not make any attempt to identify the serum folate components as they were only concerned with serum folic acid. This study is open to criticism as reduced forms of folate principally $5\text{-}CH_3H_4PteGlu$ are the predominant circulating

forms of folate and not folic acid (Herbert et al, 1962; Noronha & Silverman, 1962; Bird et al, 1965).

Zinc deficiency adversely affects the folic acid status of rat (Williams & Mills, 1973). Iron deficiency is usually associated with folate deficiency in pregnancy anaemia (Diez-Ewald & Molina, 1972; Izak et al, 1973).

Folic acid significantly increases renin activity in plasma (Yamamoto et al, 1972). Histidine was found to increase serum folate level in epileptics (Arakawa et al, 1972). Chida et al (1972) suggested that folic acid might play a certain role in desaturation and/or hydroxylation of long chain fatty acids in the brain of growing rats.

Since the discovery of folate binding proteins in milk and human serum and tissue (Johns et al, 1961; Metz & Herbert, 1967; Markkanen, 1968; and Salter & Ford, 1968), a considerable volume of research into the proportionate distribution of the folic acid activity bound to the serum proteins has been accomplished. By both ultra-filtration of plasma (Neal & Williams, 1965; Elsborg, 1972) and sephadex gel chromatography (Markkanen & Peltola, 1971) it was found that protein bound folate accounts for about 50% of the total folates in serum. α-macroglobulin, transferrin and albumin have been found to be the folate-binding proteins in serum and the binding capacity is in this order of magnitude (Markkanen et al, 1972a; Markkanen et al, 1972b). Reports contradicting the validity of the presence of protein folate complex in human serum have also appeared (Metz et al, 1968; Mantzos et al, 1974). Elsborg (1974) has claimed albumin to be the predominant folate binding protein whereas Jacob and Herbert (1974) presented evidence against transferrin as a

binder of folate. So it appears that there is still a lot of contradictions and confusion as regards the presence of folate bound proteins in serum and the type of protein which plays the predominant role in this sort of binding.

Further investigations of the pregnancy and pathological conditions have revealed special activity in the metabolic events of folates for transferrin. Binding of folate to transferrin was found to increase during the advancement of pregnancy (Markkanen et al, 1973a), in liver diseases and hyperthyroidism, and under the influence of diphenylhydantoin (Markkanen et al, 1973b) and also in pernicious anaemia (Markkanen et al, 1974a). It was also observed that there was a simultaneous decrease in the folate binding of α-macroglobulin and albumin with the increase of transferrin binding. In pregnancy it was also found that within a week after delivery, the total protein binding of folate as well as that of transferrin decreased remarkably. The peculiar behaviour of transferrin folate binding during pregnancy and after delivery may be a factor for folate deficiency in pregnancy, it may ensure adequate folate content of mother's milk or it may retain folate for the mother's haematopoietic needs. In the disease states as mentioned earlier and in hydantoin therapy it has been found in addition to increased transferrin binding of folate that below the folate level of 3 mg/ml in serum all folates were practically protein bound and in some cases there was increased binding of folate with γ-globulin although in healthy subjects this binding was usually absent as it was also absent in myeloma. No explanation has been offered as to the absence of free serum folate at low level of total serum folate and as to the

selective nature of Y-globulin folate binding. The inflammation of liver in the liver diseases has been thought to cause stimulation of folate transferrin binding. In pernicious anaemia it was concluded that changed ratios of folate binding were an indication of disorders in folate metabolism. Markkanen et al (1974b) have further reported that binding to transferrin in women of fertile age seemed to exceed that in men. In the middle and late phases of the menstrual cycle the binding of folate to Y-globulin and albumin increases to some extent. Oral contraceptives affect the protein binding of folate.

SECTION 1. PART 2.

Because of the multifarious roles with which the folates are associated a tremendous amount of research has been going on for more than thirty years on different aspects of folic acid and its derivatives. Biochemists, physiologists, nutritionists, enzymologists, microbiologists, physicians, etc. all are engaged to reveal more mysteries and facts about their roles as coenzymes and vitamins, about how they are transported and metabolized, about how they cause deficiency disease states.

Absorption and transport of folates have been studied and are still being studied by utilising various techniques on various systems. A voluminous literature has grown up, but the most baffling point is that literature is full of contradictions as regards mechanism, site of transport and extent of metabolism.

Attempts have been made here to summarize what is known about the absorption and transport of folates, both in man and experimental animals enumerating causes and effects of malabsorption and the clinical syndromes associated with the malabsorption and deficiency.

i) ABSORPTION OF PTEROYLPOLYGLUTAMATES.

As stated earlier, food folate exists as a mixture of free and conjugated forms of 5-methyl, 5-formyl and 10-formyl-tetrahydropteroyl glutamates; the conjugated forms accounting for about 75% of the total folates present.

Pteroyl-heptaglutamate is a large molecule of molecular weight 1215. At pH 7 it is strongly electro-

negative and highly water soluble and lipid insoluble. By established concepts of cell membrane transport, this molecule would be expected to be excluded by the cells of most animal species. Even E. coli will not incorporate pure glutamyl peptides of chain lengths greater than three (Gilvarg & Katchalski, 1965).

Folate in human blood circulates in the free or unconjugated form(Herbert et al, 1962) presumably to facilitate entry into body cells. With but few exceptions (Blakley, 1957) it is the monoglutamate form of folate which is preferentially or exclusively utilised as cofactor for cellular enzymes (Stokstad & Koch, 1967).

Early studies using S. faecalis as a test organism suggested that very little folate could be absorbed from yeast (Spray, 1952). Cooperman and Luhby (1965) on the other hand suggested that pteroylpolyglutamates can be absorbed intact. However the majority of authors agree that pteroylpolyglutamates are utilizable from yeast and other foods and they are not absorbed intact (Bethell et al, 1947; Swendseid et al, 1947; Rosenberg et al, 1969; Butterworth et al, 1969; Hoffbrand, 1971).

Evidence that deconjugation does, in fact, occur in vivo was obtained from the experiments (Bethell et al, 1947; Swendseid et al, 1947) in which purified yeast conjugates when fed to normal subjects the resulting urinary excretion product was found to be microbiologically active, i.e. free folates containing not more than 3 glutamic acid residues. Elevation of serum folate active for both L.casei and S. faecalis was observed when normal volunteers were fed purified yeast folate proving that conjugated folates have

been converted to monoglutamates during absorption (Rosenberg et al, 1969; Rosenberg & Godwin, 1971). In this experiment, no conjugated folate, as evidenced by the failure of deconjugating enzymes to release additional microbiologically active folate in serum following in vitro incubation, was detectable in the serum of any subjects. Other laboratories have reported similar observations employing either yeast folate or synthetic crystalline pteroyl heptaglutamates as the test dose (Perry & Chanarin, 1968; Hoffbrand & Necheles, 1968; Bernstein et al, 1970a).

Baugh et al (1970) by injecting variously labelled C^{14} pteroylpolyglutamates into isolated loops of dog jejunum with the nerve and blood supply intact, collected blood samples through a cannula placed in the mesentric vein and found polyglutamates were cleaved to monoglutamates. In vitro studies by Rosenberg et al (1969) using everted rat gut sacs also showed hydrolysis of folic polyglutamate during passage from mucosa to serosal surfaces.

Butterworth, Baugh and Krumdieck (1969), using ^{14}C-labelled polyglutamates in man, provided additional support for the in vivo deconjugation of ingested pteroylpolyglutamates by demonstrating the appearance in serum of ^{14}C-labelled PteGlu after oral ingestion of ^{14}C-$PteGlu_N$ labelled either in the pteroate moiety or the first glutamic acid. When the label was placed in the second glutamic acid of the peptide chain, plasma folate was not radio-active. Label appeared as $^{14}CO_2$ exhaled in the breath, suggesting that the terminal glutamates had been cleaved off and rapidly metabolized.

The absorption of liver folate from the rat jejunum

and ileum was found to be highly efficient in comparison with crystalline PteGlu as indicated by rapid and pronounced serum folate elevation (Grossowicz et al, 1973) and these findings were in conformity with the observations in humans reported recently (Grossowicz et al, 1972).

Although there are two reports suggesting the absorption of intact $PteGlu_n$ (Cooperman & Luhby, 1965; Banerjee & Chatterjea, 1966) and it has been reported that a part of $PteGlu_7$ is absorbed as such (Perry & Chanarin, 1968) the consensus of other experiments is sufficient to suggest that polyglutamate is converted to the monoglutamate form before release into the circulation (Bethell et al, 1947; Swendseid et al, 1947; Rosenberg et al, 1969; Butterworth et al, 1969; Baugh and Krumdieck, 1969; Baugh et al, 1970; Rosenberg & Godwin, 1970; Bernstein et al, 1970a; Baugh et al, 1971), and the free folic acid is not methylated at the 5 position during this process (Bernstein et al, 1970a; Baugh et al, 1971).

Some reports have appeared as regards the absorption of natural polyglutamates in comparison with PteGlu. Claims have been made that the polyglutamate forms of folate are absorbed and utilized to about one third of the extent of monoglutamate forms (Perry & Chanarin, 1968; Hoffbrand & Peters, 1970; Perry & Chanarin, 1972). Jandl and Lear (1956) also found limited absorption of yeast folates. It is likely that the compounds with the greatest number of glutamate moieties are least well absorbed (Butterworth et al, 1969). In the chick heptaglutamate appears to be fully utilized (Jukes, 1955).

Wide variations in the availability of pteroylpoly-

glutamates from different foods, for instance, folate from lima beans, bananas, peas and liver is better absorbed than equivalent amounts from cabbage, lettuce, tomato and pumpkin have been reported (Retief, 1969; Tamura & Stokstad, 1973). Apart from differences due to variation in the chemical composition of the polyglutamates, difference may also arise because of other substances in the foods. Cellulose has been reported to impede folate absorption by complexing the vitamin in insoluble form (Luther et al, 1965). There may also be folate conjugase inhibitors.

The enzyme responsible for the removal of glutamic acid residue from the pteroylpolyglutamate has been called 'folate conjugase' (Bird, Binkley, Blood, Emmett & Pfiffner, 1945). The alternative names 'pteroylpolyglutamate hydrolase' (Hoffbrand & Peters, 1969) and 'gamma glutamyl-carboxypeptidase' (Blakley, 1969; Bernstein, Gutstein & Weiner, 1970b) are also used. The enzyme which hydrolyses the peptide chain to the monoglutamate form, has not however, been purified, and it is possible that two or more enzymes are concerned for instance, one that hydrolyses the higher polyglutamates to the triglutamate stage, and a second which takes the tri- to the monoglutamate form (Mims & Bird, 1950). The enzyme conjugase is present in plasma (Wolff et al, 1949), throughout the gastrointestinal mucosa and pancreas, duodenum and jejunum (Booth, 1967; Baker et al, 1969; Bernstein et al, 1970a). Jejunum is actually believed to play a key role in activating folate absorption. Experimentally it has been demonstrated that the isolated rat jejunum was capable of releasing free folate from its conjugate (Rosenberg et al, 1969). All the folate which traversed the intestinal wall

into the serosal fluid was free or unconjugated folate. Rosenberg and Godwin (1970), employing synthetic ^{3}H-pteroyl-heptaglutamate as substrate for digestion by intestinal mucosal fractions identified ^{3}H PteGlu as the product of digestion. The similar sort of finding was attained at by using ^{14}C $PteGlu_3$ (Baugh & Krumdieck, 1969).

It seems likely that conjugases would be located on the mucosal surface of the intestinal cell, much as are disaccharidases and certain peptidases. If this were true, the large folate molecule could be hydrolyzed before passage across the mucosal surface of the intestinal cell.

Surprisingly, if the mucosal enzyme does have an absorptive function, it is not situated in the brush border of the mucosal cell, the usual subcellular site of an absorptive enzyme, but is concentrated in the mucosal cell lysosomes (Hoffbrand & Peters, 1969). The low pH optimum (4.5) of the intestinal conjugase (Hoffbrand & Peters, 1969; Rosenberg & Godwin,1971) suggests the acidic lysosomal vacuole as a possible site for hydrolysis of the conjugate. Such a hypothesis could include passage of the polyglutamate into the lysosomal vacuole by a process which would not necessitate transport across the cell membrane. However, the capability of mature mammalian intestine to incorporate large molecules by pinocytosis is by no means established.

A third hypothesis supposes that the known continuous sloughing of intestinal villous cells releases enzyme into the lumen. The enzyme activity in the lumen of the jejunum resulting from desquamated gut epithelial cells was found to be very small (Santini et al, 1962; Klipstein, 1967). The pH of the lumen, which is about 6.5, is also

unfavourable for the effective cleavage of the peptide chain. Moreover, the normal folate-polyglutamate absorption in pernicious anaemia patients, where the pH in the gut, is unlikely to fall much below neutrality, suggests that the cleavage of the peptide chain does not occur significantly in the gut lumen (Hoffbrand, 1971) but at some other site. The conjecture as regards the actual site of folate conjugase activity, whether intraluminally, at the brush border, or within the intestinal cell, is yet to be resolved. Despite the controversy over the site of folate conjugase activity it has been established that a normal jejunum is necessary for the absorption of polyglutamates (Baker et al, 1969). It has been stated earlier that folic polyglutamate is hydrolyzed to folic acid during absorption and that folic acid is not methylated at the 5 position during this process (Herbert et al, 1962). Suggestions have also been made that if the $PteGlu_n$ are already partly reduced, it is likely that they are fully reduced and methylated in the mucosa so that the major compound entering portal blood after ingestion of a wide variety of dietary folate compounds is $5\text{-}CH_3H_4PteGlu$ (Chanarin & Perry, 1969; Perry & Chanarin, 1970; Hoffbrand, 1971).

Baker et al (1965) observed that diopterin when ingested by humans were converted to $5CH_3H_4PteGlu$ but teropterin circulated unaltered after ingestion. Baugh et al (1971) reported that diglutamates might cross intestinal mucosa of dog unaltered.

ii) ABSORPTION AND UPTAKE OF MONOGLUTAMATES.

After digestion or hydrolysis of conjugated folate, the next event in absorption involves the transport of monoglutamic folate either present as such in foods or released from conjugates by intestinal enzymes. It is theoretically possible that monoglutamic folate derived from conjugates is transported by a different mechanism than is monoglutamic folate ingested as such. There are, of course, no experimental data suggesting separate transport pathways for mono-, di-, or the larger pteroylglutamates. At present it is often assumed that concepts derived from the study of monoglutamic folate absorption are applicable to the understanding of the absorption of conjugated folate as well. Perhaps the best evidence against separate pathways of assimilation comes from reports of an isolated and specific gastro-intestinal defect for monoglutamic folate absorption by Luhby *et al* (1961), and Lanzkowsky *et al* (1969). The severe deficiency of folate which results suggests that the major portion of folate derived from the conjugate in food has no separate pathway by which to traverse the intestine and so to prevent the deficiency. Synthetic $PteGlu_7$ and synthetic PteGlu have been reported to be absorbed with nearly the same efficiency (Rosenberg & Godwin, 1971). Pteroylglutamic acid absorption was originally studied by measuring urinary excretion of folate microbiologically with *S. faecalis* after an oral dose of the compound (Denko, Grundy, Wheeler, Henderson, Berryman, Friedmann, & Youmans, 1946; Swendseid, Bird, Brown & Bethell, 1947). Normally, only small amounts of microbiologically active compound are

excreted on unsupplemented diets. When oral PteGlu is given, excretion varies from 2% of a 500 μg dose (Clark, 1953) to almost 50% of a large 5 mg dose (Doig & Girdwood, 1960). When both folic acid and folinic acid were ingested in normal subjects it was claimed that they appeared as folic acid in the urine indicating kidney as the site of conversion of monoglutamates of folate to the unreduced pteroylmonoglutamate because both folic acid and folinic acid after oral administration were found in 5-CH_3H_4PteGlu form in circulation (Baker et al, 1965).

A more reliable method for routine clinical purposes was subsequently developed, in which the rise in serum pteroylglutamic acid level was measured with S. faecalis following an oral dose of pteroylglutamic acid (Denko, 1951; Spray & Witts, 1952; Chanarin, Anderson & Mollin, 1958). The patient was saturated with folic acid before the test to ensure that absorbed folate was not rapidly removed from plasma by folate deficient tissues.

There are significant problems of interpretation with both serum rises and renal excretion following oral folate doses. Dependence upon microbiological assays presents difficulties of specificity and accuracy. The degree of tissue saturation significantly affects results. Renal excretion of folate is influenced by the distribution of bound and circulating folate and by renal function. Equally as important is the fact that neither serum rises nor renal excretion allows for direct and accurate quantitation of the amount of folate absorbed.

The introduction of tritium labelled PteGlu in 1960 represented a most significant advance in the study of

folate absorption. For the first time it became possible to perform balance studies. Anderson et al (1960) first reporting clinical investigations of the compound in man, showed that mean normal absorption (based on faecal excretion of radioisotope) was 79% of an oral dose of 200 µg of tritiated PteGlu. Without a preloading dose of unlabelled PteGlu, urinary excretion averaged only 6.3% of the 200 µg oral dose but this increased to a mean of 41% following a pre-loading injection of 15 mg. of PteGlu. Faecal excretion may minimize absorption results because of entry and then loss in bile. Administration of oral doses of either 40 µg per Kg or 15 µg per Kg of tritiated PteGlu to human subjects preceded by 15 mg loading doses of PteGlu was found to result in urinary excretion of 41% and 48% of the doses respectively (Klipstein, 1963; Kinnear, Johns, MacIntosh, Burgen & Cameron, 1963). Radioactivity excreted in the urine of rats was 6.8%, 5.9% and 30.7% of the oral dose of ($2^{14}C$) PteGlu in the first 24 hours after doses of 3.1, 32 and 320 µg/kg respectively (Blair & Dransfield, 1971). Yoshino (1968a) observed urinary excretion of tritiated PteGlu in normal subjects ranging from 37.6% to 57.9% with a mean of 48.4% when an oral dose of 40 µg tritiated PteGlu per Kg of body weight was given 30 minutes after an injection of 15 mg. of non-radioactive PteGlu. Yoshino (1968c) also observed that when tritiated PteGlu was intravenously injected in a dose of 20 µg per Kg of body weight of normal subjects, the urinary excretion of 25% of the dose followed after 12 hrs. of injection. In rats 12% of the intravenously administered dose of 50 µg per kg of body weight was found to be excreted after 24 hours.

Plasma radioactivity rises have been shown after an oral dose of tritiated PteGlu (Halsted et al, 1967; Helbock, 1968). Peak levels are achieved at 1 hr., which is similar to the experience with microbiologically determined serum rises of folate following unlabelled PteGlu. Much higher plasma levels are achieved if the patient is preloaded with PteGlu. Findings with intravenous administration of tritium labelled PteGlu, on the other hand, suggest a gradual fall of the plasma folic acid level from a period of 3 minutes to 1 hour after the injection of 15 - 20 μg/Kg of body weight (Johns et al, 1961; Sheehy et al, 1963; Yoshino, 1968c).

Studies in man have also been performed by using the double-lumen tube technique, i.e. perfusing a given segment of small intestine and measuring folate absorption by the difference in concentration between folate infused and withdrawn (Hepner, Booth, Cowan, Hoffbrand & Mollin, 1968).

Investigation of the localization of the intestinal absorption of folic acid has been undertaken by many workers. A jejunal location is strongly suggested clinically by the high incidence of folate deficiency in diseases involving the proximal small bowel (Cox et al, 1958). Abnormal folic acid absorption is characteristic of disease of proximal jejunum (Klipstein, 1963). Baker et al (1969) performing intestinal and jejunal resection showed that polyglutamate deconjugation was essentially a feature of the jejunum but PteGlu can be absorbed both by jejunum and ileum. Folate malabsorption is particularly well documented in coeliac disease, or gluten-sensitive enteropathy (Kinnear et al,

1963) which is a disease primarily involving the proximal small intestine. On the other hand, folate deficiency does not develop in patients with ileal disease or following ileal resection (Cox et al, 1958). All these bear testimony to the fact that the jejunum is the principal site for absorption of folates. Nearly all the available experimental evidence bearing on the question of absorptive site has been obtained using crystalline PteGlu. Herbert and Shapiro (1962), Yoshino (1968b), Hepner (1969) Smith et al (1970b) and Selhub et al (1973), utilising rat intestine found the absorption of folic acid to be occurring principally in the jejunum. Using flat sheets of rat jejunum or ileum mounted between lucite chambers, Olinger et al (1973) found mucosal to serosal transfer of folic acid across ileal mucosa to be significantly greater than across jejunal mucosa. They also observed that both jejunal influx and uptake of folic acid was greater than ileal influx and uptake. The higher folic acid transferred across the ileum than jejunum was explained by the hypothesis that PteGlu while passing through the jejunal sac was partly converted to $5CH_3H_4$PteGlu, and a significant part of the $5CH_3H_4$PteGlu remained in the mucosa, this had been supported by the presence of dihydrofolate reductase in jejunum but not in ileum and also by the appearance of $5CH_3H_4$PteGlu on the serosal side when jejunum but not ileum was used. So it leads to the fact that total mucosal loss of folate by jejunum is higher than that by ileum, i.e. jejunum is the principal site for folate absorption.

Cohen et al (1964), Burgen and Goldberg (1962), and Elsborg (1974) however, were unable to demonstrate any

difference in the absorption rate of folic acid in jejunum and ileum. Smith et al (1970b) reported that PteGlu was absorbed over the major portion of the small intestine at similar rates. Using 5-CH_3H_4PteGlu as the test compound, Strum et al (1971) demonstrated that mucosal to serosal transfer of the test compound was similar in all segments of the rat intestine.

In the only study performed in man, absorption of perfused crystalline folic acid was shown to occur in the proximal small intestine with negligible absorption taking place in the ileum (Hepner et al, 1968). Although there may be species variations, the evidence from clinical and experimental sources favours preferential absorption of folate in the jejunum.

Contradictions exist concerning the chemical state in which folic acid is transferred through the intestinal wall. It has been suggested that in man PteGlu is metabolised in the intestinal mucosa and appears on the serosal site of the gut as 5CH_3H_4PteGlu (Baker et al,1965; Cohen, 1965; Chanarin & Perry, 1969; Perry & Chanarin, 1970).

Oral doses of reduced forms of folate monoglutamates such as H_2PteGlu, H_4PteGlu and 5-CHO-H_4 PteGlu in man are followed by a rise in plasma 5-CH_3H_4PteGlu (Baker et al, 1965; Cohen, 1965; Chanarin & Perry, 1969; Perry & Chanarin, 1970; Pratt & Cooper, 1971; Whitehead et al, 1972; Nixon & Bertino, 1972b). Strum et al (1970); Olinger et al (1973) and Selhub et al (1973) using everted sacs of rats and Cohen (1965) using everted sacs of hamsters observed reduction amd methylation of PteGlu but these were not the prerequisites for transport. Perry and Chanarin

(1973) using the everted rat gut technique with PteGlu, H_2PteGlu, H_4PteGlu in the mucosal medium demonstrated the recovery of 10CHO-PteGlu, 10-CHO H_4PteGlu, 5-CHO H_4PteGlu and 5CH_3H_4PteGlu from serosal fluid. 5-CH_3-H_4PteGlu has been shown to be the final product of PteGlu absorption and conversion.

Reports suggesting that no alteration in the folate molecules occurred during intestinal absorption in men have appeared (Whitehead & Cooper, 1967; Butterworth, Baugh & Krumdieck, 1969; Bernstein et al, 1970a; Melikian et al, 1971; Leeming et al, 1972). Liver has been suggested to be the principal site of conversion of PteGlu to 5-CH_3-H_4Pte-Glu.

Baugh et al (1970, 1971) demonstrated that PteGlu had been absorbed as such by the intestinal mucosa of dog and reduction and methylation were not essential for the absorption process.

Smith et al (1970b)and Blair and Dransfield (1971) have also reported that PteGlu is absorbed by rat intestine without metabolism.

A detailed report incorporating the studies on the transport of PteGlu and its conversion to 5-CH_3-H_4PteGlu in rat jejunum and ileum has revealed some very interesting and important observations on PteGlu uptake and metabolism (Olinger et al, 1973). ^{3}H PteGlu was incubated in vitro on the mucosal side of rat jejunum. Of the folate transferred to the serosal side, the percentage identified as 5-CH_3-H_4PteGlu was inversely related to the initial mucosal PteGlu concentration: at 7, 20 and 2000 nM, 44%, 34% and 2%, respectively was converted to 5-CH_3H_4PteGlu. The total

folate and 5-CH_3H_4PteGlu increased with the increasing concentration, although the percentage of 5-CH_3H_4PteGlu flux decreased. PteGlu was found to be transported across the jejunum in, at least, two forms: the unaltered PteGlu and converted 5-CH_3-H_4PteGlu but reduction was not found to be essential to the absorption of PteGlu. Specific activity of dihydrofolate reductase, the enzyme responsible for converting PteGlu to H_4PteGlu was found to be significantly greater in the jejunum than in the ileum.

Serosal folate transfer across ileal mucosa was greater than across jejunal mucosa but the percentage of 5-CH_3-H_4PteGlu flux across the ileum was much lower than across the jejunum. In the presence of methotrexate the serosal folate transfer across the jejunum improved considerably, i.e. it was equal to the folate transfer across the ileum but the 5-CH_3-H_4 PteGlu flux decreased significantly. One explanation of these findings was that ileum was not the site for reduction and methylation of PteGlu but jejunum was and it retained a significant portion of the methylated folate because methotrexate by inhibiting the dihydrofolate reductase enzyme activity reduced the formation of 5-CH_3-H_4PteGlu and consequently increased the serosal folate flux but reduced the 5-CH_3H_4PteGlu flux. This interpretation suggesting retention of reduced and methylated folate forms by the jejunum was supported by tissue accumulation studies where mucosal uptake of folate was much higher by jejunum than that by ileum and the jejunal uptake decreased significantly in presence of methotrexate.

Brown et al (1973) found a significant increase in L. casei active compounds in serum of man after oral

administration of PteGlu, 5-CHO-H_4PteGlu, 5CH=NH H_4 PteGlu 5-CH_3H_4PteGlu, 5,10CH = H_4PteGlu, H_2PteGlu and 10-CHO-H_4 PteGlu.

Transfer of 5-CH_3H_4PteGlu from mucosa to serosa in rat intestine took place without any alteration in the structure (Strum et al, 1971).

iii) THE KINETICS OF THE UPTAKE PROCESS.

The mechanism by which PteGlu and its derivatives are transported across the intestinal mucosa continues to be a subject of uncertainty. PteGlu itself is a large (mol. wt. 441), moderately water soluble molecule. As such it could cross the intestinal cell by relatively slow passive diffusion, driven in part by the pH differential inside and outside the intestinal cell. Alternatively, its passage could take place by a more efficient structure specific mechanism which may be termed active transport when driven by energy requiring cellular processes.

Much effort has been directed toward studies to establish the presence of an active transport system for folic acid in the intestine. Certain criteria are required to be fulfilled in order to establish the active transport mechanism. These are (a) temperature sensitivity, i.e. a high Q_{10} value. (b) demonstration of a saturable uptake at certain point with the increasing concentration of folate, (c) arriving at a satisfactory Michaelis-Menten Kinetics, (d) favourable balance study, i.e. reasonable serosal flux or tissue uptake at initial 'zero' concentration gradient of folate, (e) exhibition of inhibition of uptake by competitive and metabolic inhibitors.

In vivo experiments were undertaken by Burgen and Goldberg (1962) who perfused rat intestines with non-buffered

solutions of ^{3}H-PteGlu. They demonstrated that the relative absorption of PteGlu decreased as the concentration of PteGlu increased and also high serosal flux. Their data also fitted reasonably well Michaelis-Menten Kinetics with $K_s = 4 \times 10^{-5M}$ and an absorption maximum of approximately 10^{-7} moles/hour/litre. They, therefore, suggested an active saturable transport system for folic acid in the intestine.

Yoshino (1968b), Hepner (1969), and Izak et al (1972) instilled folic acid into a segment of rat intestine, and confirmed that the absorption rate for folic acid depended on the concentration of folic acid applied with a rate limiting process, suggesting that folic acid was absorbed by an active saturable mechanism. Yoshino, however, pointed out that, in absolute terms, the absorption rate for folic acid increased almost linearly with the concentration deposited, so that a passive absorption could not be denied. Halsted & Mezey (1972) contradicted the saturable uptake mechanism.

In vitro experiments with "everted sac technique", described by Wilson and Wiseman (1954), were performed on rat intestines by Herbert and Shapiro (1962), and Cohen et al (1964), who demonstrated that folic acid was concentrated on the serosal side of the intestine, at least, if the concentrations of folic acid were held as low as 10^{-8}M in the bathing solutions. These would strongly indicate that folic acid was absorbed by a special, active, mechanism, as folic acid, in these experiments presumably was transported against a concentration gradient. In contrast, a passive mechanism has also been advocated (Turner & Hughes, 1962; Spencer & Bow, 1964).

It has been claimed that the absorption of

pteroylmonoglutamate was a pH dependent partly passive process which was enhanced by a glucose stimulated solvent drag (Smith, Matty & Blair, 1970b). They also observed a saturable process existing in the jejunum over a range of 10^{-7} - 10^{-5}M of the PteGlu concentration on the mucosal side with a K_t for the process of 0.7 x 10^{-6}M. PteGlu was concentrated by the tissues with respect to the mucosal solutions at 10^{-7}M mucosal concentration. With no initial concentration gradient of PteGlu, there was no transport to the serosal side against an electro-chemical gradient. Smith (1973) has suggested that in the rat there is a rate limiting uptake into the tissue and a separate passive transport into the serosal fluid over a concentration range of 10^{-8} - 10^{-7}M. The tissues were shown to accumulate PteGlu with respect to the incubating solutions at concentrations of 10^{-7}M and below, when the PteGlu was present on both sides of the intestine with no initial concentration gradient between the mucosal and serosal solutions and when it was present initially on the mucosal side only. More recently Blair et al (1974a)utilising both jejunal sacs and rings showed saturable uptake mechanism by both tissue and serosal absorption over a concentration range of 10^{-5}M to 10^{-7}M, but their data gave a poor fit both with the Lineweaver-Burke plot and Hofstee-Edey plot. The Q_{10} values were also low at either end of the concentration range. They suggested a mechanism in which PteGlu was converted to the neutral species at the mucosal surface in an acid microclimate. Elsborg (1974) could not find any evidence of an active process involved in the absorption of PteGlu by rat intestine. On the contrary, all experiments were compatible with the

concept of a passive process, the absorption being pH dependent.

The reduction and conversion of PteGlu to H_4PteGlu and 5-CH_3-H_4PteGlu by the rat intestine tissue has been suggested to be a saturable process (Selhub et al, 1973; Olinger et al, 1973).

Hepner et al (1968) utilizing a double-lumen perfusion technique, found that the relationship between the absorption rate of PteGlu and the amount perfused was nonrectilinear, which led to the conclusion that PteGlu was transferred through the intestinal wall by a saturable, active mechanism. This view has been supported by Bernstein et al (1970a). Objections to this conclusion were raised by several investigators (Scott & Orsi, 1968; Helbock, 1968; Matty & Blair, 1968), who stressed that the claimed saturation kinetics could be explained as by precipitation of PteGlu from the perfusion solutions on the mucosal surface of the intestine, where pH is probably 5(Schanker et al, 1958). At this pH, the solubility of PteGlu does not exceed 10^{-4}mol/l, which is far below the concentration range of PteGlu perfused.

Reservations, however, must be kept in mind: investigating intestinal absorption by means of PteGlu, which is practically never found among the naturally occurring folates, only provides a clue to the mechanism of intestinal absorption of folates during physiological circumstances. So far two reports have appeared on the intestinal absorption of 5-CH_3H_4PteGlu which is the predominant circulating and storage form of folate and a major folate constituent of dietary folates. Strum et al (1971) studied the intestinal absorption of 5-CH_3-H_4PteGlu by

several different *in vitro* methods: everted sacs, mucosal scrapings, and unidirectional transmural fluxes. These investigations suggested the mechanism of 5-CH_3-H_4PteGlu absorption as a non-energy dependent, most likely passive diffusion process. The conclusion was supported by (a) the failure to demonstrate movement of 5-CH_3-H_4PteGlu against a concentration gradient in the everted sac experiments, lack of accumulation of 5-CH_3-H_4PteGlu within the intestinal mucosal scraping, and no net flux of 5-CH_3-H_4PteGlu in the unidirectional transmural flux studies; (b) the absence of saturation kinetics in that an increase in 5-CH_3-H_4PteGlu mucosal to serosal transfer was linearly related to increased mucosal concentration of 5-CH_3-H_4PteGlu.

Weir *et al* (1973) studied the intestinal absorption of the two diastereoisomers of 5-CH_3-H_4PteGlu in man comparing the urinary excretion of both orally and intravenously administered ^3H labelled compounds. In normal subjects about 50% and 100% of the intravenously administered biologically active and inactive forms were excreted in urine indicating resorption of 50% of the active form by the kidney.

Orally administered active isomer gave a mean excretion of 43% whereas the inactive form gave only 22% in the same subjects. Interpretation of these excretion levels with those of the intravenously administered levels yielded the fact that the amount of inactive forms excreted in the urine after absorption represented the total that was absorbed by intestine whereas the corresponding value for the active form was considerably lower than the actual amount absorbed since some was retained by the subject. This preferential absorption of the active isomer was attributed to the presence

of a carrier in the intestine. As 22% of the inactive isomer was also absorbed, therefore, they concluded that a certain amount of either isomer had been absorbed by the intestine by simple diffusion but under normal circumstances a second mechanism did exist which resulted in preferential treatment of the active form.

iv) THE EFFECT OF INHIBITORS.

The poor absorption of yeast $PteGlu_n$ in comparison with PteGlu in man has been ascribed to the presence of 'folate conjugase inhibitors' in food. DNA and RNA have also been reported to be the inhibitor of the enzyme, but the role has also been disputed. Although this sort of inhibition plays an important role in $PteGlu_n$ absorption and utilization, kinetics with these inhibitors have not been investigated.

As stated in this section, part 2(iii), the inhibition of transport with inhibition of energy yielding reactions by metabolic inhibitors and inhibition with competitive inhibitors are the two important criteria for active transport. Several investigators have made use of metabolic and/or competitive inhibitors in the intestinal transport studies in order to establish the nature of transport mechanism. In case of metabolic inhibition both Km and Vmax decrease but in the case of competitive inhibition Vmax remains the same but Km increases.

Absorption of PteGlu by rat duodenal-jejunal segments was not found to be significantly reduced by the folic acid analogue methotrexate indicating absence of any competitive inhibition of transport (Burgen & Goldberg, 1962).

Turner and Hughes (1962) failed to discern any marked difference in the absorption of PteGlu by rat and hamster jejunal sacs in the presence of metabolic inhibitors like azide, cyanide and phlorrhizin and hence concluded a passive diffusion mechanism for folic acid absorption.

The absorption of PteGlu was not also found to be affected by dinitrophenol or amethopterin by rat small intestine (Yoshino, 1968b). When the PteGlu was present on the mucosal side at 10^{-8}M, the uptake by rat jejunum tissue was found to be depressed by methotrexate at 5.10^{-4}M. At the mucosal concentration of 4.5×10^{-9}M PteGlu the inhibition of uptake was 68% by sodium fluoride (Smith, 1973). Selhub *et al* (1973) using everted sacs of rat small intestine observed that at low concentrations methotrexate inhibited the reduction of PteGlu but did not affect its transport; but at a much higher concentration it decreased the accumulation of folates in the tissue without significantly affecting the vitamin concentration in the serosal solution. Olinger *et al* (1973) also reported that methotrexate inhibited PteGlu conversion to 5-$CH_3$$H_4$PteGlu by the rat jejunum.

Strum *et al* (1971) found that anaerobic condition and dinitrophenol failed to inhibit $5CH_3H_4$PteGlu transfer into intestinal sac of rats. They also reported the absence of competitive inhibition by folate analogues like PteGlu and methotrexate. So, it appears that contradictions prevail as regards the effect of inhibitors on folate uptake by rat intestine as they exist in respect of mode and site of intestinal folate transport.

v) INHIBITORY ROLE OF ANTI-CONVULSANTS AND ORAL CONTRACEPTIVES.

Polyglutamates, the major component of dietary folates are deconjugated by folate conjugase before they enter the blood. Folate deficiency occurs frequently in patients receiving the anti-convulsant drugs like diphenyl-hydantoin, phenobarbitone and primidone (Reynolds, 1968). Malabsorption of polyglutamyl folates and the resulting folate deficiency in this case has been attributed to the inhibitory effect of anti-convulsants,bromosulphthalein, silver nitrate (Hoffbrand & Necheles, 1968; Rosenberg, Streiff, Godwin & Castle, 1968; Reizenstein & Lund, 1973) on folate conjugase. Several investigators have failed to confirm these results, *in vitro* as well as *in vivo* (Baugh & Krumdieck, 1969; Bernstein, Gutstein & Weiner, 1970a; Houlihan, Scott, Boyle & Weir, 1972; Perry & Chanarin, 1972; Fehling, Jagerstad, Lindstrand & Westesson, 1973).

More recent evidence indicates that the cerebro-spinal fluid folate level is low on anti-convulsant therapy and decrease is proportional to the concentration of anti-convulsants found in blood and cerebrospinal fluid (Reynolds *et al*, 1972b; Reizenstein & Lund, 1973; Reynolds, 1974). PteGlu therapy does increase the serum folate concentration but not the cerebrospinal folate in the presence of anti-convulsants indicating decreased transport of folic acid from blood to brain (Spaans, 1970; Levitt *et al*, 1971;Reynolds *et al*, 1972a; Mattson *et al*, 1973; Reynolds, 1974). However, 5-CH_3-H_4 PteGlu and 5-CHO-H_4-PteGlu do readily enter the cerebrospinal fluid (Levitt *et al*, 1971; Mattson *et al*, 1973). Mattson *et al* (1973) assumed that the anti-

convulsants interfere with conversion of folic acid to 5-methyltetrahydrofolic acid. The inhibitory effect of the anti-convulsants remains still unconfirmed.

Sulphamethoxazole, like other sulphonamides, interferes with the synthesis of folic acid by competitive inhibition of incorporation of p-amino benzoic acid into folate. Thus sulphamethoxazole may be lethal for pathogenic organisms that make their own folate, but is not directly cytotoxic to humans since they are incapable of making their own folate, and rely on an exogenous supply of folate. Trimethoprim, on the other hand, interferes with folate metabolism both in man and micro organisms by inhibiting dihydrofolate reductase, the inhibition is much greater in case of bacteria than in the case of mammals (Hitchings, 1973; Burchall,1973). Davis and Jackson (1973) using therapeutic dosage of trimethoprim and sulphamethoxazole combination found no blocking of dihydrofolate reductase, i.e. H_4PteGlu was within the normal range in humans but in animals the H_4PteGlu was depressed by the same amount of dose. Inhibitory effect could, therefore, be produced in humans with larger doses.

Some workers (Shojania, Hornady & Barnes, 1968; 1969) though not all (Spray, 1968; McLean *et al*, 1969) have found low serum folate levels in women taking the contraceptive pills. Streiff and Greene (1970) thought that the apparent selective malabsorption of $PteGlu_n$ in women taking oral contraceptives might be due to inhibition of folate conjugase. In view of the findings of McLean, Heine, Held and Streiff (1970) of similar absorption of PteGlu and $PteGlu_n$ in pregnancy, these findings in patients receiving

the synthetic oestrogens and progesterones are difficult to understand. Stephens et al (1972) reported that the absorption of PteGlu and $PteGlu_n$ in controls and women taking oral contraceptives showed no difference between the two groups, provided the subjects were presaturated with PteGlu. They also reported that oestradiol, progesterone and oestrone failed to inhibit folate conjugase whether the latter was derived from human or guinea pig source. The nature of the effect of oral contraceptives on folate metabolism, therefore, also remains undetermined.

vi) FOLATE MALABSORPTION.

The most usual cause of folate deficiency in the Western hemisphere is inadequate dietary intake of the vitamin (Hoffbrand, 1971). Weir (1974) on the other hand is of the view that pure dietary deficiencies are rare in the so-called developed countries and occur predominantly in the Far East, the Caribbean and in Africa. In the more developed countries the incidence of dietary folate deficiency has receded dramatically in recent years due to routine prophylactic folic acid therapy. The other causes of the folate deficiency are malabsorption, increased utilization, anti-convulsant drug therapy, chronic alcoholism, etc. The most common factor precipitating folate deficiency throughout the world, however, is pregnancy. There are only three diseases in which malabsorption of folate is considered the major cause of the defiency - tropical sprue, coeliac disease, and the extremely rare disease of specific malabsorption of folate. In jejunal resection, Crohn's disease and partial gastrectomy, malabsorption is usually a minor cause of folate

deficiency. Malabsorption may also occur in anti-convulsant drug therapy, contraceptive pill therapy, alcoholism, intestinal stagnant loop syndrome and congestive heart failure.

Malabsorption of folate is now known to be a consistent feature of tropical sprue and of coeliac disease, both in children and adults. In these diseases there is structural and functional damage to the jejunal mucosa. Pathogenesis of folate deficiency in these diseases may be due to (i) disturbed passive absorption of folate due to disruption of the jejunal mucosal pores, (ii) destruction of the carrier system suggested for biologically active reduced folate derivatives (Weir et al, 1973), (iii) enhanced loss of folate compounds due to increased catabolism as a result of the increased jejunal mucosal cell turnover, and resultant increase in DNA synthesis (Weir, 1974) and (iv) qualitative defects in the intestinal conjugase enzyme system (Klipstein, 1972).

Particularly in tropical sprue absorption of dietary folate appears to be more impaired than absorption of PteGlu (Sheehy et al, 1961; Jeejeebhoy et al, 1968; Hoffbrand et al, 1969) even though the folate conjugase in the succus entericus (Klipstein, 1967) and in the jejunal mucosa in both tropical sprue and coeliac disease (Hoffbrand et al, 1969) are normal.

It has been proposed that the selective malabsorption of $PteGlu_n$ in sprue is due to inhibition of jejunal conjugase. No definite inhibitors have been identified in this disease, though a naturally occurring anti-folate compound (Butterworth, 1968) and a bacterial or viral

inhibitor (Klipstein, 1968) have been proposed to have this action. More recently, Bernstein et al (1970a) have suggested that products of bile salt degradation by bacteria may be conjugase inhibitors but this seems unlikely since, as the predominant effect of small intestinal bacteria is to raise serum folate, not to cause folate deficiency.

Sufficient folate can usually be absorbed from small as well as large doses of PteGlu to cause a satisfactory haematological response and to saturate body folate stores in patients with either tropical sprue or coeliac disease (Hoffbrand, 1971). Impaired intestinal absorptive capacity in tropical sprue eventually results in depletion of certain nutrients, the most prominant manifestation of which is megaloblastic anaemia.

Malabsorption of PteGlu and of dietary folate occurs if the jejunum is resected (Chanarin & Bennett, 1962; Baker et al, 1969). Sufficient absorption of PteGlu but not of dietary folate occurs from the remaining portion of the small intestine after jejunal resection.

Folate deficiency occurs frequently in patients with active Crohn's disease. A number of factors are involved - poor diet, excess utilization of the vitamin, and probably malabsorption in some patients. This is probably partly due to involvement of the jejunum by the disease but may also be due to impaired function of the small intestine not actually involved by the disease (Hoffbrand, Stewart, Booth, & Mollin, 1968).

Mild folate deficiency occurs frequently in post-gastrectomy patients; when megaloblastic anaemia due to deficiency occurs, however, the predominant factor is always

poor diet. A minor degree of malabsorption of PteGlu and of dietary folate has been reported in a proportion of these patients (Cox et al, 1958; Chanarin, 1969).

Specific malabsorption of folate has been reported (Luhby et al, 1961; Lanzkowsky et al, 1969) in patients showing relapsing megaloblastic anaemia requiring therapy with large doses of PteGlu by mouth, mental retardation, epileptic convulsions and ataxia. Absorption of PteGlu, $5\text{-}CH_3\text{-}H_4PteGlu$ and $PteGlu_n$ was found to be impaired.

Malabsorption of PteGlu has been reported in a few patients with intestinal blind-loop syndrome (Cooke et al, 1963). Colonic bacteria in the upper jejunum might render folate unavailable for absorption. Hoffbrand et al (1971) have demonstrated that lactobacilli had been capable of consuming folate in the small intestine of the patient. The predominant effect of an abnormal upper intestinal flora in the stagnant-loop syndrome, however, is to raise serum folate.

Folate deficiency occurs frequently in patients receiving the anti-convulsant drugs. Malabsorption of $PteGlu_n$ resulting from the inhibition of folate conjugase has been proposed to be one of the many theories for folate deficiency (Hoffbrand & Necheles, 1968; Rosenberg et al, 1968; Reizenstein & Lund, 1973). Several other workers disputed this theory (Baugh & Krumdieck, 1969; Bernstein et al, 1970a; Houlihan et al, 1972; Perry & Chanarin, 1972; Fehling et al, 1973). Malabsorption of PteGlu has been reported in rat (Hepner, 1969) and in humans (Meynell, 1966). Hepner et al,(1970) suggested that drugs do this by inhibiting intestinal Na-KATPase. Alternative mechanisms

for anti-convulsant induced malabsorption has been suggested (Benn et al, 1971). They suggested that anti-convulsants cause malabsorption of Pte-Glu by raising the pH in the lumen of the duodenum and jejunum above the optimum for PteGlu absorption. Contraceptive induced malabsorption of folate still remains to be confirmed.

There is little information in the literature on the absorption of PteGlu in inflammatory bowel disease. Cox et al (1958) and Hoffbrand et al (1968) have observed abnormal absorption in some cases of regional enteritis. Franklin and Rosenberg (1973) demonstrated that the patients with ulcerative colitis and granulomatous colitis frequently exhibited impaired folic acid absorption. The authors were of the view that it was not a pH effect.

There is no evidence that folate deficiency induces folate malabsorption. Intestinal structure and function has been described as normal in most persons who developed folate deficiency due to dietary intake (Gough et al, 1963; Winawer et al, 1965), but abnormal in others whose deficiency was associated with excessive alcoholic intake (Forshaw, 1969; Bianchi et al, 1970). Klipstein et al (1973) using rats, on the other hand, established the fact that folate deficiency alone could result in structural changes of the proximal small intestine but the intestinal function remained normal. It is likely, however, that folate deficiency contributes to mal-absorption of folate in human, at least, with tropical sprue. It is also probable that folate absorption is reduced in other situations where the small bowel suffers general non-specific damage as in kwashiorkor and starvation (Hoffbrand, 1971). A number of mechanisms are responsible for folate deficiency

in alcoholics - poor nutrition, liver damage, and possible inhibition of folate coenzymes. Malabsorption of PteGlu (Halsted et al, 1967; 1971) has been documented in recently drinking alcoholics without significant liver disease. The combination of dietary folate deficiency and prolonged ethanol uptake results in intestinal malabsorption of folates without any effect on the morphology of the jejunal mucosa (Halsted, Robels & Mezey, 1973).

vii) OTHER FOLATE TRANSPORT SYSTEMS.

Apart from intestinal folate transport studies, other folate transport systems, e.g. transport through bile, lymphocytes, placenta, isolated mucosal epithelial cells, micro-organisms and leukemia cells have also been studied by several investigators. A brief review of these studies is being furnished here.

Evidence of the rapid appearance of human portal blood folate in bile has been reported (Pratt & Cooper, 1971). Lavoie and Cooper (1974) infused tritiated PteGlu intravenously into patients and observed the prompt excretion of [^{3}H]PteGlu in bile indicating rapid transfer of PteGlu from the plasma to the liver cell. Of the ^{3}H radio-activity infused, some appeared in bile as breakdown products of PteGlu, some as biologically active folates like 5-CHO-H_4PteGlu, other formyl folates, 5-CH_3-H_4PteGlu and unchanged PteGlu. The presence of radioactive 5-CHO-H_4PteGlu was much higher than the radioactive 5-CH_3-H_4PteGlu. The largest fraction of radioactive material appearing in the bile was not a recognized form of folate but it contained both the pteridine and p-aminobenzoate portions of folate. Watanabe (1962) observed a

similar radioactive fraction in the bile of rats after injection of [^{3}H] PteGlu and presumed it to be a 'pteridine'. The small recovery of radioactive folate coenzymes during the period of collection indicated that most of the PteGlu presented to the liver entered the intracellular folate pools and was diluted by endogenous non-radioactive folate. Blair and Bransfield (1971) isolated some unidentified metabolites in urine of rats after oral administration of [2-^{14}C] PteGlu.

Cerebrospinal fluid folate levels are approximately three times higher than serum levels (Herbert & Zalusky, 1961) and the relationship between serum and cerebrospinal folate remains constant in the presence of folate deficiency (Reynolds et al, 1972b). Folic acid therapy fails to improve the low cerebrospinal fluid folate level induced by anti-convulsant (Spaans, 1970; Levitt et al, 1971; Mattson et al, 1973) but 5-CH_3H_4PteGlu on the other hand, ameliorates the anti-convulsant induced low cerebrospinal fluid folate level (Levitt et al, 1971; Mattson et al, 1973; Chanarin et al, 1974). These findings suggest that there is a rapid transport of 5-CH_3H_4PteGlu between plasma and cerebrospinal fluid folate in man.

Uptake of folic acid by the liver leads to its conversion into formyl and methyl polyglutamates in rats (Shin et al, 1972; Houlihan & Scott, 1972; Thenen et al, 1973), in guinea pigs (Corrocher et al, 1972) in sheep (Gawthorne & Smith, 1973; Osborne-White & Smith, 1973) and in monkeys (Brown et al, 1974).

Goresky et al (1963) have studied the renal reabsorption of folic acid in detail. Uptake of folate by the tubular cells appears to be a saturable process inhibited

by methotrexate.

Das and Hoffbrand (1969) cultured both mature and immature lymphocytes with [^{3}H] PteGlu and [^{14}C] CH_3-H_4PteGlu and measured the uptake of folates. The uptake by immature lymphocytes was five times greater than that by mature ones. The uptake of both the compounds exhibited saturation kinetics, temperature sensitivity and inhibition by methotrexate. 5-CH_3H_4PteGlu was likely to be decomposed as no antioxidant has been reported to be used (Blair & Pearson, 1974; Blair, Pearson & Robb, 1975). Hoffbrand et al, (1973) reported preferable uptake of PteGlu to $PteGlu_3$ by human marrow cells. Corcino et al (1971) reported energy-dependent folate uptake by human marrow cells.

Studying the effect of methotrexate on placental folate transport in rats McClain and Siekierka (1974) suggested active transport process for the placental folate. Chen and Wagner (1974) studied the transport of PteGlu and 5-CH_3-H_4PteGlu in hog's chroidplexus in the presence of competitive inhibitors as well as under the effect of low temperature and obtained results indicating an energy independent carrier mediated process.

Folate transport in rabbit reticulocytes is mediated by an energy dependent carrier mechanism which disappears with reticulocyte maturation (Bobzien & Goldman, 1972).

Momtazi and Herbert (1973) studied folate absorption by intestinal epithelial cells obtained by controlled mechanical stress. Uptake was found to be pH dependent and it was markedly enhanced by glucose in cells from the upper but not from the lower half of the small bowel. Much more

[^{3}H] PteGlu was taken up by individual cells from the upper than from the lower half of the small bowel. Percentage of uptake remained essentially unchanged as the concentration of [^{3}H] PteGlu in the medium was increased over 57 fold.

Leslie and Rowe (1972) utilizing isolated small intestinal cells from rat for PteGlu transport study observed that cells failed to accumulate PteGlu, but they were capable of binding PteGlu, the pteroic acid moiety appearing to be the structural determinant for binding. The binding of PteGlu by the cells was found to be a saturable process. Isolation of the brush border of these cells demonstrated that these membranes contained a protein with a high affinity for PteGlu. Hepner and Herbert (1969) appeared to have observed saturation kinetics for the absorption of PteGlu by isolated guinea pig jejunal cells.

Transport of folate compounds into L1210 murine leukemia cells is an active, carrier-mediated process. (Sirotnak, Kurita & Hutchison, 1968; Goldman, Lichtenstein & Oliverio, 1968; Nahas, Nixon & Bertino, 1972). Lichtenstein et al (1969) carrying out uptake of PteGlu studies in L1210 leukemia cells observed PteGlu was rapidly metabolized in that cell system and suggested an energy dependent efflux mechanism from their findings. Goldman (1971) utilizing the same system suggested a carrier transport mechanism for methotrexate and naturally occurring folates and also extended these findings to the Ehrlich ascites tumour cell as well. Jacquez (1966) on the other hand concluded that PteGlu uptake in Ehrlich ascites cells was due to passive diffusion. Investigating the effect of p-chloromercuriphenylsulphonate on the uptake of folates

into L 1210 cells, Rader et al (1974) reached the conclusion that L 1210 cells contained two systems for transport of folate compounds: (i) mercurial sensitive transport system for 5-CH_3-H_4PteGlu and amethopterin and (ii) mercurial-insensitive transport system for PteGlu. This finding was in accord with the results of Nahas et al (1972). Nixon et al (1973) utilizing murine lymphoma cells L 1210 and L 1210R (a methotrexate resistant subline) studied the metabolic change of diastereoisomers of 5-$\left[^{14}C\right]$ CH_3 -(9,3',5'-^3H) H_4 folate occurring during its uptake by the cells. About 81% to 85% of the total $\left[^{14}C\right]$ CH_3 groups were transferred to non-folate compounds but out of total cell ^3H, more than 87% remained identified as 5-CH_3-$\left[^3H_3\right]$ H_4 folate indicating that the labelled $5CH_3H_4$PteGlu was first demethylated and then re-methylated in the metabolic pool with the non-labelled methyl group. Some tritiated compounds, perhaps $\left[^3H\right]$ -$PteGlu_n$ coenzymes, were detected in the analyses of cell extracts, but these were of little quantitative significance. Pediococcus cerevisiae, a folinic acid auxotroph, possesses an active transport system for this compound and that neither folate (PteGlu) nor its analogues (amethopterin or amino-pterin) compete with this system (Mandelbaum-Shavit & Grossowicz, 1970). Nichol (1959; 1968) searching for bacterial mutants sensitive to aminopterin, isolated a strain of P. cerevisiae which could grow on relatively low concentrations of PteGlu and accumulated the labelled compound. Zakrzewski and Grzelakowska-Sztabert (1973) observed that mutant 8081/s (a mutant of P. cerevisiae 8081) exhibited a concentrative, saturable and energy dependent uptake of Pte-Glu and PteGlu was metabolized inside the cells.

Polyglutamates of reduced folate derivatives of unknown composition were also accumulated. Folinic acid and amethopterin inhibited the uptake. Mandelbaum-Shavit and Grossowicz (1973) also made similar type of observations while studying the uptake of ^{14}C-PteGlu by another mutant of P. cerevisiae. They observed a temperature dependent, energy dependent and pH dependent uptake of ^{14}C-PteGlu and the uptake was shown to be inhibited by iodoacetate, 2,4 dinitrophenol, potassium fluoride and sodium azide. The uptake obeyed saturation kinetics with an apparent Km of 6.6×10^{-6}M and Vmax of 4.0×10^{-10} mol. per min. per mg. At the steady state the intracellular concentration of PteGlu was 120 fold higher from that of the medium. Reduced folinates like 5-CHOH_4PteGlu and 5-CH_3H_4PteGlu, amethopterin and aminopterin were shown to compete for the PteGlu carrier. Recently Henderson and Huennekens (1974) have shown that transport of PteGlu, 5-CH_3H_4PteGlu, and amethopterin into Lactobaccilus casei is an active one and a single system is responsible for transport of a variety of folate compounds. Cooper (1970) has shown previously that PteGlu enters L. casei cells via a temperature-dependent, saturable system. Accumulation was prevented by sodium-iodoacetate but not by dinitrophenol. His evidence also suggested that PteGlu transport was shared by amethopterin but that a separate system might be used for 5-CH_3H_4PteGlu and 5-CHO H_4PteGlu.

SECTION 1. PART 3.

THE AIMS OF THE PRESENT STUDY.

As has emerged from the survey of literature, PteGlu is the only folate compound, the parent folate which has been studied extensively for the last two decades. Despite voluminous literature that has grown on the transport, uptake and metabolism of Pte-Glu and related compounds involving *in vivo* and *in vitro* studies on man, animals and microorganisms, the mode of folate transport mechanism across the mucosal epithelium of the intestine in man and animal still remains to be unanimously established. The existence or otherwise of a specific folate transport mechanism is the fundamental problem which emerges unanswered from the literature. Investigators are still engaged in those areas of literature which seemed contentious and to seek new information in directions which might be expected to lead to crucial mechanistic tests.

Although most previous studies have been devoted to the absorption of either PteGlu or $PteGlu_n$, literature is slim as regards the studies on $5CH_3H_4PteGlu$, the predominant circulating and storage forms of folate (Noronha & Silverman, 1962). Strum *et al* (1971), using *in vitro* techniques, have investigated most of the transport criteria discussed above. They report that absorption of labelled $5\text{-}CH_3H_4PteGlu$ is uniform throughout the small intestine, and does not occur against a concentration gradient. No statistically significant net flux was observed, and the mucosal to serosal transfer was insensitive to anaerobic condition, DNP, glucose and sodium concentration. No inhibitory effect

was observed in presence of PteGlu, methotrexate and 5-CHO H_4PteGlu. On the basis of these observations it was concluded that the absorption of 5-CH_3H_4PteGlu in the intestine did not involve a specific transport system and could probably be acccounted for by passive diffusion.

Weir et al (1973) have made the unique observation on the biologically active form of $5CH_3H_4$PteGlu for micro-organisms that it was preferentially absorbed from the intestine, and preferentially reabsorbed by the kidney in man. They suggested the existence of a transfer system in human intestine specific for active form of 5-CH_3H_4PteGlu. This interpretation has, however, been recently criticised (Blair & Matty , 1974).

The results quoted by Strum et al (1971) have all been furnished in terms of the serosal transfer, the uptake and metabolism by the intestinal tissue has not been taken into consideration at all, although literature provides ample evidence on the importance of the role played by tissue regarding the uptake and metabolism of PteGlu and $PteGlu_n$. The study also lacked in furnishing adequate data required to fulfil the criteria of active transport. The strategy of this study has been to investigate more fully the nature of uptake of $5CH_3H_4$PteGlu by rat intestine and its meta-bolism with a special stress on the side of intestinal tissue. One fundamental aim of this project was to look for the satur-ability of the uptake process and to study the kinetics of transport.

The phenomenon of substrate competition and exchange diffusion at membrane surfaces is well established for many biological transport systems (Wilbrandt & Rosenberg, 1961).

5CHO H_4PteGlu has been used in this study as a competitive inhibitor of 5-CH_3H_4Pte-Glu transport.

A series of experiments were performed to investigate the possibility of counter-transport at the mucosal surface and establish a possible relationship with the 5-CH_3H_4PteGlu transfer in the intestine. Effect of metabolic inhibitor was also studied.

pH, which is a very important factor in determining the folate transport has been given its due emphasis in this study. Uptake studies at various pH values were undertaken. Water uptake was studied simultaneously with the 5-CH_3H_4PteGlu of the uptake in order to correlate the two transports. Temperature dependence uptake studies were also done to look for any temperature sensitivity, another criterion of active transport. Attempts were also made to look for accumulation of 5-CH_3H_4PteGlu against electrochemical gradient.

Leslie and Rowe(1972) stated the existence of a membrane protein with a strong binding affinity for folate. This can be interpretated as circumstantial evidence for a folate carrier transport system but the inability of viable mucosal cells to accumulate folate disputes the carrier mediation. Consequently experiments were undertaken using isolated mucosal cells prepared by the method of Kimmich (1970) to establish the 5-CH_3H_4PteGlu transport characteristics. Histological studies were performed to characterize cell anatomy.

Section 2 of this thesis deals with the general description of the physiological and chemical techniques used throughout this study. The methodological details

peculiar to specific experiments are included where necessary, in Section 3, together with the results. Section 4 comprises a detailed discussion of the results obtained along with the conclusion.

SECTION 2.

MATERIALS AND METHODS.

Part 1. Materials.

Part 2. Physiological techniques.

Part 3. Chemical techniques.

Part 4. Viability study methods.

SECTION 2. PART 1.

i) ANIMALS.

The animals used were 13 - 15 weeks old adult male Wistar rats, weighing 180 - 230 g, supplied from the Department of Pharmacy, The University of Aston in Birmingham. Food was diet 41B, supplied by Pilsbury, Edgbaston, Birmingham. Composition of the diet is given in Table 2.1. The rats were kept in the animal house of the Department of Pharmacy in standard animal cages for about a week during experimental period and were allowed food and water ad libitum. The rats were transferred from the animal house to the laboratory the day before use, and housed in standard animal cages overnight. The rats were deprived of the food for about 20 hours overnight prior to sacrifice but allowed water ad libitum in this period.

ii) FOLATES.

The mixture of epimers about C_6 of 5-(^{14}C H_3) tetrahydrofolic acid as its barium salt was obtained from the Radiochemical Centre, Amersham, Great Britain. Pteroyl-L-glutamic acid and methotrexate were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks, Great Britain. 50%-50% dl-5-CH_3H_4PteGlu were prepared by the method of Blair and Saunders (1970). 5-$CHOH_4$PteGlu (Ca-leucovorin) was a gift from the Lederle Laboratories, Pearl River, New York, U.S.A.

As the 5-CH_3H_4PteGlu is an unstable compound its stability was determined by thin layer chromatography and autoradiography as described in Section 3, part 1,iv).

TABLE 2.1.

Composition of Heygates Diet 41B.

	%
Crude protein	17.069
Crude oil	2.732
Crude fibre	4.352
Digestible oil	2.114
Digestible fibre	1.723
Arginine	0.801
Lysine	0.877
Methionine	0.309
Cystine	0.261
Tryptophan	0.192
Histidine	0.326
Leucine	1.089
Tyrosine	0.362
Isoleucine	0.710
Phenylalanine	0.674
Threonine	0.555
Valine	0.837
Glycine	0.981
Calcium	1.3
Phosphorus	0.72
Ca:P	1:0.6
Sodium	0.576
Chloride	0.154

Vitamins:	
B_{12}	14.09 µg/Kg
E	19.25 mg/Kg
Thiamine	6.201 "
Riboflavin	3.499 "
Niacin	55.1 "
Pantothenicacid	14.99 "
Choline	1311 "
Biotin	0.904 "
Folic acid	0.838 "
Pyridoxine	5.95 "
Inositol not less than	220 "
A	10,220 I.U/Kg
D_3	2,556 "

Iron	65 mg/Kg
Manganese	32 "
Copper	7 "
Iodine	4.18 "
Cobalt	0.89 "
Zinc	8.29 "

iii) PHYSIOLOGICAL SALINES.

One of the prerequisites for a successful *in vitro* method is the use of solutions isotonic to plasma, which contain the electrolytes found in the plasma in similar quantities. The commonly used physiological buffers are Krebs Ringer solution (Krebs and Henseleit, 1932) which are constituted to closely approximate the ionic composition of the mammalian plasma. This medium would seem to provide a physiological extracellular environment and so insure the metabolic integrity of the surviving cells. The Krebs Ringer Phosphate Buffer (Umbreit, Burris & Stauffer, 1951) was used throughout with all the experiments with sac and cells, the constitution of which is furnished below: -

100 parts of 1) 0.90% NaCl (0.154M)
4 parts of 2) 1.15% KCl (0.154M)
3 parts of 3) 1.22% $CaCl_2$(0.11M)
1 part of 4) 2.11% KH_2PO_4(0.154M)
1 part of 5) 3.82% $MgSO_4$, $7H_2O$ (0.154M)
12 parts of 6) 0.1M phosphate buffer pH 7.4

(17.8 g of Na_2HPO_4, $2H_2O$ + 20 ml N HCl when diluted to 1L give 0.1M phosphate buffer). This solution, with the addition of glucose (28 mM) is referred to as "the standard incubation medium" throughout the text.

Tris chloride buffer medium. This medium (Leslie & Rowe, 1972) consisted of 120 mM sodium chloride, 3 mM dipotassium hydrogen phosphate, 1 mM magnesium chloride, 1 mM calcium chloride, and 20 mM Tris chloride buffer (pH 7.4) containing (1 mg/ml) hyaluronidase and (1 mg/ml) bovine serum albumin. This medium was used as an isolation medium for mucosal cells.

iv) SCINTILLATORS AND OTHER CHEMICALS.

The liquid scintillators used were NE 220 (Nuclear Enterprises, Edinburgh, U.K.), Dioxan Cocktail 'D' (Fisons, U.K.), a toluene based scintillator containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis (5-phenyloxazol-2-yl) benzene (Koch-Light) made up to 1 litre with A.R. toluene and C^{14} absorber P (Fisons, U.K.) consisting of methanol 22% (v/v), 2 phenylethylamine 33% (v/v) toluene 40%(v/v), water 5% (v/v) and scintillator 2-(4-tert-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (7g/L).

Other chemicals were (1-^{14}C) hexadecane (The Radiochemical Centre, Amersham, U.K.), sodium ascorbate (British Drug Houses Ltd., Poole, U.K.), 2-mercaptoethanol (Koch-Light), Tryptan Blue (BDH Chemical Ltd., Poole, U.K.). Cellulose (MN 300 uv) polygram precoated plastic sheets were purchased from Macherey Nagel & Co., Duren, Germany. X-ray films (Ilford, Industrial G, Ilford, U.K.) were used for autoradiography.

SECTION 2. PART 2.

The history of the development of techniques for the study of intestinal absorption reflects the history of medical sciences. Although physiological investigations of absorption most probably date back to the seventeenth and eighteenth centuries, serious investigations started developing slowly during the nineteenth century. Thiry (1864) heralded a new era in physiological investigation by the description of a surgically prepared intestinal fistula which permitted quantitative experiments on absorption in an unanesthetized animal. Since then several techniques have developed over more than a century for investigations into intestinal physiology, the history of which has been well documented (Wilson, 1962; Parsons, 1968).

The kinds of methods used for the investigation of intestinal absorption and transport may be classified as follows:

A. Methods for demonstrating the occurrence of absorption *in vivo*. These are essentially the methods for tracing the fate in the animal of the products of absorption.

1. Techniques based on the recovery from urine of absorbed substrate or derivatives. This is a method for measuring the intestinal absorption of carbohydrates in intact animals, and in particular in human subjects based on the comparison of the rate of excretion of the substance in the urine when given orally with that occurring after intravenous injection.
2. Tolerance-curve studies. In this method the absorption, of an exogenous substance is determined by

measuring the concentration of that substance in the blood stream after making allowance for the removal of the substance from the pool of body fluid by processes such as metabolism by tissues or by renal excretion.

3. Measurement of appearance of absorbed material in tissues of body. This is a measure of the absorption from the intestine of intact animals obtained by following the appearance of the material in an organ or part of the body either directly or by indirect means.

B. Methods for the determination of net rates of absorption in intact animals in which the input to the absorbing surface is controlled by the physiological activity of the experimental animal. In these cases the rate of appearance of substrate at the absorbing cells of the intestinal tract is thus determined by physiological factors which are not necessarily directly under the control of the experimentor.

1. Balance study method.
2. Intragastric administration of test dose.

C. Methods for the determination of the net rate of absorption by the mucosal surface. In these methods the experimentor directly controls the access of the substrate to the absorbing cells.

1. <u>In vivo</u>
   (a) Intubation methods
   (b) Studies on chronic fistulous animals.

(c) Acute experiments on intestinal segments *in situ*.

i) Closed segments.

ii) Infused segments.

2. *In vitro*.

(a) Whole segments surviving *in vitro*.

i) Mesentric Vascular Perfusion.

ii) Circulation techniques.

iii) Everted sacs.

(b) Portions of whole wall of separated mucosal layers.

i) Tissue accumulation method.

ii) Short circuit current measurements method.

D. Methods that form the basis of investigation of the cellular and subcellular mechanisms underlying the processes of intestinal transport. These methods include the use of various tissue preparations *in vitro* such as:

1. Rings of whole wall.
2. Preparations involving short-circuit current measurements.
3. Preparations of mucosal tissue.
4. Isolated mucosal epithelial cell preparations.
5. Brush border preparations.

Other methods belonging to this category include:

6. Methods for fractionation of subcellular components.
7. Methods for cytochemical localization of enzymes.
8. Methods for intracellular localization of substrate during absorption.

a) Microscopic methods.

b) Direct analysis of tissue.

c) Qualitative and quantitative autoradiographic techniques.

E. Methods for collecting absorbate.

A. In vivo.

1. Methods for sampling venous effluent.

a) Portal circulation.

b) Systemic circulation.

2. Cannulation of the lymphatics.

B. In vitro.

1. Tissue accumulation.

2. The collection and examination of fluid in contact with the serosal surface of surviving segment.

3. Direct collection of the fluid extruded from the mucosal cells.

4. Fluid emerging from the vascular effluent of segments of intestine artificially perfused through the mesentric vascular bed may be collected.

The author has used everted sacs and isolated cell preparations for the absorption studies of $5\text{-}CH_3H_4PteGlu$ in rat jejunum. The merits and demerits of these techniques will be dealt with at appropriate places, i.e. where the preparations have been described in detail.

i) THE EVERTED SAC PREPARATION AND TRANSPORT EXPERIMENTS.

In the present experiments everted sacs and isolated mucosal cells were used. The everted sac preparation has become a standard technique in intestinal physiology since its initiation for research into intestinal function (Wilson & Wiseman, 1954). The preparation is simple and the technique allows reproducibility. In this method where the normal vascular circulation is absent the oxygenation of the mucosal cells are achieved by exposing the surface of the everted sac to a relatively large volume of well oxygenated and stirred solution containing the substrate. The transport of substrate into the sac contents (serosal fluid) can then be followed, without the need for elaborate apparatus to circulate fluid through the intestinal lumen. The method is free of the disadvantages of *in vivo* methods which include uncontrollable extraneous, e.g. hormonal, influences and false assumptions about the site of action where pharmaceutical agents have other physiological actions at sites other than intestine, e.g. renally active agents.

Recently papers have appeared in literature that criticise the everted sac preparation for assessing intestinal function (Levine *et al*, 1970; Mohiuddin & Olubi, 1970). On examination of the histological appearance of everted sacs of rat and hamster intestine during incubation they have concluded that large complexes of epithelial cells are desquamated into the lumen and villi begin to disintegrate. Extrusion zones began after a few minutes of incubation at 37^{o} and the complete destruction of the mucosal epithelium took place after 1 hour. Histological studies undertaken by the author

have been detailed in Section 4. Minimal distortion of villus pattern of the sac was observed after 30 minutes incubation in presence of glucose. This is compatible with the observations of Bhanthumnavin et al (1974). The polysaccharide layer at the extremity of brush border was also visible when stained.

The use of anaesthetics for killing rats causes less structural damage (Levine et al, 1970) than the method of decapitation. However, since anaesthetics cause substantial reductions in sodium movement and transmural potential via the uncoupling of oxidative phosphorylation (Dinda & Beck, 1969) the lesser amount of structural damage is probably only a consequence of less cellular activity and as such is not a reliable index of functional integrity.

The approach in this study has been to develop preparations which seem both viable and functionally intact by standards of what seem to be the most competent reports in the literature. At the same time, the possible drawbacks of in vitro preparations have been borne in mind. The pale "mushy" appearance of the sac associated with mucus and cellular material in the incubation medium, as described by (Levine et al, 1970) has rarely been observed within $1\frac{1}{2}$ hours of incubation at $37^{\circ}C$, although the effect may be conspicuously induced by incubating the tissue at higher temperatures. No gross damage was observed during the incubation periods undertaken in this study here and the preparations retained a pink appearance. Other viability studies (Section 2, part 4) were performed on the sacs to ensure their functional capacity for in vitro studies. A well stirred nutritive bathing medium was

provided to compensate for the absence of normal vascular circulation in such studies. The everted sac preparation of small intestine of rat as described by Wilson and Wiseman (1954) and modifications of the method (Barry, Matthews & Smyth, 1961; Turner & Hughes, 1962) was used in order to prepare four or more sacs from the same animal.

Rats after a starvation period of about 20 hours were stunned and decapitated. The abdomen was opened by a mid-line incision and the intestine cut at the duodenum just below the pylorus. The entire length of intestine was removed and washed through with ice-cold 0.9% saline until the washings were clear. The mesentery was stripped off manually.

The gut was transferred to a beaker of chilled saline and slipped on to a 300 mm. polished stainless steel rod of 1.5 mm. diameter. The duodenal end was tied with a ligature 2 cm. from the end of the rod and the rest of the intestine was everted over the ligature and slipped off the end of the rod. The existing ligature was cut and the everted section of the intestine was suspended from a glass hook clamped to a stand by means of a ligature in ice-cold saline kept continuously oxygenated by passage of 95:5% (v/v) O_2: CO_2 gas mixture. Different sizes of sacs were prepared for different purposes, e.g. 7 cms. sacs for transport studies, 6 cms. sacs for efflux studies and 3 cms. sacs for oxygen uptake measurements. The sacs were prepared from the jejunum.

To prepare a sac, the gut was lifted from the saline by the glass hook, a ligature tied (second ligature) at a distance (a little longer than the sac size required)

below the first ligature, the gut cut just above the second ligature. The remaining length of intestine was re-attached to the hook and again completely immersed in the saline. The freed segment was taken, a second ligature loosely tied at the free end. The segment was then freed of the excess saline clinging to it by blotting with moist tissue paper or filter paper. It was then weighed on a torsion balance. The sac was then transferred to a glass plate covered with moist tissue paper and appropriate Krebs-Ringer Phosphate buffer along with 28 mM glucose (about 0.5 ml. for a 7 cm. sac) previously oxygenated was injected. This was done via a 1 ml. syringe with a very blunt hypodermic needle. The needle was introduced into the open end of the sac and the loose ligature was tied around the sac with the needle in it. After the serosal contents have been introduced the needle was slipped out and the ligature tightened. The sac was then re-weighed on a torsion balance. The sac was then transferred to 25 ml. conical flask containing 6 mls. of ice-cold phosphate buffer until all other sacs were made. Incubation period was varied as necessitated by the experimental nature. Mixture of O_2/CO_2 (95:5) was allowed to pass through the incubation medium throughout the whole incubation period and the shaking incubator was oscillated at 100 cycles/minute in a Griffin 100 series constant temperature water bath, amplitude 3 cms. Incubation was usually done at $37^{o}C$, sometimes at $27^{o}C$.

After incubation, each everted sac was lightly blotted with moist tissue paper or filter paper, and then suspended from the torsion balance. The weight taken was the final weight after incubation. The sac was then cut

and the serosal fluid was collected in a graduated centrifuge tube. The cut end of the sac was blotted and the cut sac re-weighed.

Therefore, during the transport experiment with sac four weighings were available: -

weight of empty sac	W_1
weight of sac + serosal content	W_2
weight of sac after incubation	W_3
weight of sac after drainage of serosal content.	W_4

The total amount of fluid transferred across the sac wall was calculated as $W_3 - W_2$. Tissue uptake or gut uptake of fluid was given by $W_4 - W_1$. Serosal fluid transfer was calculated as $(W_3 - W_2) - (W_4 - W_1)$.

In case of 5-methyltetrahydrofolic acid transport experiments at different temperature, pH, time and concentration variations, four to six 7 cm. sacs were made starting 7 cm. from pylorus from one rat. The sacs were filled with 0.5 ml. of appropriate serosal fluid, e.g. Krebs Ringer Phosphate when concentration gradient needed to be established between the serosal and mucosal medium and Krebs Ringer Phosphate mixed with required concentration of 5-methyltetrahydrofolic acid (labelled 5-methyltetrahydrofolic acid diluted with unlabelled if required) where no concentration gradient was necessary between the serosal and mucosal medium. The sacs after being prepared were transferred to 6 ml. of incubating medium consisting of Krebs Ringer Phosphate mixed with 28 mM glucose and appropriate concentrations of 5-methyltetrahydrofolic acid

(labelled compound diluted with unlabelled as and when required) with sodium ascorbate (3 mg/ml) contained in 25 ml. conical flasks. The flasks were kept immersed in ice-cold water until all were ready for incubation. The sacs were incubated and weighed exactly in the same manner as stated earlier. The sacs were washed with 5 ml. of 10^{-5}M non-radioactive 5-methyltetrahydrofolic acid for about 30 secs. at room temperature.

Since a time lag was created in between the making of 1st and last sac, maximum possible care was taken to ensure that same treatment was done to the sacs used for one full experiment. If six sacs were prepared from each animal and were to be incubated for 15, 30 and 60 mins, then the treatment was done in the following way. In case of first rat the sacs were prepared as follows:
First two sacs (Sacs No. 1 and 2 starting from duodenal end) were prepared firstly and transferred to conical flasks fixed for 15 mins. incubation, the second two sacs (Sacs No. 3 and 4) prepared after that and transferred to the conical flasks fixed for 30 mins. incubation and the last two sacs (Sacs No. 5 and 6) were prepared in the last order and transferred to conical flasks meant for 60 mins. incubation. In case of the second animal, the second two sacs were prepared in the first order and meant for 60 mins. incubation, the third two sacs were prepared in the second order and meant for 15 mins. incubation, and the first two sacs were prepared in the last order and meant for 30 mins. incubation. In case of third rat, the third two sacs were prepared in the first order and meant for 30 mins. incubation, the first two sacs were prepared in the second order and

meant for 60 mins. incubation and the second two sacs were prepared in the first order and meant for 15 mins. incubation.

Determination of serosal and tissue uptake of 5-methyltetrahydrofolic acid will be described under headings Chemical Techniques and Methods of Assay in Section 2, Part 3.

In efflux experiments the 6 cm. everted sacs were first loaded with radioactive 5-methyltetrahydrofolic acid by incubating the sacs for 20 minutes in the incubation medium consisting of Krebs Ringer Phosphate with 28 mM glucose and sodium ascorbate (3 mg/ml) containing 0.5×10^{-6}M 5-methyltetrahydrofolic acid (labelled compound) at 37°C and pH of 7.2. The O_2/CO_2 (95:5 v/v) was passed throughout the incubation period and incubator shaken at a rate of about 100 oscillations/minute.

After incubation the sacs were flushed with warm oxygenated saline for 45 seconds. The sacs were then incubated in 1 ml. of the different efflux media, e.g. Krebs-Ringer Phosphate with 28 mM glucose and sodium ascorbate (3 mg/ml) which served as the control efflux medium. Other efflux media were the same medium containing 10^{-5}M non-radioactive 5-methyltetrahydrofolic acid, folic acid, 5-formyltetrahydrofolic acid and methotrexate. Incubation temperature was 37°C. 100µl of the efflux medium was withdrawn after 5, 15 and 30 minute intervals for counting.

ii) THE PREPARATION OF MUCOSAL EPITHELIAL CELLS.

The logical development in the study of intestinal function *in vitro* was stated by Wilson (1962) to be the use of suspensions of isolated epithelial cells. The multiple transport processes carried out by the mucosal epithelial cells of the small intestine have been recognized and appreciated by biochemists and physiologists alike. Of the many other methods available for producing cellular preparations, including crude cell 'breis' (Wilbrandt & Laszt, 1933; Dickens & Weil-Malherbe, 1941; Newey, Smyth & Whaler, 1955; Sols, 1956) and isolated cell preparations (Paterson & Zbarsky, 1958; Harrer, Stern & Reilly, 1964; Huang, 1965; Perris, 1966) some lead to poorly respiring cells. The most recent methods which successfully produce adequately respiring cells (Harrison & Webster, 1969; Webster & Harrison, 1969; Kimmich, 1970; Reiser & Christiansen, 1971; Evans, Wrigglesworth, Burdett & Pover, 1971) have been utilized for various metabolic and transport studies. Isolated mucosal epithelial cell suspensions have been used very recently for folate uptake and metabolic studies (Leslie & Rowe, 1972; Momtazi & Herbert, 1973). Other methods cited in literature for folate uptake and metabolic studies are mucosal scrapes and isolated intestinal villi preparations (Rasmussen, Waldorf, Dziewatkowski & DeLuca, 1963; Schultz, Fuisz & Curran, 1966).

Isolated mucosal epithelial cells have been prepared by the method of Kimmich (1970), modified by Leslie and Rowe (1972).

Male Wistar rats weighing around 200 g. were killed

by stunning and decapitation. The jejunum was cut some 7 cm. past the pylorus and removed from the rest of the intestine, flushed with ice-cold saline and everted as described for the sacs before. The everted jejunum was cut into 4 to 5 cm. segments and incubated in about 50 ml. of a standard Tris buffer medium containing 1 mg/ml of bovine serum albumin and 1 mg/ml hyaluronidase in a plastic beaker for about 30 minutes at 37°C in Griffin 100 series water bath shaking at 100 oscillations per minute. O_2/CO_2 (95:5 v/v) gas mixture was passed throughout the incubation period.

The epithelial cells adherent to the underlying mucosa were removed by gentle agitation by placing the intestinal sheets in a plastic trough containing the ice-cold medium devoid of hyaluronidase. The whole cell suspension, i.e. normally sloughed off cells plus the cells removed by gentle agitation as above was then centrifuged at about 4°C at setting 5 - 6 for 2 - 3 minutes in a bench centrifuge (M.S.E. Ltd). The supernatant fluid was discarded and cell pellet was gently resuspended and dispersed in 10 ml. of enzyme-free ice-cold incubation medium and centrifuged as before for another 2 - 3 minutes. The process was repeated twice in order to ensure complete removal of the hyaluronidase. The final cell pellet was re-suspended in 2 - 4 ml. of the ice-cold enzyme-free incubation medium.

The cell pellet isolated in this way was dispersed by gently shaking the test tube and the cells were incubated in 10 ml. of Krebs Ringer Phosphate medium containing 28 mM of glucose, 10^{-5}M 5-methyltetrahydrofolic acid (radioactive

compound mixed with non-radioactive compound) and sodium ascorbate (3 mg/ml) contained in a plastic beaker. The medium was oxygenated with O_2/CO_2 (95:5 v/v) gas mixture throughout the incubation period, and the water bath shaken at 100 oscillations/minute. The temperature of incubation was set in accordance with the experimental condition, e.g. at 37°C and at 27°C whenever necessary. Cells were withdrawn at appropriate intervals by pipette in duplicate and transferred to 5 ml. of 0.9% ice-cold saline in polythene centrifuge tube, stirred and then centrifuged for 1 - 2 minutes. The clear supernatant was removed and then the cells were treated in the same manner another three times. The cells were then freeze-dried to constant weight.

The determination of 5-methyltetrahydrofolic acid from cell washings and uptake by cells has been described under heading Chemical Techniques and Methods of Assay in detail in Section 2, Part 3.

Although the use of isolated mucosal epithelial cells is the most logical development for carrying out the *in vitro* transport studies it is susceptible to certain criticisms in respect of their physiological integrity. The isolated cells prepared above have lost their *in vivo* polar orientation and apposition to one another (Leslie & Rowe,1972). It is quite possible that this is critical for a transport system which according to Burgen and Goldberg (1962) has a luminal-serosal directionality, i.e. is largely one-way path. The loss of physiological orientation of the cells would lead to complete disruption of transport process.

It seemed very probable that the absence of glyco-

proteins and lipoproteins observed by Leslie and Rowe (1972) indicated the removal of the glycocalyx associated with microvilli of the brush border. Further discussion on this has been done in Section 4.

SECTION 2. PART 3.

i) TISSUE COMBUSTION.

Sacs used for the 5-methyltetrahydrofolic acid uptake experiments after being emptied of the serosal fluid were freeze-dried. Portions of the sac were weighed into previously weighed filter paper baskets (Fig. 2.1), the total weight not exceeding 250 mg.

The combustion apparatus was a modification of that of Kalberer and Rutschmann (1961) (Fig. 2.1). The sidearm contained 25 ml. of absorbing solution consisting of manoethanolamine, 140 ml. made to 1 litre with A.R. methanol. The monoethanolamine must be colourless; if necessary it was redistilled before use (b.p. 170° at 760 mm.). The absorbing solution was cooled thoroughly in solid CO_2 in acetone, and the apparatus purged with oxygen. The sample was placed in the platinum basket, or a nichrome spiral, the wick ignited, and the sample plunged into the flask; the stopper was held firmly in place during combustion, then with a spring, with the side-arm containing absorbing solution still in the solid carbondioxide in acetone. The flask was allowed to cool, the absorbing solution tipped into the flask, and left for an hour. The solution was then returned to the side arm, and the apparatus dismantled.

The radioactivity was determined by adding 3.0 ml. of the absorbing solution to 7.0 ml. of scintillator consisting of 2,5-diphenyloxazole(4 g.), and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (100 mg) dissolved in 1 litre of AR toluene. If the scintillator/absorber solution was not homogeneous, A.R. methanol was added dropwise to make

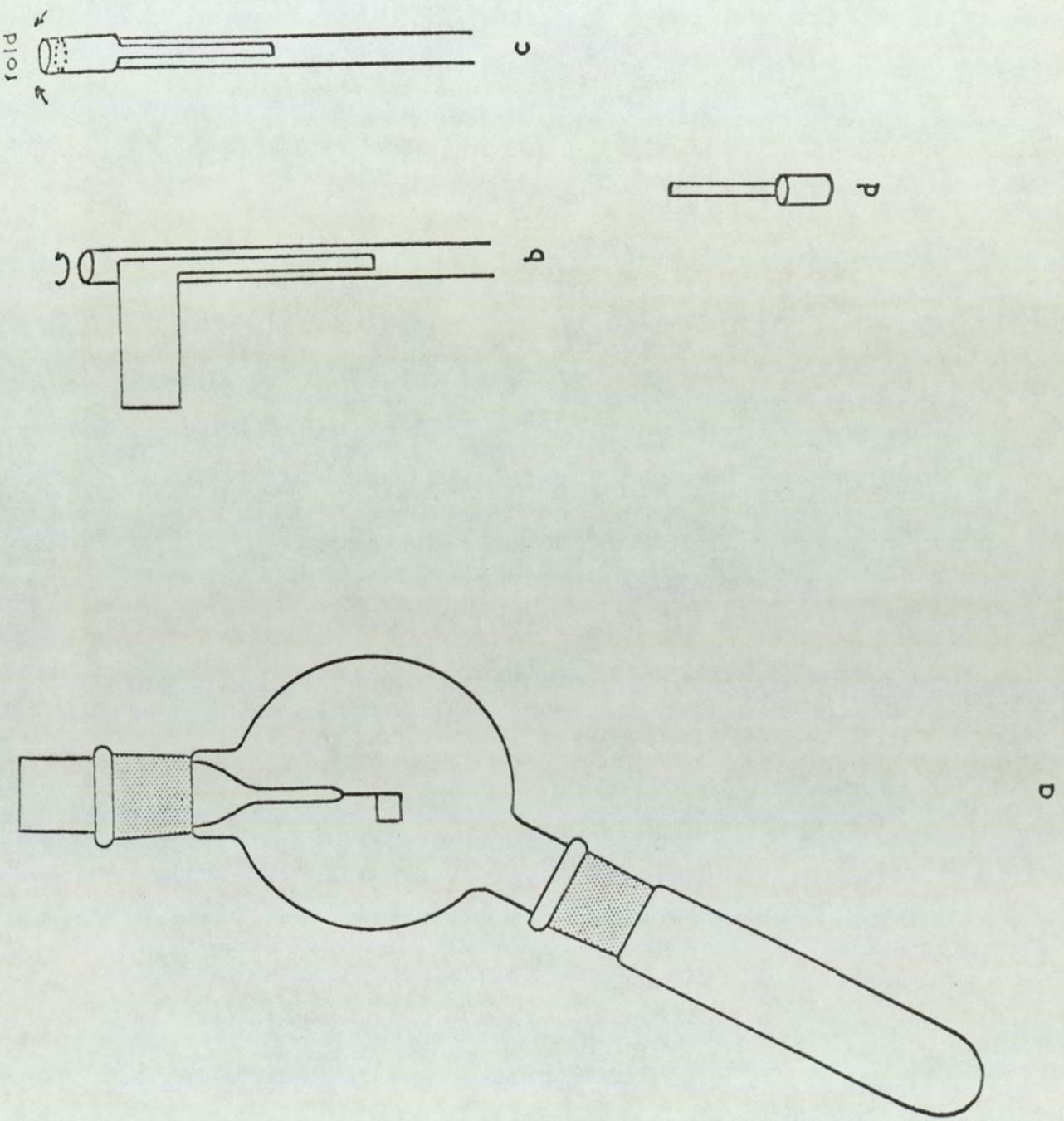


FIGURE 2.1. a) The whole tissue combustion apparatus. b), c) and d) show the method of making a paper basket.

it homogeneous. Glass scintillation vials were used throughout for counting radioactivity and all counting was done with a Nuclear Enterprises NE8305 Scintillation Spectrometer operating at 0^{o}. Background counts were routinely determined for each set of samples, and counting efficiencies for each sample being found by internal standardisation with n- (1-^{14}C)-hexadecane.

Use of coloured monoethanolamine, laboratory grade methanol, or storage of samples for a few days prior to counting resulted in yellow samples whose counting efficiency was very low and could not be accurately assayed.

Standardisation procedure.

After determination of sample counts, a quantity (5 - 15 mg) of n- (1-^{14}C) - hexadecane (specific activity 1.1 or 1.016 μCi/g) was accurately weighed into each vial. Samples were then recounted.

In all cases samples were counted for a time sufficient for 10,000 counts to accumulate. Radioactivity was found from the expression

$$\text{activity (m}\mu\text{Ci)} = \frac{Afw\ (c-b)}{(s - c)}$$

where A = activity of hexadecane, μCi/g,

f = dilution factor

w = weight of hexadecane added, mg,

c = sample counts, c/s,

b = background counts, c/s

s = sample + standard counts, c/s.

All calculations were done on an Olivetti Programma 101

computer using the program (Beavon, 1973).

ii) USE OF BIOLOGICAL OXIDISER.

The Beckman Biological Material Oxidiser operates on principle similar to tissue combustion apparatus as described before, though the combustion technique is automated and considerably refined. Samples are burnt in an electrically heated combustion chamber under a continuous stream of oxygen, and the oxidation is carried out over a high temperature catalyst bed. The final effluent gas consists entirely of CO_2 and water vapour which may, if desired, be collected separately for simultaneous determination of tritium and carbon-14. The equipment operates on an automatic 4 minute cycle which allows the full combustion and preparation of a tissue sample for the next burning. In order to determine the 5-methyltetrahydrofolic acid uptake by isolated mucosal epithelial cells, cells after incubation with 5-methyltetrahydrofolic acid (labelled compound diluted with unlabelled as and when necessary) for the specified period as warranted by the experimental conditions were washed and centrifuged as detailed in the isolation procedure before. After the final wash the clear supernatant was removed and the cell pellet was transferred to a glass boat, an important accessory of the Beckman Biological Material Oxidiser, which was used for the combustion of the cells under study. The cells contained in the boat were freeze-dried and the weight of the boat plus freeze-dried cells was taken.

Before actual burning of samples, the Biological Oxidizer was connected to an oxygen supply and switched on.

The temperature was set to 700°C on the catalyst bed side and to 900°C on the combustion side. Oxygen was allowed to flow at a rate of 300 ml/min.

When the temperatures were reached, the Biological Oxidiser was primed by burning several times about 150 mg. of mannitol contained in boat, which in turn was placed in a cool ladle and the ladle inserted into the combustion tube. Burning started, as soon as the start button was pushed. The ladle was held in place by a retaining spring between the ladle and the unit. The complete burning cycle took four minutes. After the priming, the boat ladle with the boat was removed and the carbon-14 trap with ^{14}C cocktail (^{14}C absorber P, Fisons) 15 ml. in volume was attached to the catalyst bed end of the combustion tube. A boat in a cool ladle, containing 150 mg. of mannitol was inserted into the combustion tube and the start button pressed. CO_2 produced was absorbed in the cocktail, this served as the blank sample. After the cycle was completed, the ^{14}C trap with the combusted sample was removed and put in the Trap Hanger. The hot ladle with the boat was removed from the Combustion Tube. A second trap with fresh cocktail was attached as before and a second blank sample was ignited. While the sample was burning, the previous combusted sample was passed into a counting vial. The trap was washed three times with methanol from a wash bottle, refilled with cocktail and placed on the trap hanger. The cell samples were combusted exactly in the same manner as the blank ones. Some known weights of n(1-^{14}C) hexadecane were burnt. Samples were then counted in a Nuclear Enterprises NE 8305 Scintillation Spectrometer along with some known

weights of n(1-^{14}C)-hexadecane in the same cocktail. The empty boats when cool were weighed and the difference of boat plus cells and empty boat gave the weight of the cells burnt.

Some sac tissues were also combusted and counted by the same method.

Determination of the cell content of 5-methyltetrahydrofolic acid.

Some weighed amounts of n-hexadecane were oxidised and $^{14}CO_2$ produced was collected in the absorber and control. The theoretical d.p.s. was calculated for each weighed amount of the sample from the specific activity of the compound. The ratio of the observed c.p.s. to that of the calculated theoretical d.p.s. gave the counting efficiency. If the counting efficiency was derived to be 20% then

at 100% efficiency of counting 1nCi = 37 d.p.s.

∴ at 20% " " " 1nCi = 7.4 d.p.s.

The cell counts obtained after subtraction of the background count were calculated as follows:

∴ 7.4 c.p.s. = 1 nCi

∴ Cell counts, say x = $\frac{x}{7.4}$ nCi

When the cell activity was thus calculated from the counts, then the cell content of 5-methyltetrahydrofolic acid could be calculated from the original activity of external solution in relation to the amount of 5-methyltetrahydro-

folic acid present. The relation can be furnished as follows: -

If in the mucosal solution

XnCi corresponds to Y µg of 5-methyltetrahydrofolic acid.

$$\therefore \quad \frac{x}{7.4} \quad nCi = \frac{Y}{X} \times \frac{x}{7.4} \quad \mu g$$

iii) FLUID SAMPLES.

The fluid samples, e.g. the working solution, i.e. the mucosal solution, the serosal solution collected as described in uptake experiments, the washings from the cells after completion of incubation with 5-methyltetrahydrofolic acid and the efflux medium after scheduled incubation time in the efflux experiment were counted in the following way. 100 µl of each solution was pipetted with Eppendorf pipette and transferred to NE 220 scintillator or Dioxan 'D' cocktail and then counted in the Nuclear Enterprise Scintillation counter. Counting efficiency was about 70% with amplifier gain at 30, lower discriminator setting of 0.8V and upper discriminator setting of 5.0V.

After counting they were either standardized by internal standardization method as described in the tissue burning experiments method or the 5-methyltetrahydrofolic acid content of the solutions were determined as described in the cell combustion procedure by efficiency determination.

iv) THIN LAYER CHROMATOGRAPHY.

Thin layer chromatography was done on MN 300 UV cellulose plates, 20 x 20 cm, pre-spread on plastic foils (Polygram foils; Macherey-Nagel). This was done to determine the metabolic fate of the 5-methyltetrahydrofolic acid in the serosal fluid, in the tissue, in the efflux medium and in the external incubation medium after incubation.

In case of liquid samples, the solutions collected were freeze-dried. The residue was taken up in 0.5 ml. of 14% (v/v) aqueous ammonia (containing 1% v/v 2 mercapto-ethanol) and centrifuged. The clear supernatant was then applied to the plate by a micropipette fitted with a syringe. After drying, the plate was developed in subdued light in solvent 1 or 2 (Table 2.2).

A spot or band of pure marker compound was routinely applied. Spots or bands were located as absorbing or fluorescing areas under 254 or 365 n.m. u.v. light. Chromatographic run usually took 140 - 150 minutes in case of solvent system 1 and 40 - 45 minutes in case of solvent system 2.

In case of sac tissue, sac was freeze dried and then homogenized in 5 ml. of 5% trichloroacetic acid and the solution was centrifuged. Spot or band was given by micro syringe from the supernatant and the thin layer chromatography was developed as described earlier.

Spots were identified by comparison with standard marker compounds or by reference to Rf values reported in the previous literature.

TABLE 2.2.

Chromatography solvents.

Solvent number	System
1	n-Propanol/ammonium hydoxide (s.g.0.880)/ water, 200:1:99 v/v. Made immediately before use.
2	0.1M phosphate buffer, pH 7.0

v) AUTORADIOGRAPHY.

Autoradiography was done with X-ray film (Ilford industrial 'G' or Kodak 'Kodirex'). This was used for the detection and location of labelled compounds in chromatograms. The t.l.c. plates were thoroughly dried, then were affixed to glass plates with 'sellotape'. Then in the dark room, the X-ray film was opened and laid over the cellulose surface and aligned with the bottom and one side edge of the plate, a second clear glass plate placed on top, and the whole 'sandwich' held together with four stout rubber bands. The plates were put in black paper or polythene envelopes, then in light-tight cardboard boxes and kept in the refrigerator at about $-20^{\circ}C$ for a few days, i.e. time required for the autoradiography to be developed. The sensitivity of the method was approximately 10 nCi/cm^2 in 24 hours.

After exposure, the sandwich was dissembled in the dark room and the film developed. Development was for about 10 minutes in Kodak DX 80; then 1 minute in a stop bath of aqueous acetic acid (3% v/v); and finally fixed in Kodak FX40 for at least 10 minutes. The films were washed thoroughly and air dried. Bands or spots were located by autoradiography and Rf determined.

SECTION 2. PART 4.

The incorporation of *in vitro* methods into intestinal physiology provides conditions where experimental variables can be more precisely controlled in the investigation of their effects on normal intestinal function. Incubation conditions can be standardised and other physiological parameters, e.g. blood flow, that affect gut function in the intact animal, are excluded. In the *in vitro* case, the preparations must be shown to be viable under the artificial conditions provided and demonstrated to be still functional, during the experimental period. Deficiencies of *in vitro* methods have been detailed in Section 2 (2) in connection with the preparation of sacs and isolated cells. Here certain parameters commonly accepted as demonstrating viability were investigated, including oxygen uptake, transmural potential, water transport and some histological criteria.

i) OXYGEN UPTAKE.

Tissue oxygen uptake was determined for the everted sac preparation and isolated intestinal mucosal epithelial cells in phosphate buffer using standard manometric methods. (Umbreit, Burris & Stauffer, 1951).
3 - 4 cms. everted sacs were prepared as described in the previous section and placed in 15 ml. Warburg flasks. The flasks contained 3 ml. of Krebs Ringer Phosphate buffer (pH 7.0). About 0.2 ml. of 10% KOH was added to the centre well. The flask side-arm stopper with gas vent was fitted by greasing properly. The attachment joint on the manometer was greased. One filter paper strip was added to

alkali in the centre cup and then the flask was attached to the manometer. The manometer fitted with the flask was then placed in constant temperature bath. The whole setting was then shaken for about 5 minutes to see whether the greasing was all right.

Oxygen was then introduced into the manometer from an oxygen cylinder through the closed side of the manometer (with stopcock and gas vent in the sidearm stopper open). The oxygen flow was controlled in such a way as to prevent the forcing out of the manometer fluid, i.e. the Brodie's solution. When adequate oxygen has been introduced the stopcock as well as the sidearm gas vent was closed. Equilibration was done with shaking for 10 - 15 minutes with all channels closed. The closed side of the manometer was finally adjusted (with stopcock open) to zero point (by adjusting the screw clamp on the fluid reservoir of the manometer). The stopcock was closed and the reading taking started. A thermobarometer was also fitted and adjusted in the same way except that there was no sac inside the flask. The temperature of the bath was maintained at 37°C and the rate of shaking was about 90 oscillations per minute.

At the start of reading taking the level of the liquid in both the arms of the experimental Warburg respirometer and the thermobarometer were at 'zero'. After 10 minutes the liquid went up in the closed arm and down in the open arm of the experimental respirometer. The change of level of liquid in the thermobarometer was negligible. The closed arm was then again adjusted to zero, consequently the liquid level in the open arm further went down and this was due to the oxygen consumption in the flask. The flask

reading was then noted from the open arm of the manometer. Actual change in mm. was obtained after thermobarometer correction. Similarly the flask readings were taken after 2 minutes interval up to one hour of incubation.

Calculation of oxygen uptake from manometer readings.

Oxygen uptake is given by the following relation:

oxygen uptake = alteration of reading on open arm of manometer X flask constant.

or $x = hK$

now $K = (Vg)\ (c) + (\Delta ml)\ (ml\ fluid)$

where Vg —— flask volume which is known

c —— a constant dependent on temperature only and known.

Δml —— another constant dependent only on α & T also known.

ml of fluid also known.

So K (flask constant) can be calculated from known values and h is calculated for each incubation period from the flask reading after thermobarometer correction. Since h and K are known, x is then easily calculated for each incubation period. Plotting of x against incubation time gives a linear graph for a period of 40 minutes. Oxygen uptake for 40 minute period is then obtained from the graph.

After the oxygen uptake experiment was over the sacs were freeze-dried and weighed, oxygen uptake in terms of dry weight was then found out.

Oxygen uptake by the isolated mucosal epithelial cells was determined in the same way as described above.

ii) TRANSMURAL POTENTIAL.

The electrical potential difference across the wall of the everted sac was measured by a modification of the method of Barry et al (1964). The everted sac was tied at one end and the other end was tied over the neck of a glass funnel. The sac was filled with 0.5 ml. of incubating solution and suspended in a boiling tube full of the same solution. Polythene tubes containing 1% agar in saturated KCl acted as salt bridges which lead to calomel half cells which were connected to Vibron Electrometer (Model 33 B2). One electrode was inserted into the funnel, the other in a beaker containing the medium, the beaker and boiling tube was connected by salt bridge. The mucosal solution was gassed throughout the incubation period with O_2-CO_2 (95:5 v/v). The test tube was incubated in a water bath at 37^{o} and the potential difference was measured at 15 minute intervals.

iii) WATER TRANSPORT.

Water transport was measured in the everted sac. The method has been described earlier in section 2 part 2(i). Tissue water uptake and serosal water transport were determined separately.

iv) HISTOCHEMICAL STUDIES.

Histochemical studies (Ito, 1965) were carried out both with everted sac and isolated cells.

The everted sac was incubated as usual for half an hour and then frozen in liquid nitrogen, sections cut and slides were made. One slide was fixed with formaldehyde by exposing it to formaldehyde vapour and the other was washed with absolute alcohol by placing on parallel glass rods. Both the slides were then washed with tap water for half an hour and then oxidized in 0.5% aqueous periodic acid for ten minutes. The slides were then washed again under running water for 5 minutes. Schiffs reagent was then drained in the slides and kept at rest for ten minutes. These were again washed for 30 minutes in a glass trough under gentle flow of water. They were then stained with haematoxylin and then washed with alkaline water. The slides were then treated with absolute alcohol followed by xylene. The slides were then viewed under microscope.

In case of cells, these were isolated as usual and then applied to slide with a pasteur pipette and dried at room temperature. The rest of the procedure was exactly the same as described for sacs.

v) CELL COUNTS.

Cell viability was also determined by counting the cells in the presence of 2% solution of tryptan blue (Girardi, McMichael & Henle, 1956). A 2% solution of tryptan blue was prepared in demineralized water and centrifuged at 1500 r.p.m. for 10 minutes. A 0.5 ml. of the supernatant was added to 1 ml. of the suspension to be counted. The cell count was performed immediately after mixing. An improved neubauer counting chamber was used for counting. In this method the damaged cells absorb the dye, whereas viable cells remain colourless.

SECTION 3.

RESULTS

Part 1. Viability studies.

Part 2. 5-CH_3H_4PteGlu uptake studies.

SECTION 3. PART 1.

i) OXYGEN CONSUMPTION.

The detailed method for the measurement of oxygen consumption by everted sacs and isolated mucosal epithelial cells has been described in Section 2, part 4.

Oxygen consumption of the sacs was found to be nearly linear over 40 minutes at a rate of 5.21 µl/mg dry weight/hr $\pm$ sem 0.11 for four sacs from two rats.

Oxygen consumption by isolated cells at pH 7.0, 6.0 and 5.0 was found to be linear for about 40 minutes with values of 3.24 $\pm$ sem 0.39, 1.42 $\pm$ sem 0.02 and 1.40 $\pm$ sem 0.02 µl/mg dry weight/hr. for four, two and two determinations respectively. Each determination comprised cells from one rat. At pH 3.0 and 4.0 oxygen uptake was nil.

These results indicate that the tissue preparations maintain aerobic metabolism for periods well in excess of the experimental incubation times of this study which were 30 minutes or less with the exception of one experiment where 5-CH_3-H_4PteGlu uptake was determined for 60 minutes.

ii) TRANSMURAL POTENTIAL.

The ability of the small intestine of the rat to maintain a transmural potential difference of several millivolts has been recognised for a number of years (Barry et al, 1964). The potential is thought to be the result of uphill sodium movement by the epithelial cells and is related to the transport and metabolism of hexose sugars (Smyth, 1966). It follows that the maintenance of the transmural potential difference must reflect the activity

of some of the primary transport systems of the intestinal epithelium and therefore provides a convenient index of the functional viability of the *in vitro* gut.

The measurement of transmural potential difference has been described in detail in Section 2, part 4.

Transmural difference measurements gave mean values of 3.43 mV $\pm$ sem 0.25 (21 sacs from 11 rats) after 30 minutes. The result suggests that the functional integrity of the mucosal epithelium in the everted sac used in the present study is satisfactory.

iii) WATER UPTAKE MEASUREMENTS.

Isotonic fluid transport across the mucosal surface is a feature of viable *in vitro* intestinal preparations. The fluid uptake mechanism is not fully clear, but is generally thought to occur as a corollary of active solute transport at the mucosal surface. Consequent upon the water absorption both serosal fluid volume and gut fluid volume increase. Fluid uptake is thought to occur as a consequence of energy dependent transport processes.

The use of anaesthetic may possibly affect the viability of intestinal preparations from the sacrificed animal and as a result can reduce the gut fluid uptake (Levine *et al*, 1970). The observations made by Levine and her co-workers sound interesting and therefore a comparative study of the fluid uptake by everted sacs prepared from animals sacrificed in two ways was made.

Section 2, part 2, has dealt with the detail of preparation of sacs and water uptake measurements.

The two different methods of sacrifice are being

furnished: -

1.) Etherisation. The animals were placed in a glass vacuum dessicator, the lower section of which contained a quantity of cotton wool soaked in diethyl ether. As soon as the animal became unconscious it was removed from the killing jar, the animal was killed by decapitation and the intestine was excised as described before.

2.) Stunning. The rat was placed gently on the surface of laboratory stool with minimum of disturbance. The animal was then swung suddenly by the tail and stunned with a single blow against the stool, the spine was severed at the base of the skull, and the intestine removed as usual. Unduly excited or alarmed animals were discarded.

Figure 3.1 presents the total fluid uptake, tissue fluid uptake and serosal fluid transfer against time for animals killed by stunning and decapitation. Both total uptake and serosal transfer tend to become constant after 1 hour whereas gut fluid uptake becomes constant after 30 minutes. Serosal fluid transfer appears to be higher than tissue fluid uptake.

Figure 3.2 represents a comparison of the total fluid transport of jejunal everted sacs from animals sacrificed by the two methods. Each column shows the mean and standard error of the number of animals given in parentheses.

These results suggest that everted sacs prepared from etherised animals had a significantly lower fluid transport capacity than similar sacs prepared from stunned and decapitated rats.

Literature does not provide any detailed report

on the effects of environment, handling, and the mode of sacrifice on the behaviour of subsequently prepared *in vitro* intestinal preparations although the importance of such study cannot be exaggerated. The use of anaesthetics is often justified on the grounds that stunning and decapitation cause shock and which in turn gives rise to low blood flow and consequent anoxia during the period prior to the perfusion of the severed intestine. Under anaesthesia, the intestine may be perfused with the blood flow still intact, a method in conformity with the recommendation of Fisher and Parsons (1949). Anaesthetics are said to have unknown effects on the metabolism of the intestine and since the whole operation takes several minutes one must work with tissue which has been exposed to unknown levels of a potentially toxic material. The suppression of oxidative metabolism in the brain is thought to result from the anaesthetic effect of ether and Taylor (1963) has noted a significant decrease in glycolysis in everted sacs prepared from animals killed with ether. Matthews (1966) has observed that the transport of D-Xylose is inhibited *in vitro* by phenobarbitone.

Literature provides evidence that both methods of killing have been used by many authors in a variety of *in vitro* intestinal studies. In the light of the results obtained it was thought that the use of ether probably results in a suppression of at least some of the energy dependent transport processes of the jejunum and, as a result, stunning and decapitation was adopted as the method of killing.

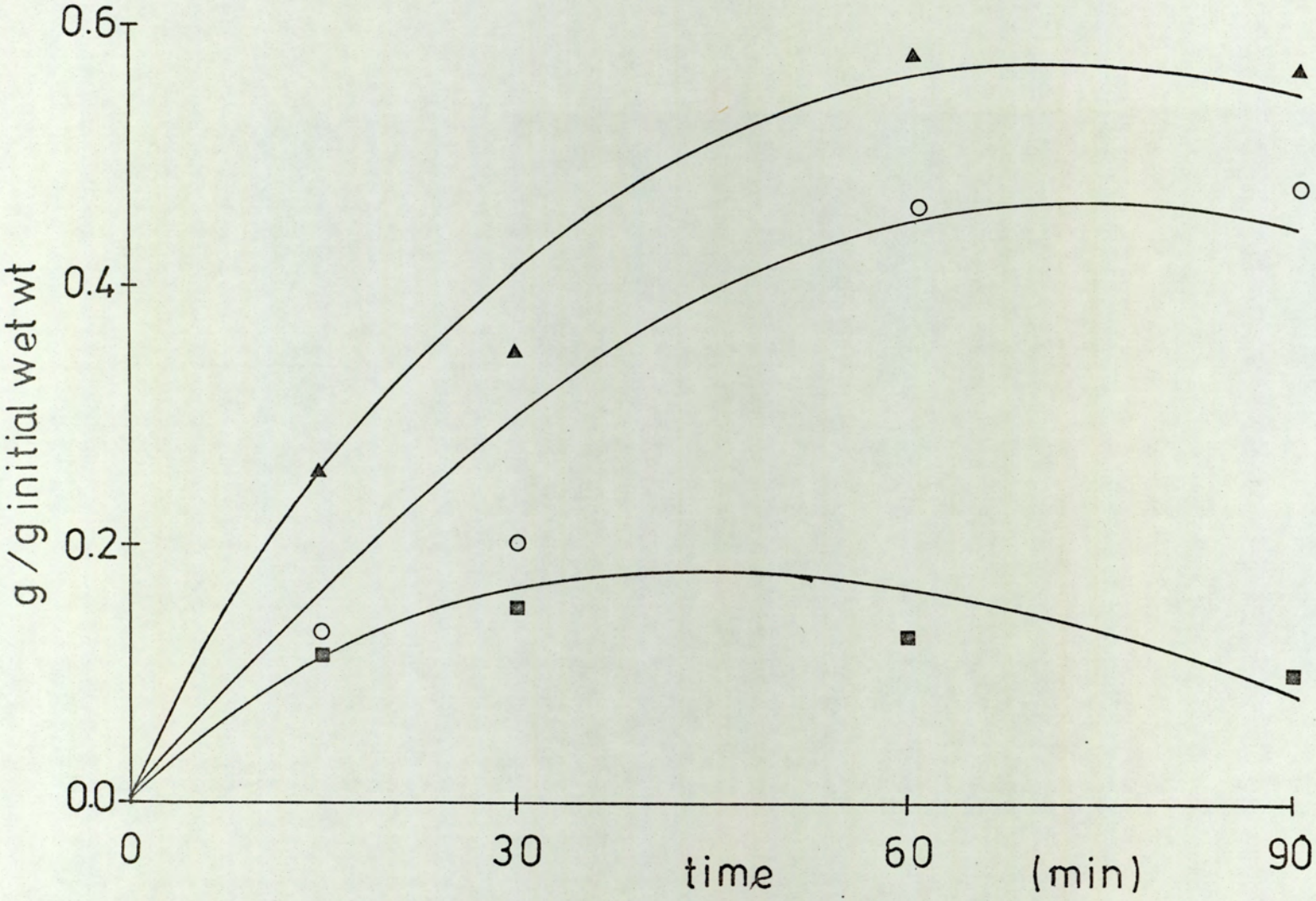


FIGURE 3.1. Time course of total fluid uptake (▲), serosal fluid transfer (○) and tissue fluid uptake (■) in everted sacs derived from animals killed by stunning and decapitation.

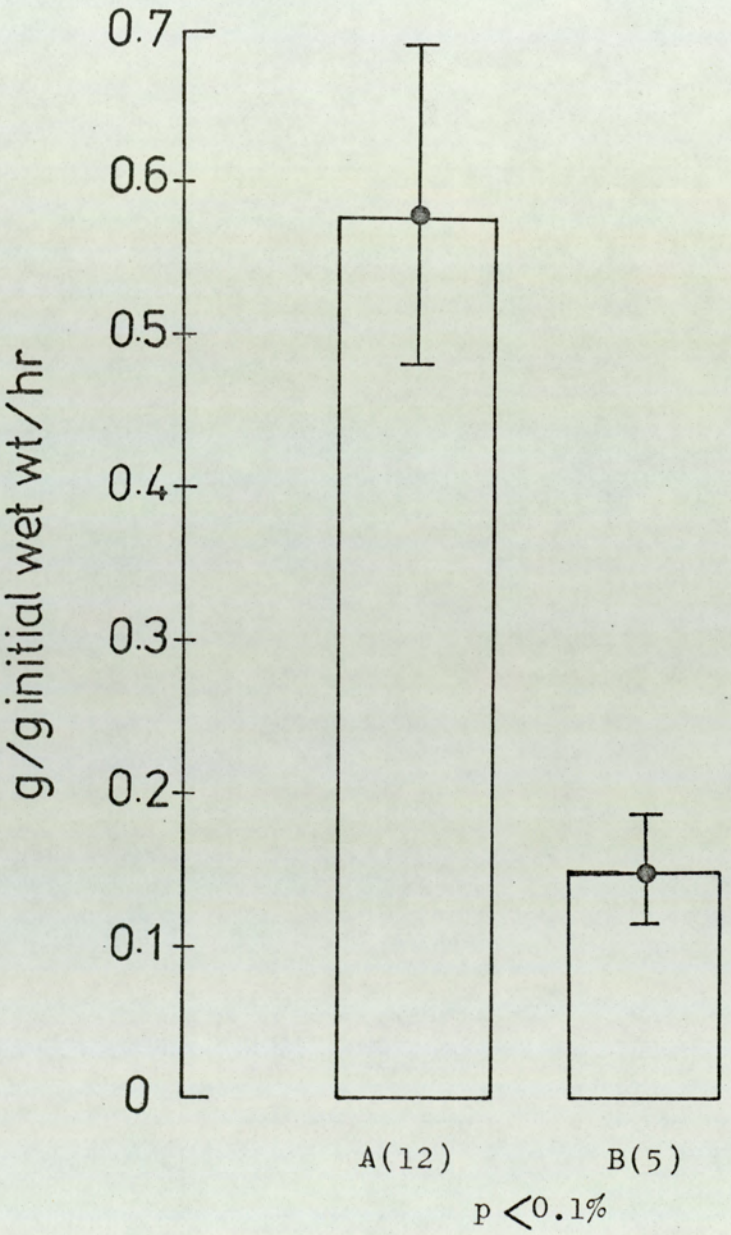


FIGURE 3.2. Comparative rates of total water transport by everted sacs from rats sacrificed by stunning and decapitation. (A) and by ether anaesthesia (B).
( ) no. of rats used is in parentheses.

iv) STABILITY OF 5-CH_3H_4PteGlu.

Stability of 5-CH_3H_4PteGlu was studied by thin layer chromatographic and autoradiographic analyses. A solution of 5-CH_3H_4PteGlu was prepared in Krebs Ringer Phosphate buffer (pH 7.2 - 7.4) the concentration being 1 mg/ml. To the solution was added sodium ascorbate the antioxidant (3 mg/ml). The said solution was kept in the cold chamber of the refrigerator at -20°C. The stability of this compound was determined by thin layer chromatography during the whole storage period. Thin layer chromatograms were run on cellulose plates (MN 300, 0.1 mm) in solvent No. 2 (Section 2, part 3). Standard solution of freshly prepared 5-CH_3H_4PteGlu was run at the same time, using fluorescent indicator plate. The Rf value of the chromatogram obtained from the stock solution was compared with that of the freshly prepared compound and it was found that the only absorbing chromatogram developed from the stock solution had the same Rf value of 0.86 as that of the freshly prepared one. Continuation of the thin layer chromatographic run of the stock solution along with a parallel freshly prepared 5-CH_3H_4PteGlu solution exhibited the same sort of behaviour, at least, for one month, i.e. the Rf value of the chromatogram from the stock solution always corresponded to that of the freshly prepared one proving the fact that 5-CH_3H_4PteGlu remains stable, at least, for a month in presence of sodium ascorbate if stored at -20°C.

Stability of labelled 5-CH_3H_4PteGlu can be better examined by autoradiography method. It is very convenient to detect extremely minute quantity. The detailed method has been described in Section 2, Part 3. Figure 3.3

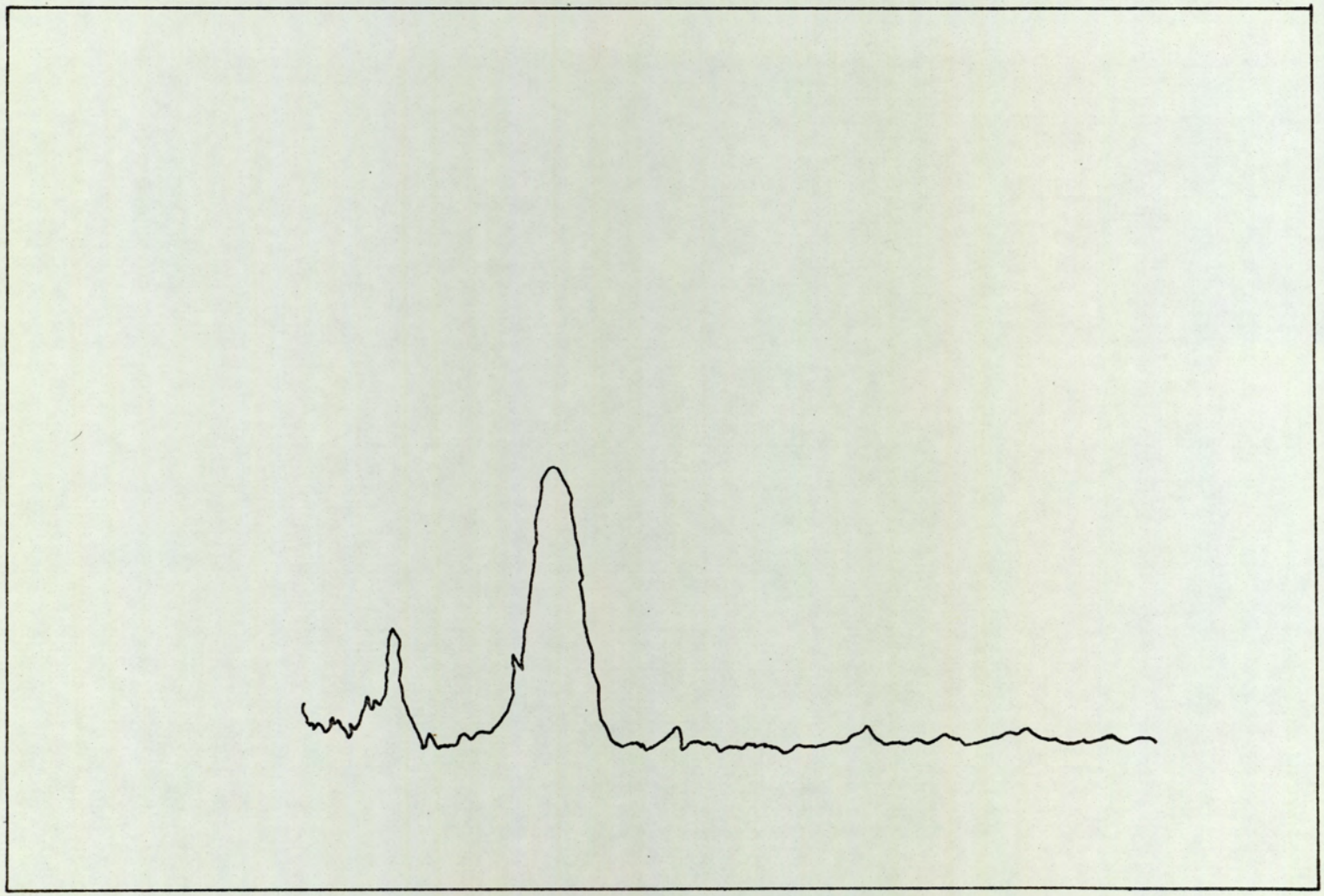


FIGURE 3.3. Microdensitometer trace for autoradiogram of freshly prepared labelled 5-CH_3H_4PteGlu run in phosphate buffer solvent.

FIGURE 3.4. Microdensitometer trace for autoradiogram of labelled 5-CH_3H_4PteGlu stored at -20°C for about two months run in phosphate buffer solvent.

illustrates the autoradiogram obtained from freshly prepared labelled 5-CH_3H_4PteGlu run in Solvent No. 2. Figure 3.4 represents the autoradiogram of a labelled 5-CH_3H_4PteGlu solution stored at -20°C for about two months. Development of only one band represented by the peak bears testimony to the fact that 5-CH_3H_4PteGlu remains stable for about two months in presence of antioxidant when stored at -20°C. A minor peak accounting for about 10% of the radioactivity appears in both the figures 3.3 and 3.4 corresponding to the solvent front. This may be the original impurity which according to the manufacturer's specification accounted for about 3% of the total radioactivity.

SECTION 3. PART 2.

i) UPTAKE OF 5-CH_3H_4PteGlu WITH TIME.

In experiments where the initial mucosal 5-CH_3H_4PteGlu concentration was 1.10^{-5}M and that of serosal solution was zero the total uptake was found to be linear for about 60 minutes. Figures 3.5, 3.6 and 3.7 show the result of a typical experiment. Table 3.1 shows the total, serosal and tissue uptakes of 5-CH_3H_4PteGlu and water. Both net water and 5-CH_3H_4PteGlu uptake increase with time for one hour incubation period. Tissue and serosal 5-CH_3H_4PteGlu uptake also increase with time and tissue uptake appears to be higher than that of serosal uptake. Serosal water uptake rises with time whereas tissue water uptake seems to reach saturation at 30 minutes interval. Figure 3.5 shows that there is a rapid uptake of 5-CH_3H_4PteGlu at the early period of incubation and this may be attributed to some unknown rapid uptake mechanism in tissue as tissue uptake at 15 mins. interval is considerably higher in comparison to serosal uptake.

The relative amounts of 5-CH_3H_4PteGlu and water uptake are given in Table 3.2. From Table 3.2 it appears that both total and serosal 5-CH_3H_4PteGlu transport in terms of water transport rise up to 30 mins. incubation time, after that both fail to show any rise in relative uptake although Table 3.1 shows increase in both 5-CH_3H_4PteGlu and water uptake. So it transpires that both the total and serosal 5-CH_3H_4PteGlu and water transport rise proportionately, i.e. balances each other at 60 mins. period. In case of tissue uptake there appears to be no change in the relative uptake of 5-CH_3H_4PteGlu and water up to 30 mins, but, in fact, both the uptakes increase as appears from Table 3.1. So again there

is the balancing of 5-CH_3H_4PteGlu uptake by water uptake. But after a further 30 minutes, i.e. at 60 mins. incubation period, the relative uptake shows a considerable rise; this is due to both rise in the 5-CH_3H_4PteGlu uptake and fall in the water uptake (Table 3.1).

Table 3.3 deals with the comparison of final mucosal, serosal and tissue concentrations of 5-CH_3H_4PteGlu of the same experiment represented by Table 3.1. Although the ratio of final tissue concentration to final mucosal concentration of 5-CH_3-H_4PteGlu seems to be higher than that of the final serosal concentration to final mucosal concentration of 5CH_3H_4PteGlu, the ratio in either case is not high enough to advocate concentration by tissue or serosal medium.

In another set of experiments performed under the same conditions sacs were incubated with 10^{-5}M 5-CH_3H_4PteGlu in both mucosal and serosal solutions, i.e. no initial concentration gradient between the mucosal and serosal solutions (Table 3.4). Both total and tissue 5-CH_3H_4PteGlu uptake are high but the serosal uptake is small. A ratio of 1.09 ± 0.19 sem for the concentration of 5-CH_3H_4PteGlu in the final serosal to that of the final mucosal solutions was found for an incubation period of 15 minutes. Calculation of the ratios of serosal to mucosal concentrations of a dianion at equilibrium using this value for the potential difference was made using the Nernst equation. A ratio of 1.14 was calculated giving good agreement with that obtained by measurement.

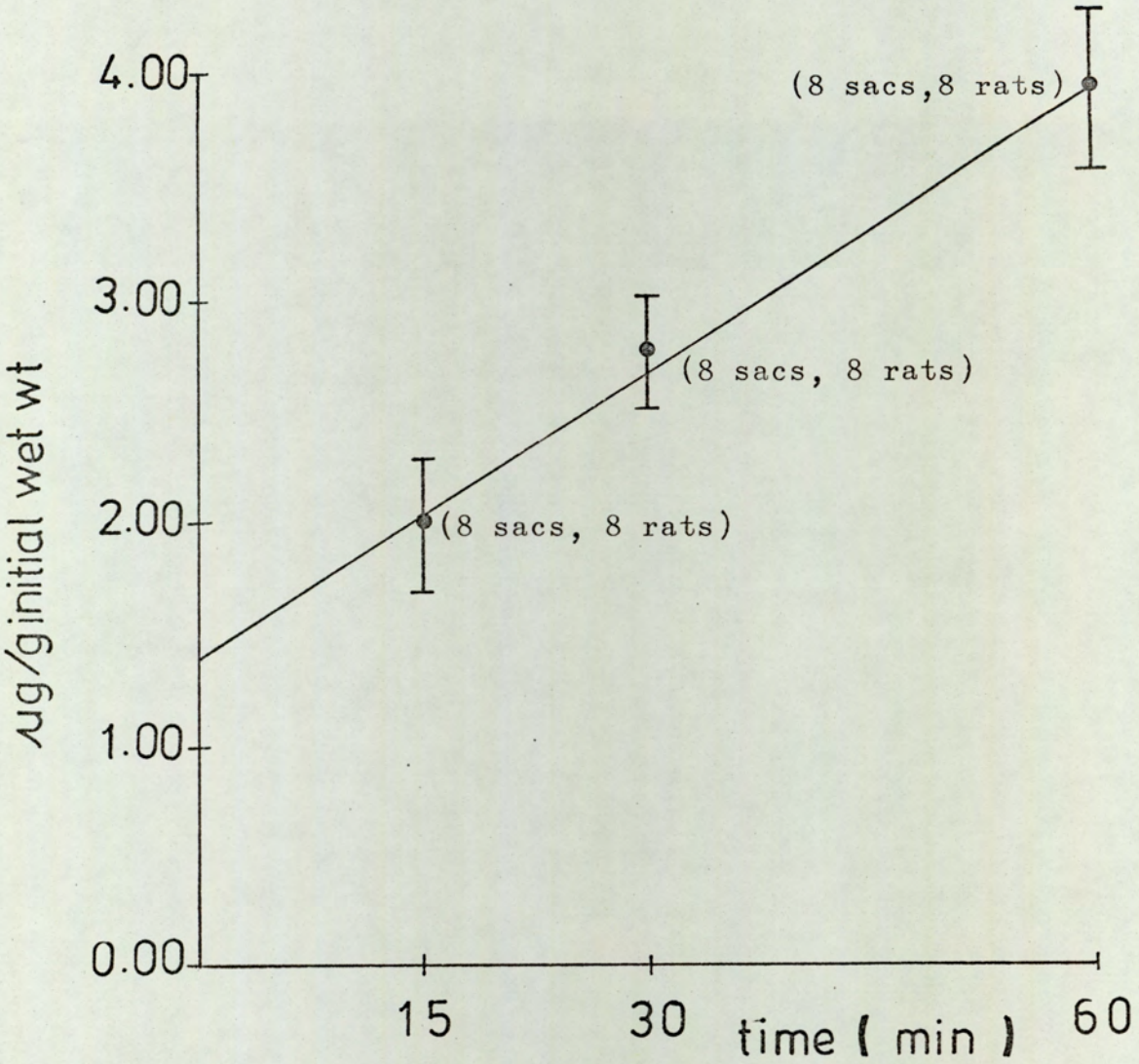


FIGURE 3.5. Time dependent total uptake of 5-methyltetrahydrofolic acid by rat jejunal sacs at a concentration of 10^{-5}M at 37°C and at starting pH of 7.2. Results are mean with sem.

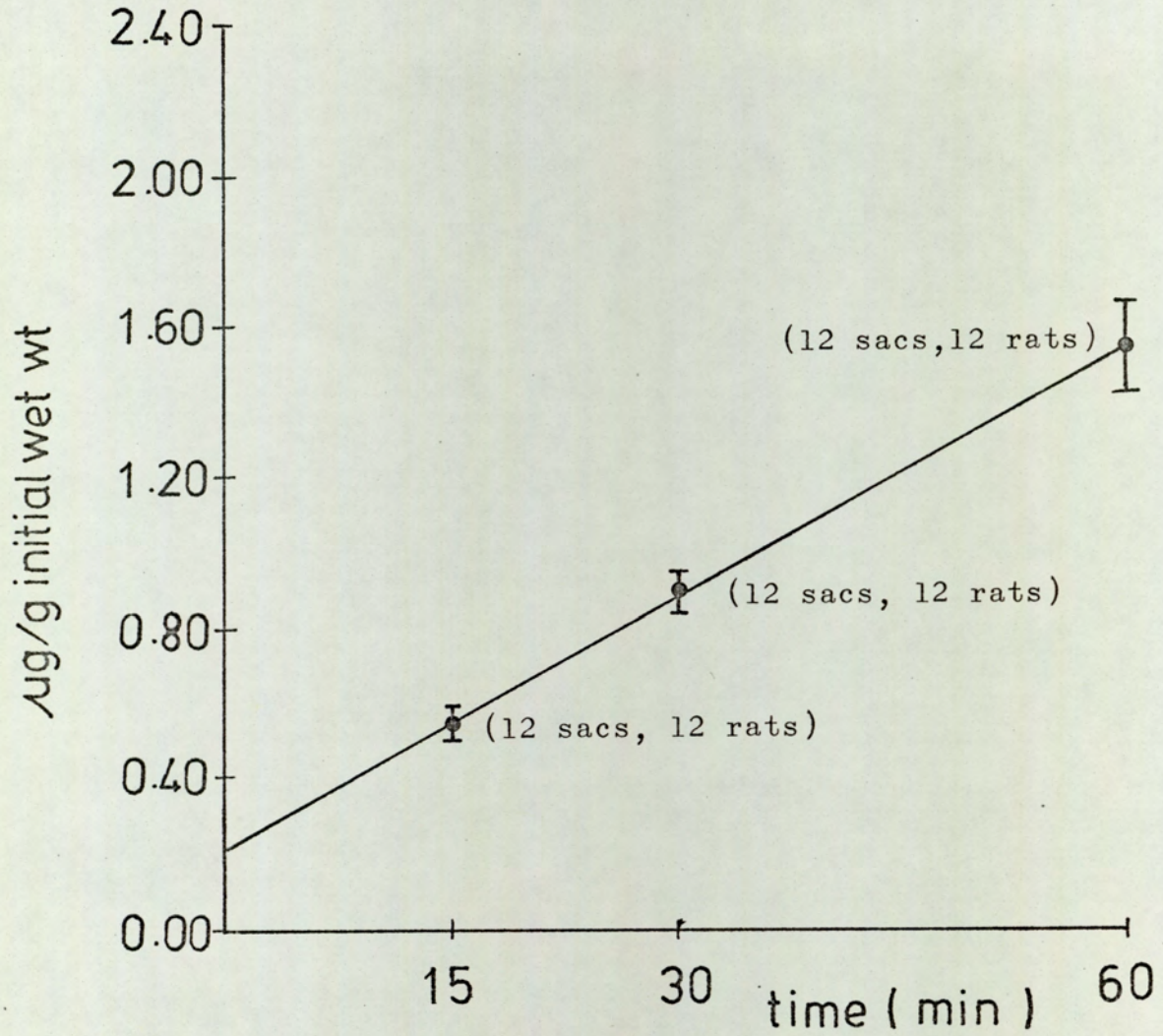


FIGURE 3.6. Time dependent serosal uptake of 5-methyltetrahydrofolic acid by rat jejunal sacs at a concentration of 10^{-5}M at 37°C and at starting pH of 7.2. Results are mean with sem.

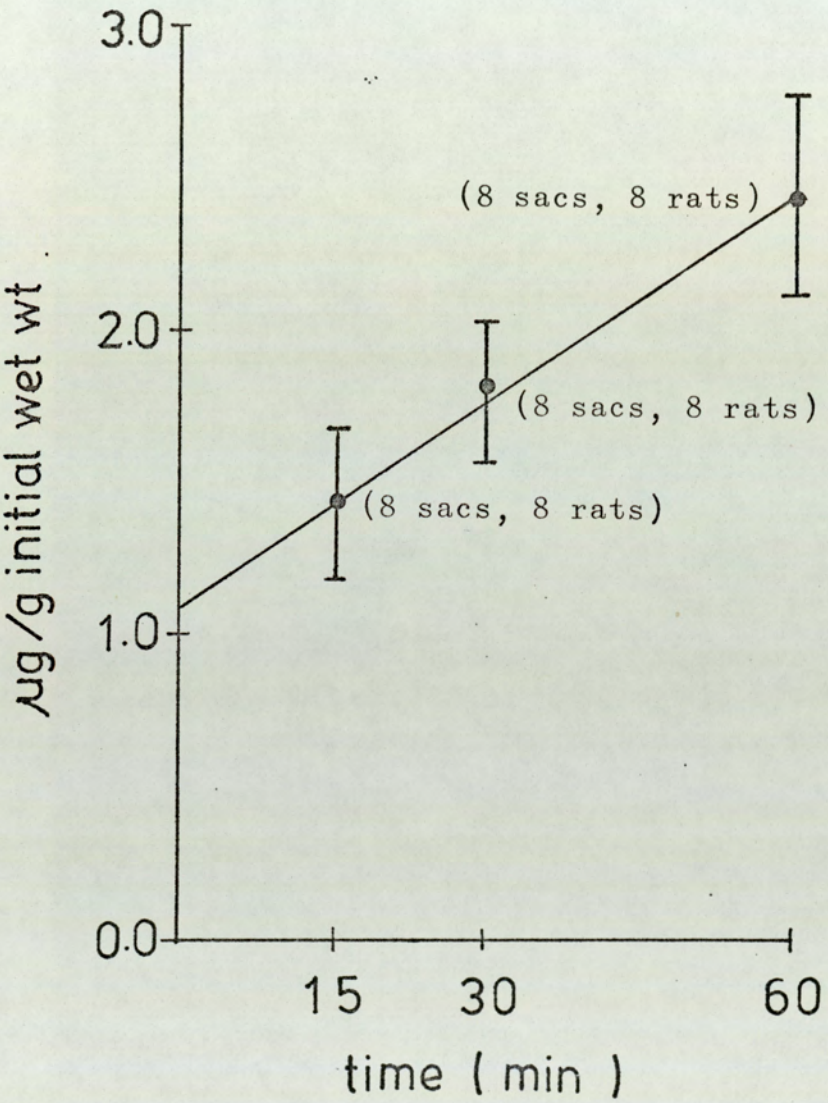


FIGURE 3.7. Time dependent tissue uptake of 5-methyltetrahydrofolic acid by rat jejunal sacs at a concentration of 10^{-5}M at 37°C and at starting pH of 7.2. Results are mean with sem.

TABLE 3.1.

$5CH_3H_4$PteGlu and water transport with time.

7 cms. rat jejunal sacs were filled with 0.5 ml. of Krebs Ringer Phosphate with 28 mM glucose and incubated in 6 ml. of the same medium containing 10^{-5}M 5-CH_3H_4PteGlu and sodium ascorbate (3 mg/ml) in addition at 37°C and at pH of 7.2. Values are mean with standard error of the mean. Wet wt. represents initial wet weight.

( ) indicates no. of sacs used.

[ ] indicates no. of rats used.

Incubation Time	Total Uptake.		Serosal Uptake.		Tissue Uptake.	
	$5CH_3H_4$PteGlu in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.	$5CH_3H_4$PteGlu in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.	5-CH_3H_4PteGlu in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.
15 mins.	1.99 ± 0.35 (8) [8]	0.25 ± 0.02 (12) [12]	0.55 ± 0.04 (12) [12]	0.13 ± 0.01 (12) [12]	1.46 ± 0.26 (8) [8]	0.11 ± 0.01 (12) [12]
30 mins.	2.76 ± 0.32 (8) [8]	0.35 ± 0.04 (12) [12]	0.90 ± 0.06 (12) [12]	0.20 ± 0.02 (12) [12]	1.81 ± 0.28 (8) [8]	0.15 ± 0.02 (12) [12]
60 mins.	3.91 ± 0.46 (8) [8]	0.58 ± 0.10 (12) [12]	1.56 ± 0.12 (12) [12]	0.45 ± 0.10 (12) [12]	2.46 ± 0.35 (8) [8]	0.13 ± 0.01 (12) [12]

TABLE 3.2

Transport of 5-CH_3-H_4PteGlu (10^{-5}M) in terms of water transport.

Results are mean with sem.

Incubation Period.	Total 5-CH_3H_4PteGlu transport in terms of total water transport in 10μM.	Serosal 5-CH_3H_4PteGlu transport in terms of serosal water transport in 10μM.	Tissue 5-CH_3H_4PteGlu transport in terms of tissue water transport in 10μM.
15 mins.	1.93 ± 0.35	0.94 ± 0.11	3.68 ± 1.08
30 mins.	2.24 ± 0.47	1.28 ± 0.24	3.23 ± 0.90
60 mins.	2.03 ± 0.29	1.27 ± 0.17	4.30 ± 0.69

TABLE 3.3.

Final serosal and tissue concentration of 5-CH_3H_4PteGlu have been compared with final mucosal concentration of 5-CH_3H_4PteGlu. This is the same experiment as in Table 3.1. Values are given as the means with the standard error of the mean.

Incubation Time	Serosal Concentration (μM) / Mucosal Concentration (μM)	Tissue Concentration (μM) / Mucosal Concentration (μM)
15 mins.	0.19 ± 0.02	0.33 ± 0.07
30 mins.	0.32 ± 0.03	0.41 ± 0.08
60 mins.	0.46 ± 0.04	0.59 ± 0.10

TABLE 3.4

$5\text{-}CH_3H_4PteGlu$ uptake by everted sacs at 'zero' initial concentration gradient of $5\text{-}CH_3H_4PteGlu$ between the mucosal and serosal solutions. 7 cm. rat jejunal sacs were filled with 0.5ml of Krebs Ringer Phosphate with 28 mM glucose containing 10^{-5}M $5\text{-}CH_3H_4PteGlu$ and sodium ascorbate (3 mg/ml) and incubated in 6 ml. of the same medium at 37°C and starting pH of 7.2. Values are shown as the means with the standard error of the mean.

( ) indicates no. of sacs. [ ] indicates no. of rats.

Incubation Period	$5\text{-}CH_3H_4PteGlu$ uptake in µg/g initial wet wt. of sac.		
	Total	Serosal	Tissue
15 min.	3.97 ± 0.65 (5) [3]	0.78 ± 0.47 (5) [3]	3.19 ± 0.46 (5) [3]

ii) TRANSPORT STUDY AT A DIFFERENT TEMPERATURE.

Transport was also studied at 27°C but this time the concentration of 5-CH_3H_4PteGlu in the mucosal medium was 10^{-5}M and that in the serosal medium was zero (Table 3.5). The Q_{10} value for total 5-CH_3H_4PteGlu transport at 37°C and at 27°C is 1.8 for 30 mins. incubation period.

iii) TRANSPORT STUDY AT DIFFERENT INITIAL pH.

Since PteGlu and 5-CH_3H_4PteGlu can exist in aqueous solution as the neutral species, monoanion, dianion and trianion the relative amounts of which vary with the pH of the solution, it is important to consider which form or forms are concerned in the transport. Studies on the transport of weakly acidic drugs across the rat intestinal wall suggest that only the neutral non-charged molecule is transported and that this form is transported through the lipoid barrier (Pohland et al, 1951; Schanker et al, 1958; Hogben et al, 1959).

These studies have also shown that the mucosal surface of the rat intestine is more acid than the bulk phase of the bathing solution ("microclimate") having a virtual pH of 5.3 when the bathing solution is at pH 7.4. Matty and Blair (1968) suggested that folic acid should be transported as the neutral compound across the lipoid barrier. At pH 5.0 only about 1% of folic acid in aqueous solution is present in the neutral form, and with increasing alkalinity, even less. This should also be valid for 5-CH_3H_4PteGlu. Therefore, any absorptive process will be limited by conditions which exist within the microclimate which in its turn will be influenced by mucosal or intraluminal conditions.

Uptake studies at different pH values were undertaken to determine pH value of optimum uptake of 5-CH_3H_4PteGlu and correlate this with the solubility and characteristic of the form of the compound present. Table 3.6 illustrates the 5-CH_3H_4PteGlu uptake studies at different pH values of the incubation medium. The maximum total uptake was observed at initial pH of 6.2. Higher as well as lower pH in the incubating medium decreased the absorption rate. (Fig. 3.8).

TABLE 3.5

$5\text{-}CH_3H_4PteGlu$ uptake at 27°C. 7 cm. rat jejunal sacs were filled with 0.5 ml. of Krebs Ringer Phosphate containing 28 mM glucose and incubated in 6 ml. of the same medium containing 10^{-5}M $5\text{-}CH_3H_4PteGlu$ at 27°C and at starting pH 7.2. Values are given as the means with the standard error of the means. Wet wt. represents initial wet weight. ( ) indicates no. of sacs used. [ ] indicates no. of rats used.

Incubation Period	Total Uptake		Serosal Uptake		Tissue Uptake	
	$5\text{-}CH_3H_4PteGlu$ in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.	$5\text{-}CH_3H_4PteGlu$ in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.	$5\text{-}CH_3H_4PteGlu$ in µg/g wet wt. of sac.	Water in g/g wet wt. of sac.
30 min.	1.54 ± 0.32 (5) [4]	0.16 ± 0.02 (5) [4]	0.34 ± 0.03 (5) [4]	0.12 ± 0.01 (5) [4]	1.20 ± 0.32 (5) [4]	0.04 ± 0.02 (5) [4]

TABLE 3.6.

Transport of 5-CH_3H_4PteGlu for 15 mins. incubation at different pH values. 7 cm. sacs from rat jejunum were filled with 0.5 ml. of Krebs Ringer Phosphate containing 28 mM glucose and incubated in 6 ml. of the same medium containing in addition 10^{-5}M 5CH_3H_4PteGlu and sodium ascorbate (3 mg/ml) at 37°C. Values are means with the standard error of the means.

( ) indicates no. of sacs used. [ ] indicates no. of rats used.

Initial pH.	Final pH.	Total Transport µg/g initial wet wt. of sac.	Serosal Transport µg/g initial wet wt.of sac.	Tissue Transport µg/g initial wet wt.of sac
7.2	6.7	1.99 ± 0.35 (8) [8]	0.55 ± 0.04 (8) [8]	1.46 ± 0.26 (8) [8]
6.7	6.4	2.83 ± 0.22 (6) [4]	0.46 ± 0.04 (6) [4]	2.36 ± 0.26 (6) [4]
6.2	6.0	3.30 ± 0.24 (7) [4]	0.43 ± 0.07 (7) [4]	2.87 ± 0.28 (7) [4]
5.7	5.7	1.90 ± 0.22 (6) [3]	0.44 ± 0.07 (6) [3]	1.46 ± 0.18 (6) [3]

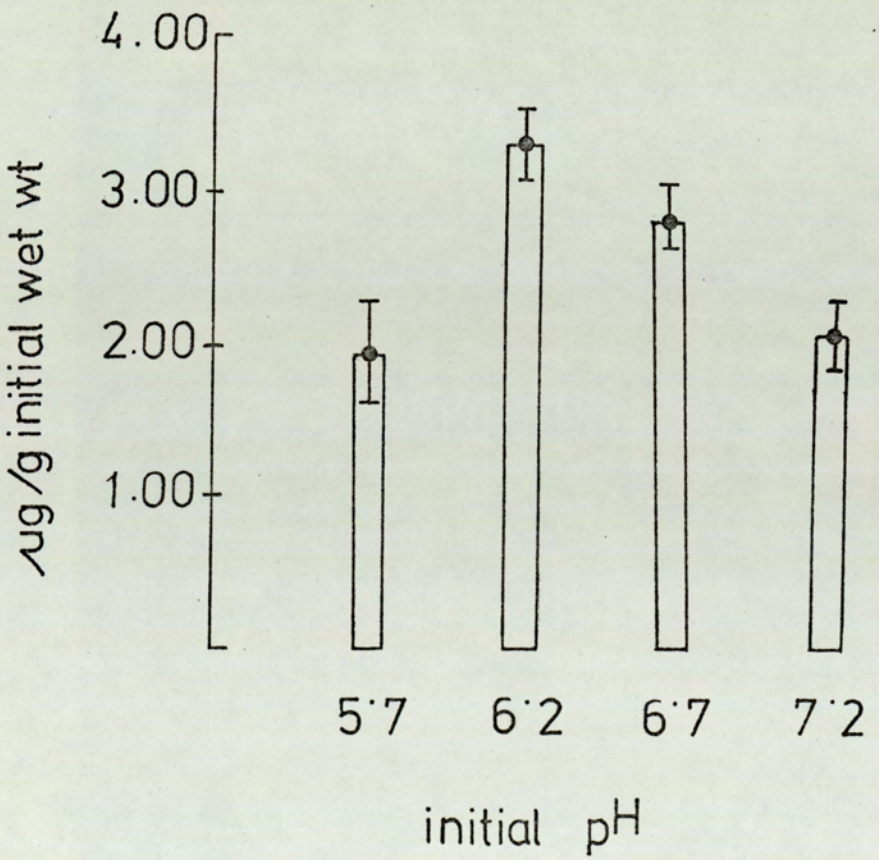


FIGURE 3.8 Uptake of 5-CH_3H_4PteGlu at different pH (initial) values at mucosal concentration of 10^{-5}M at 37°C.

iv) KINETICS OF 5-CH_3H_4PteGlu UPTAKE.

The uptake of 5-CH_3H_4PteGlu by the rat jejunal sacs was studied over the concentration range from 1.10^{-7}M to 1.10^{-4}M in the mucosal side of the sacs and zero concentration on the serosal side. Tissue uptake and serosal transfer were also studied simultaneously. The pH of the incubation medium was maintained at 7.2 and the temperature was 37°C. Table 3.7 contains the results of these experiments carried out at the concentrations cited. The results have been illustrated in Figures 3.9, 3.10 and 3.11. From the Figures it appears that the effect of a 1000-fold increment in substrate concentration (1.10^{-4} to 1.10^{-7}M) on transport of 5-CH_3H_4PteGlu from mucosa to serosa, i.e. on the total, serosal and tissue uptakes was to produce a linear increase in the uptake amount. The results show the absence of saturation kinetics within the concentration range studied. So, there is no kinetic resemblance between the processes of transport exhibited by the total, serosal and tissue 5-CH_3H_4PteGlu and the simple enzyme-mediated reaction of the general form:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow[K_3]{} E + \text{products}$$

Therefore a Michaelis-Menten equation

$$v = \frac{V.S}{K_S + S} \qquad \ldots\ldots\ldots\ldots \quad (3,a)$$

does not hold good for the total, tissue and serosal transport of 5-CH_3H_4PteGlu by rat jejunal everted sacs.

Need for fitting the datas in Lineweaver-Burk plot, Hofstee plot and Woolf plot does not arise at all.

TABLE 3.7

5-CH_3H_4PteGlu transport with concentration. 7 cms. rat jejunal sacs were filled with 0.5 ml. of Krebs-Ringer Phosphate containing 28 mM of glucose and incubated in 6 ml. of the same medium containing different concentrations of 5-CH_3H_4PteGlu along with sodium ascorbate (3 mg/ml). Incubation temperature and initial pH were 37°C and 7.2 respectively. Results are mean with standard errors.

( ) indicates no. of sacs used. [ ] indicates no. of rats used.

5-CH_3H_4PteGlu concentration	5 CH_3H_4PteGlu Uptake in µg/g initial wet wt. of sac.					
	Serosal		Tissue		Total	
	15 mins. incubation	30 mins. incubation	15 mins. incubation	30 mins. incubation	15 mins. incubation	30 mins. incubation
10^{-7}M	0.0034 ± .0007 (12) [9]	0.0095 ± 0.0027 (12) [9]	0.030 ± 0.005 (12) [9]	0.054 ± .008 (12) [9]	.034 ± .006 (12) [9]	0.064 ± 0.01 (12) [9]
10^{-6}M	0.034 ± 0.003 (12) [10]	0.11 ± 0.02 (12) [10]	0.36 ± 0.06 (12) [10]	0.68 ± 0.10 (12) [10]	0.39 ± 0.06 (12) [10]	0.79 ± 0.11 (12) [10]
10^{-5}M	0.39 ± 0.04 (24) [20]	0.68 ± 0.06 (24) [20]	1.22 ± 0.15 (20) [16]	1.42 ± 0.16 (20) [16]	1.57 ± 0.18 (20) [16]	2.07 ± 0.21 (20) [16]
10^{-4}M	2.49 ± 0.25 (13) [9]	5.65 ± 0.96 (11) [9]	6.00 ± 0.81 (13) [9]	7.15 ± 0.81 (11) [9]	8.50 ± 0.95 (13) [9]	12.80 ± 1.08 (11) [9]

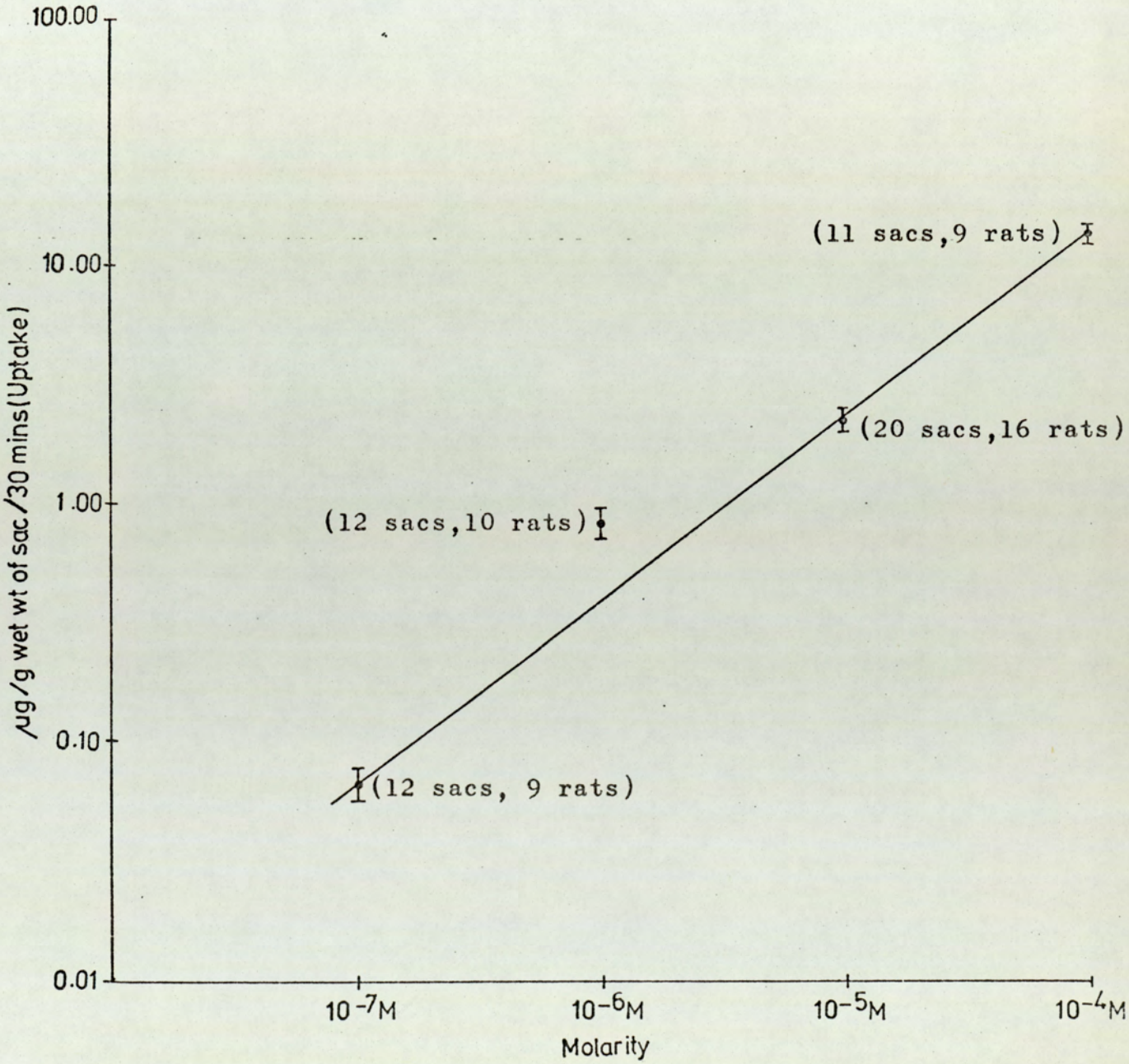


FIGURE 3.9 Total 5-CH_3H_4PteGlu uptake by rat jejunal sacs incubated at different concentrations of 5-CH_3H_4PteGlu at 37°C and at initial pH of 7.2. Values are means with sem. Wet wt. represents initial wet weight.

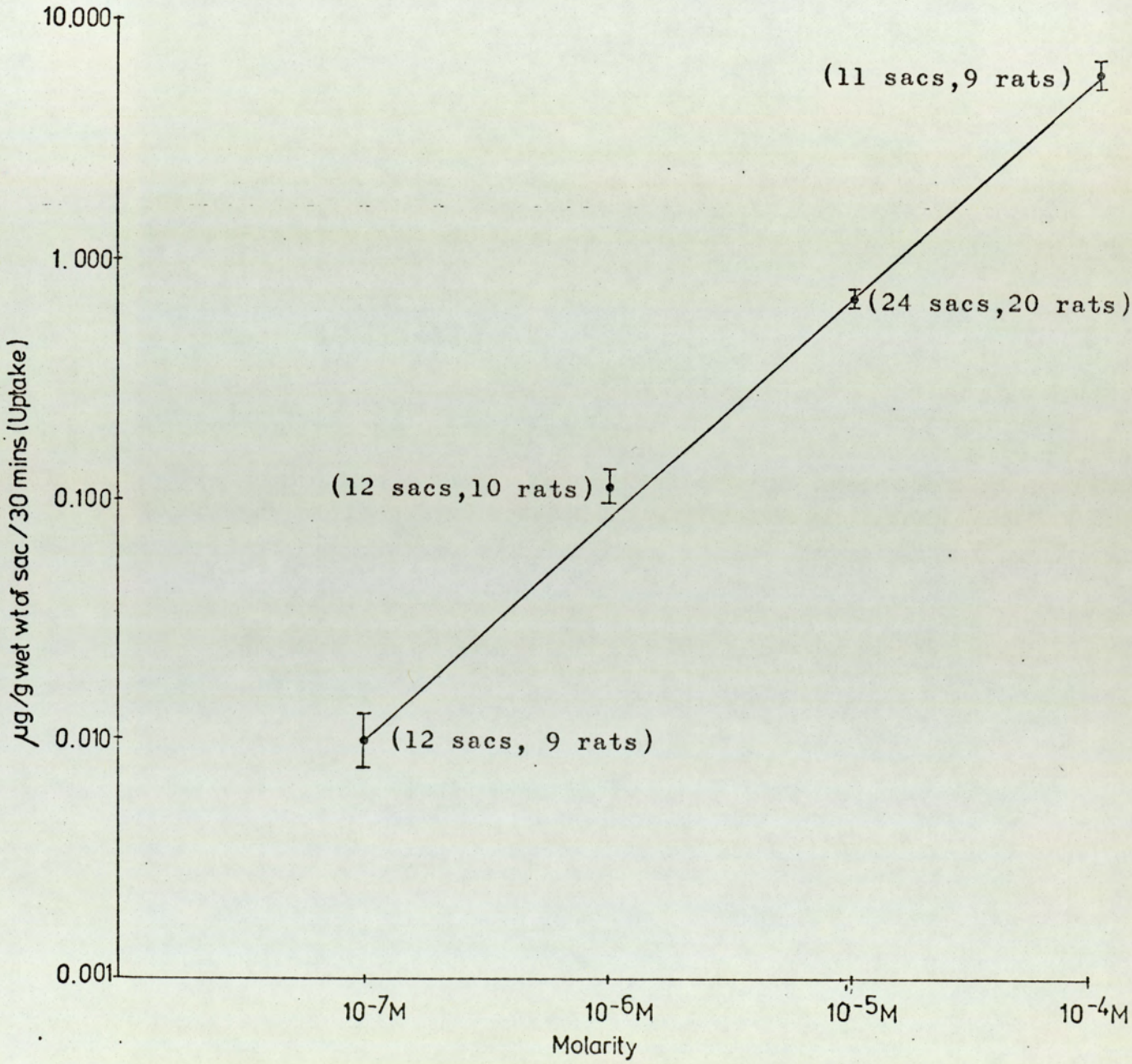


FIGURE 3.10 Serosal 5-CH_3H_4PteGlu uptake by rat jejunal sacs incubated at different concentrations of 5-CH_3H_4PteGlu at 37°C and at initial pH of 7.2. Values are mean with sem. Wet wt. represents initial wet weight.

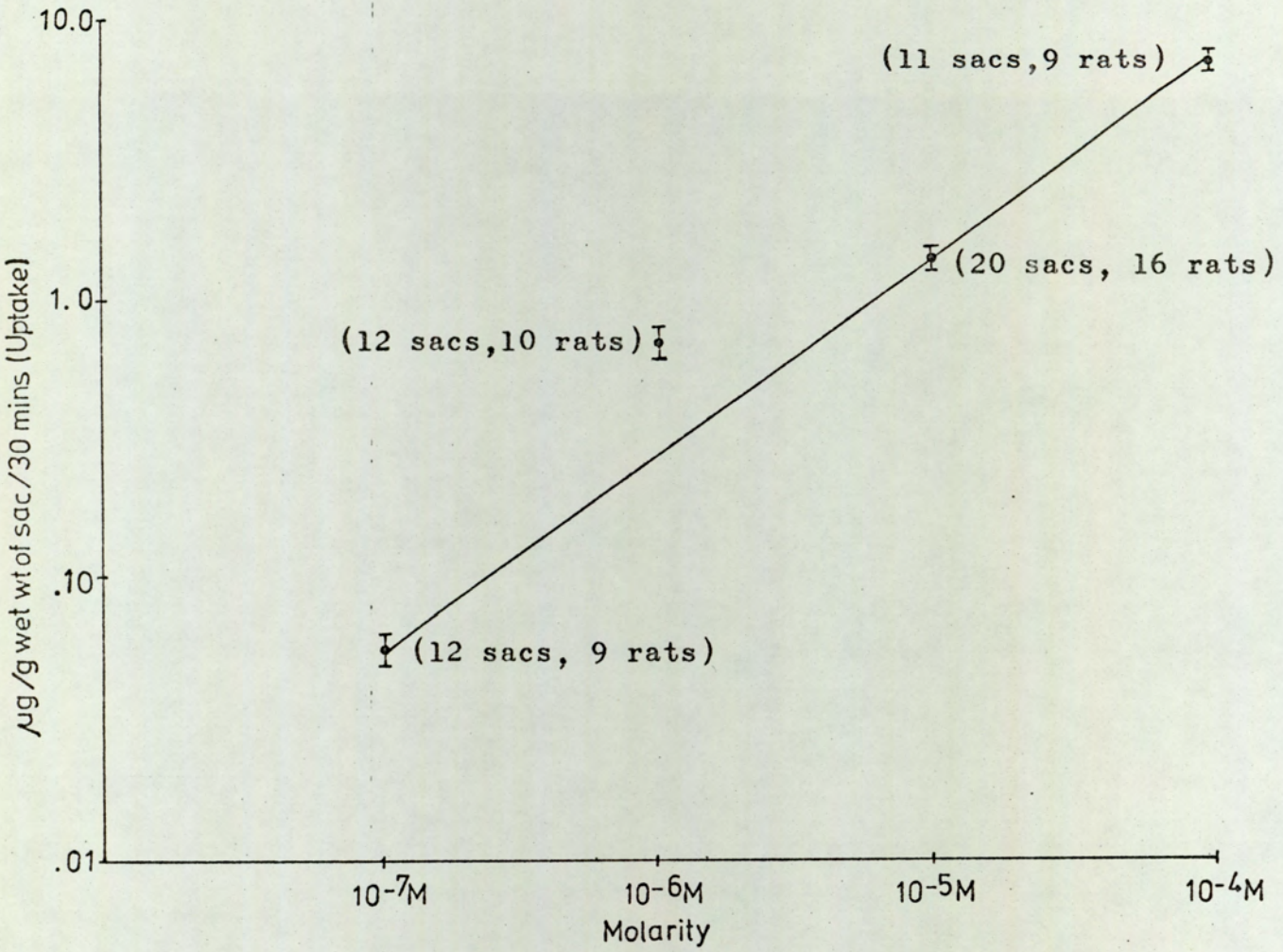


FIGURE 3.11. Tissue 5-CH_3H_4PteGlu uptake by rat jejunal sacs incubated at different concentrations of 5-CH_3H_4PteGlu at 37°C and at initial pH of 7.2. Values are shown as means with sem. Wet wt. represents initial wet weight.

v) EFFECT OF INHIBITION.

In vitro intestinal transport of 5-CH_3H_4PteGlu has been established to be a process showing absence of saturation kinetics and the temperature coefficient is also low (Section 3, Part 2ii). Here the everted sac technique has been used to examine the effect of inhibitors of both metabolic and competitive nature on total uptake, tissue uptake and serosal transfer of 5-CH_3H_4PteGlu.

In these experiments, 6 sacs (7 cm. initial wet length) were prepared from the jejunum of each rat. Of the six, 2 were maintained as controls under normal incubation conditions, i.e. incubated in 6 ml. of the medium containing Krebs Ringer Phosphate, 28 mM glucose, sodium ascorbate (3 mg/ml) and also radioactive 10^{-6}M 5CH_3H_4PteGlu solution, in addition, 2 were incubated in the same medium as above in presence of non-labelled 10^{-5}M5CHO-H_4PteGlu and 2 were incubated in the same medium as above, but this time in presence of 2 x 10^{-3}M Dinitrophenol which is a metabolic inhibitor. The sacs were incubated under the usual conditions for 30 minutes, after which all six sacs were removed and assayed for serosal transfer and tissue accumulation as described by the methods in Section 2, Part 3. The serosal water transfer was measured by the method described in Section 2, Part 2. Figures 3.12, 3.13 and 3.14 illustrate the total 5-CH_3H_4PteGlu uptake, the tissue 5-CH_3H_4PteGlu uptake and serosal 5-CH_3H_4PteGlu transfer respectively in the controlled conditions as well as in the presence of inhibitors. Table 3.8 contains the data for 5-CH_3H_4PteGlu uptake in controlled condition as well as in the presence of inhibitors. Table 3.9 incorporates the data for

5-CH_3H_4PteGlu uptake in terms of water uptake.

The reduction in the total, tissue uptake and serosal transfer of 5-CH_3H_4PteGlu in the presence of 5-CHO H_4PteGlu as assessed by paired "t" test appears to be statistically highly significant (Table 3.8). This is also true when the 5-CH_3H_4PteGlu transport is considered in terms of water transport (Table 3.9).

DNP in a similar manner brings about the reduction in the uptake of 5-CH_3H_4PteGlu by tissue and serosal transfer and the reduction is statistically significant (Table 3.8). But when the uptake is considered in terms of water uptake (Table 3.9), the reduction in total and tissue uptake appears highly significant but the serosal transfer is non-significant indicating a parallel reduction in the serosal fluid transfer in presence of DNP.

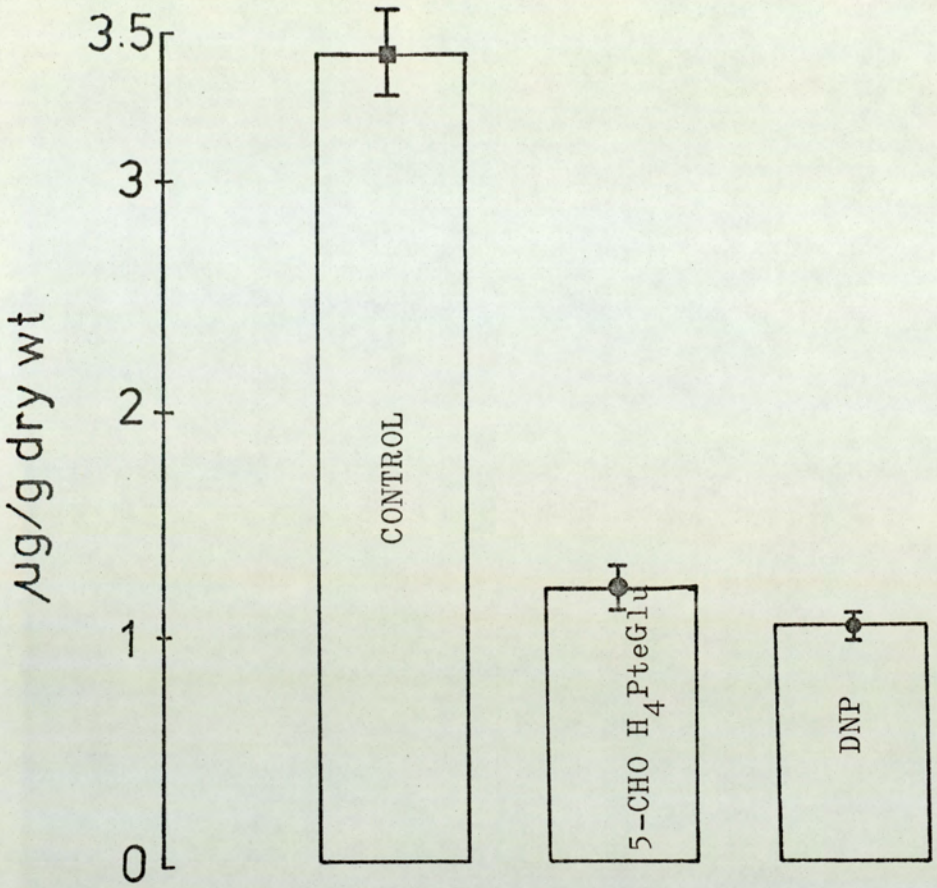


FIGURE 3.12. Inhibition of total uptake after 30 mins. incubation.

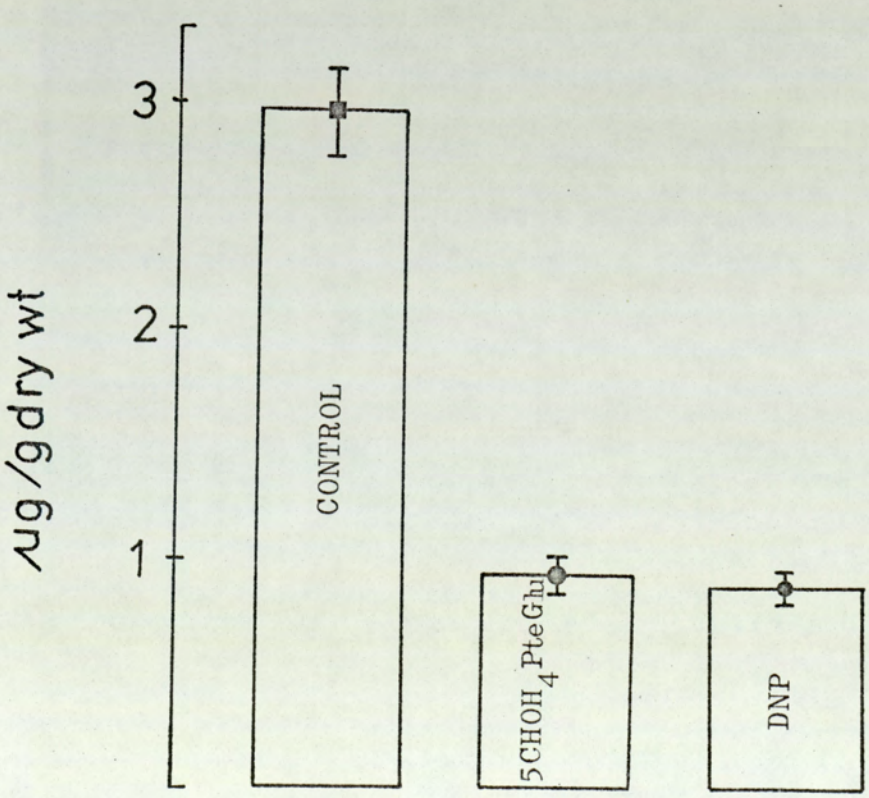


FIGURE 3.13 Inhibition of tissue uptake after 30 mins. incubation.

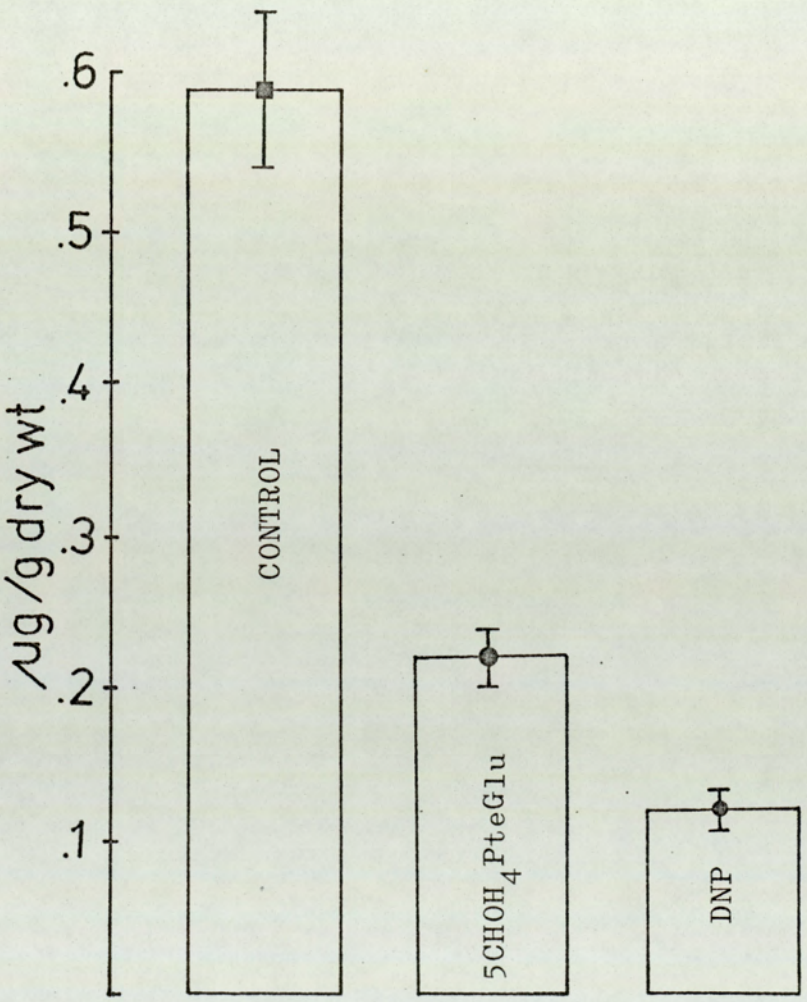


FIGURE 3.14. Inhibition of serosal transfer after 30 mins. incubation.

TABLE 3.8.

Effect of inhibition on transport of 5-CH_3H_4PteGlu by everted sacs of rat jejunum.

	Control	5-$CHOH_4$PteGlu	p*	DNP	p*
1) Total Uptake (µg/g dry wt)	3.54 ± 0.22 (8) [4]	1.17 ± 0.09 (8) [4]	<0.1%	1.03 ± 0.05 (8) [4]	<0.1%
2) Tissue Uptake (µg/g dry wt)	2.94 ± 0.21 (8) [4]	0.95 ± 0.08 (8) [4]	<0.1%	0.90 ± 0.05 (8) [4]	<0.1%
3) Serosal Transfer (µg/g dry wt)	0.59 ± 0.05 (8) [4]	0.22 ± 0.02 (8) [4]	<0.1%	0.12 ± 0.01 (8) [4]	<0.1%

* As indicated by student's paired "t" test.

Results are shown with sem.

( ) indicates no. of sacs used.

[ ] indicates no. of rats used.

TABLE 3.9.

$5\text{-}CH_3H_4PteGlu$ transport in terms of water transport in presence of inhibitors by rat jejunal everted sacs.

	Control	5-CHO H_4PteGlu	p*	DNP	p*
1) Total Uptake (μM/g dry wt)	4.31 ± 0.41 (8) [4]	1.48 ± 0.10 (8) [4]	<0.1%	2.24 ± 0.13 (8) [4]	<0.1%
2) Tissue Uptake (μM/g dry wt)	6.96 ± 0.86 (8) [4]	2.50 ± 0.38 (8) [4]	<0.5%	2.72 ± 0.22 (8) [4]	<0.5%
3) Serosal Uptake (μM/g dry wt)	1.73 ± 0.26 (8) [4]	0.67 ± 0.15 (8) [4]	<2%	1.36 ± 0.34 (8) [4]	<50%

* As indicated by student's paired "t" test.
Results are shown with sem.
( ) indicates no. of sacs used.
[ ] indicates no. of rats used.

vi) EFFLUX OF 5-CH_3H_4PteGlu FROM PRE-LOADED SACS.

A number of experiments were carried out to observe the efflux of 5-CH_3H_4PteGlu from the mucosal surface in the presence and absence of 5-CH_3H_4PteGlu and related compounds.

About six sacs (approximately 6 cms. initial length) from the rat jejunum were prepared from each animal as usual. The sacs were pre-loaded by incubating for 20 minutes in 6 ml. of standard incubation medium (Krebs ringer phosphate buffer with 28 mM glucose and 3 mg./ml sodium ascorbate) containing 0.5×10^{-6}M ^{14}C-labelled 5-CH_3H_4PteGlu. After pre-loading the sacs were washed for about 45 seconds with a gentle swirling agitation in warm oxygenated saline to remove extraneous activity, and transferred to polythene test tubes containing 1 ml. of the incubation medium (Krebs ringer phosphate buffer with 28 mM glucose and 3 mg/ml sodium ascorbate). Control tubes contained 5-CH_3H_4PteGlu free medium, treatment tubes contained non-labelled 5-CH_3H_4PteGlu and other related compounds at a concentration of 10^{-5}M. The sacs were incubated under these conditions for a further 30 minutes during which adequate stirring was done by continuous passing of O_2/CO_2 mixture (95:5 v/v) as before. At intervals of 5, 15 and 30 minutes 0.1 ml. of aliquots of the mucosal bathing medium were removed and transferred to liquid scintillator for ^{14}C determination.

In each experiment, groups of 2 or 3 sacs were incubated in the presence of 5-CH_3H_4PteGlu and paired with similar control groups incubated in 5-CH_3H_4PteGlu free medium. The results for sacs in each group were averaged to give single treatment and control values for each animal.

Differences were tested by student's paired "t" test.

Figure 3.15 and Table 3.10 show the time course mucosal radioactive efflux from sacs pre-loaded with 0.5 x 10^{-6}M $5^{14}C$-CH_3H_4PteGlu and subsequently incubated in a non-radioactive solution of 10^{-5}M 5-CH_3H_4PteGlu along with the efflux from similar pre-loaded sacs incubated in 5-CH_3H_4PteGlu free medium, i.e. Krebs-Ringer Phosphate medium containing 28 mM of glucose. At each time interval there is a consistent and significantly higher concentration of labelled compound in the treatment tubes in comparison to the control tubes, indicating that the efflux of labelled compound from the mucosal epithelium is stimulated in the presence of external 5-CH_3H_4PteGlu.

Figures 3.16, 3.17 and 3.18 illustrate similar type of experiments carried out with "treatment" solutions containing 10^{-5}M concentrations of folic acid, methotrexate and 5-CHO H_4PteGlu. The data as well as the statistical details for these experiments are contained in Tables 3.11, 3.12 and 3.13 respectively.

Similar trend of results has been observed in all the efflux experiments, i.e. at each time interval there is always a consistent and significantly higher concentration of labelled compound in the treatment tubes.

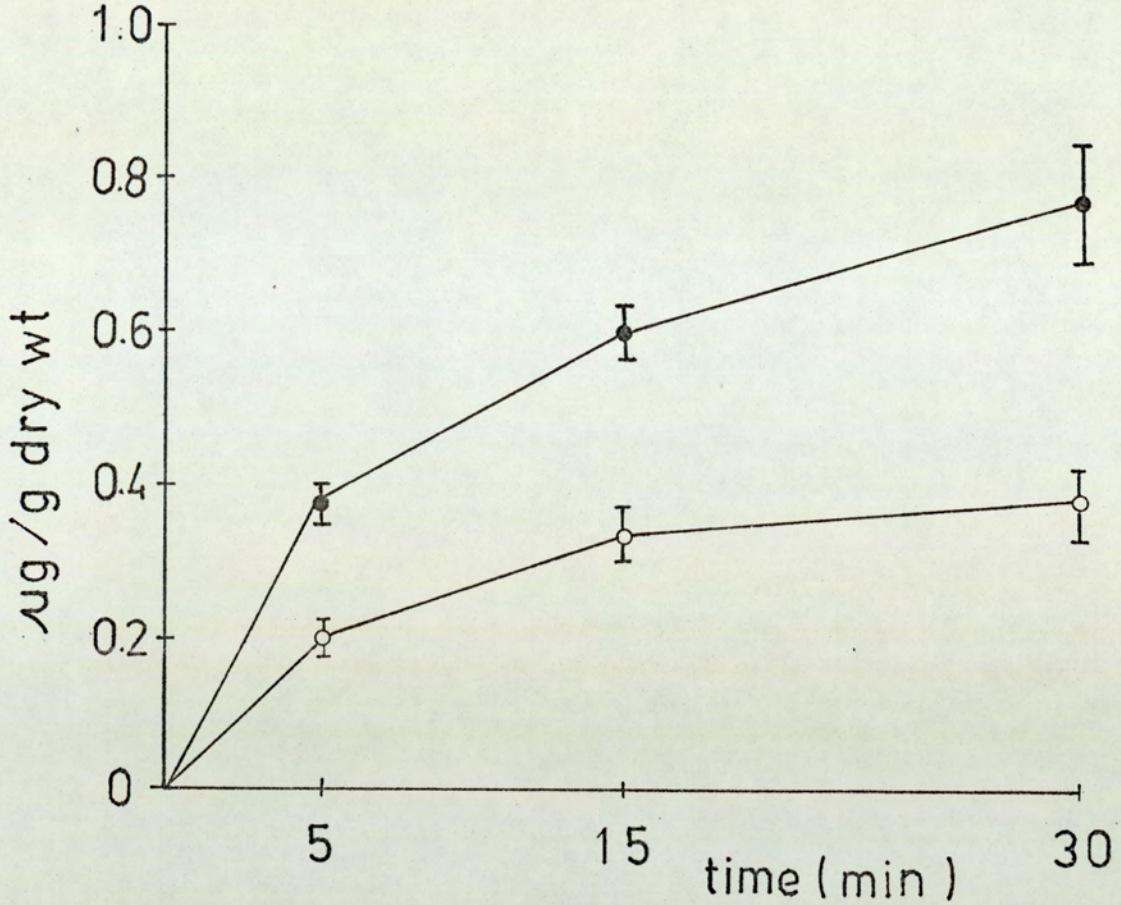


FIGURE 3.15 Mucosal radioactive efflux in the presence (● —— ●) and absence (○ —— ○) of mucosal 5-CH_3H_4PteGlu.

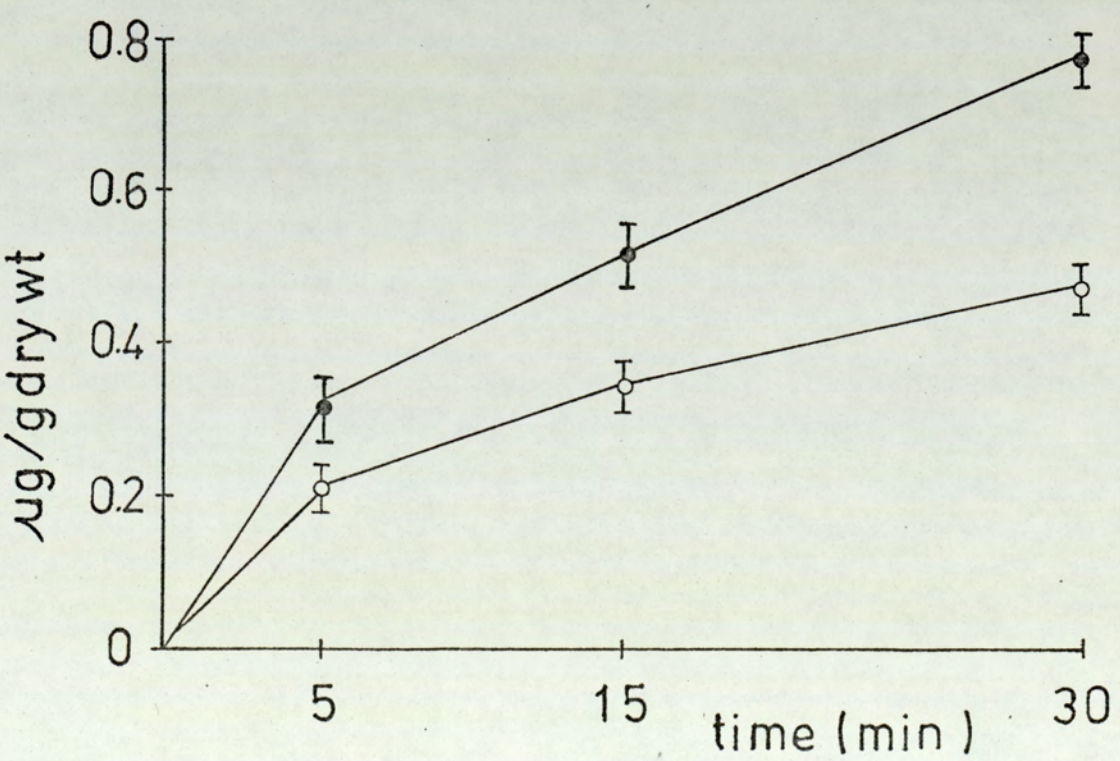


FIGURE 3.16. Mucosal radioactive efflux in the presence (● —— ●) and absence (○ —— ○) of mucosal folic acid.

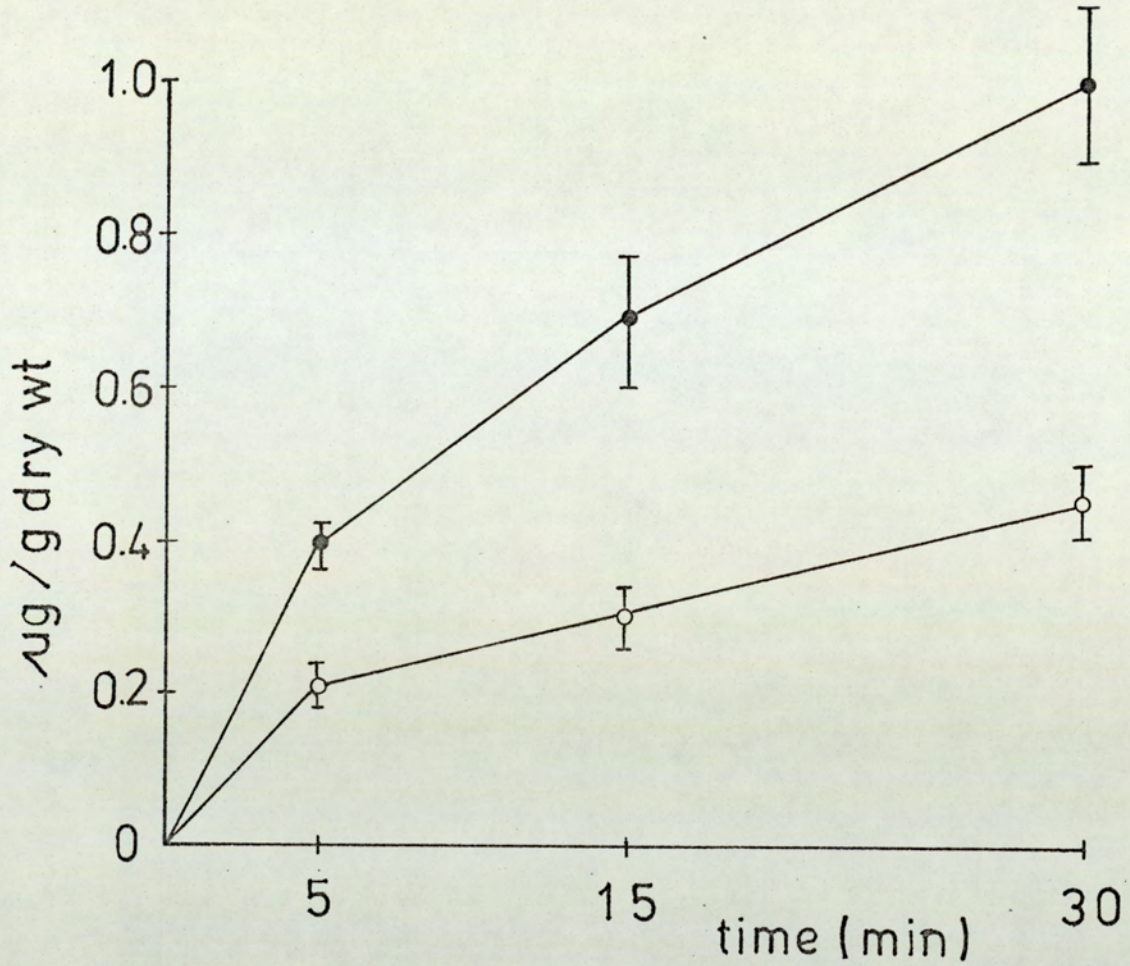


FIGURE 3.17 Mucosal radioactive efflux in the presence (● —— ●) and absence (○ —— ○) of mucosal methotrexate.

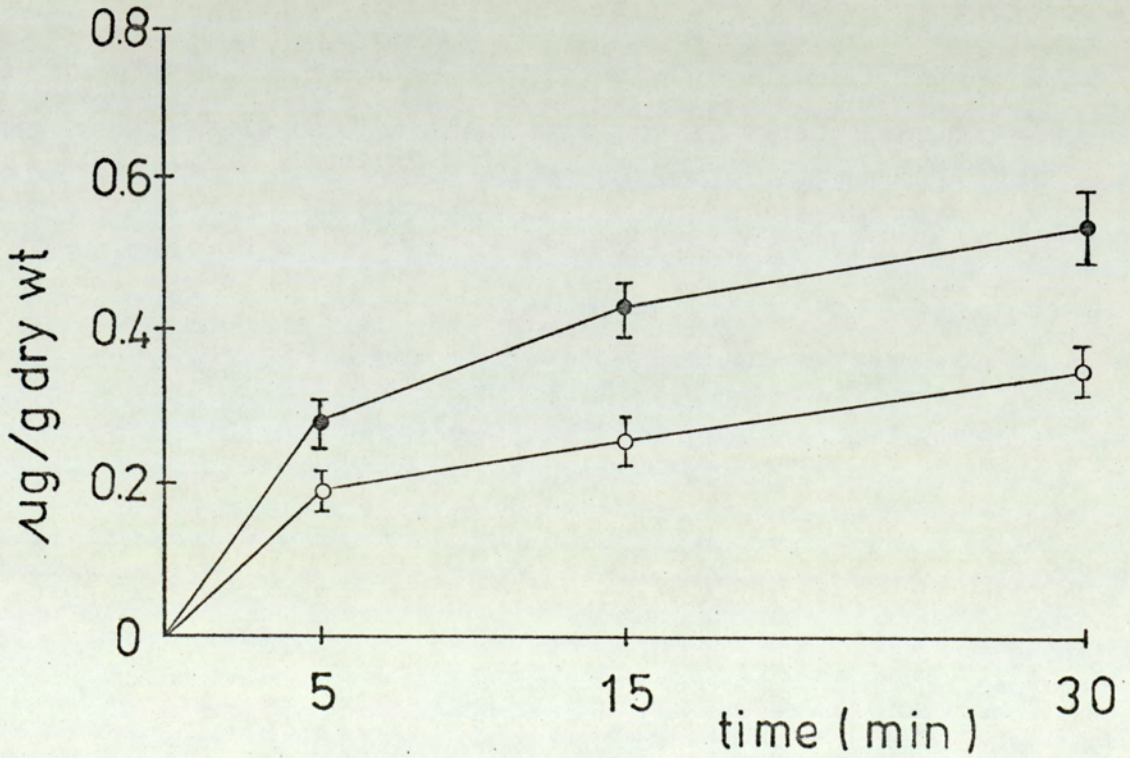


FIGURE 3.18 Mucosal radioactive efflux in the presence (● —— ●) and absence (○ —— ○) of mucosal 5-CHO-H_4PteGlu.

TABLE 3.10

STIMULATED EFFLUX.

Mucosal radioactive efflux in the presence of mucosal 5-CH_3H_4PteGlu.

Time (minutes)	Mucosal Content (µg/g dry wt)		
	5-CH_3H_4PteGlu free	10^{-5}M 5-CH_3H_4PteGlu	p*
5	0.20 ± 0.01 (4)	0.38 ± 0.01 (4)	< 0.5%
15	0.33 ± 0.02 (4)	0.60 ± 0.03 (4)	< 1%
30	0.38 ± 0.06 (4)	0.77 ± 0.08 (4)	< 2%

Values are given as mean ± s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates no. of rats used. Each individual value is derived from the mean of 2 or 3 sacs.

TABLE 3.11

STIMULATED EFFLUX.

Mucosal radioactive efflux in the presence of mucosal folic acid.

Time (minutes)	Mucosal Content (µg/g dry wt)		
	Folic acid free	10^{-5}M folic acid	p*
5	0.21 ± 0.03 (4)	0.31 ± 0.04 (4)	<2%
15	0.34 ± 0.03 (4)	0.51 ± 0.04 (4)	<5%
30	0.47 ± 0.04 (4)	0.76 ± 0.03 (4)	<2.5%

Values are given as means with s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates no. of rats used. Each individual value is derived from the mean of 2 or 3 sacs.

TABLE 3.12

STIMULATED EFFLUX.

Mucosal radioactive efflux in the presence of mucosal methotrexate.

Time (minutes)	Mucosal Content (µg/g dry wt)		
	Methotrexate free	10^{-5}M Methotrexate	p*
5	0.21 ± 0.02 (4)	0.39 ± 0.03 (4)	<0.1%
15	0.30 ± 0.03 (4)	0.69 ± 0.08 (4)	<1%
30	0.46 ± 0.06 (4)	1.01 ± 0.15 (4)	<2%

Values are given as means with s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates no. of rats used. Each individual value is derived from the mean of 2 or 3 sacs.

TABLE 3.13

STIMULATED EFFLUX.

Mucosal radioactive efflux in the presence of mucosal 5-CHO-H_4PteGlu.

Time (minutes)	Mucosal Content (µg/g dry wt)		
	5-CHO H_4PteGlu free	10^{-5}M 5-CHO-H_4PteGlu	p*
5	0.19 ± 0.01 (3)	0.28 ± 0.03 (3)	<2.5%
15	0.26 ± 0.02 (3)	0.43 ± 0.04 (3)	<2%
30	0.35 ± 0.02 (3)	0.54 ± 0.04 (3)	<1%

Values are given as means with s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates no. of rats used. Each individual value is derived from the mean of 2 or 3 sacs.

vii) SEROSAL TRANSFER AND TISSUE CONTENT.

It has been established that the efflux of labelled compound from the mucosal surface of the intestine is stimulated *in vitro* by the presence of 5-CH_3H_4PteGlu in the mucosal medium. Stimulation of efflux by the presence of 5-CH_3H_4PteGlu in the mucosal bathing medium triggered the interest as to what changes did take place in the serosal and tissue content of 5-CH_3H_4PteGlu over the incubation period of 30 minutes after the necessary loading of the sacs by radioactive 5-CH_3H_4PteGlu.

Sacs were pre-loaded as before using 0.5×10^{-6}M radioactive 5-^{14}C-CH_3H_4PteGlu in the incubation medium over an incubation period of 20 minutes. Just after loading one sac was withdrawn, rinsed with warm oxygenated saline and the serosal and tissue contents of the labelled compounds were determined as per the procedure described in Section 2, Part 3. The efflux incubation was conducted as usual, i.e. the sacs were withdrawn at intervals of 5, 15 and 30 minutes and rinsed as described before. Control sacs and test sacs were then opened and drained and the serosal transfer and tissue content of the labelled compounds were determined.

Figures 3.19 and 3.20 illustrate the serosal transfer and tissue content of the labelled compounds during "efflux" incubation both in the presence and absence of 5-CH_3H_4PteGlu in the "efflux" incubation medium. The mean and standard errors for both serosal transfer and tissue content of 5-CH_3H_4PteGlu are given in Tables 3.14 and 3.15 respectively together with the statistical significances as calculated by Student's paired "t" test.

Figure 3.19 exhibits that the serosal transfer both in the presence and absence of 5-CH_3H_4PteGlu in the efflux medium increase with time and is higher in the absence of 5-CH_3H_4PteGlu. But the difference of transfer in the presence and absence of 5-CH_3H_4PteGlu is not significant (Table 3.14). Tissue uptake of the labelled compound during the efflux rather, presents just a reverse picture (Fig. 3.20). Tissue uptake falls with time, here again the tissue content of the labelled compound is higher in the absence of 5-CH_3H_4PteGlu in the efflux medium than in its presence but the difference is not significant (Table 3.15).

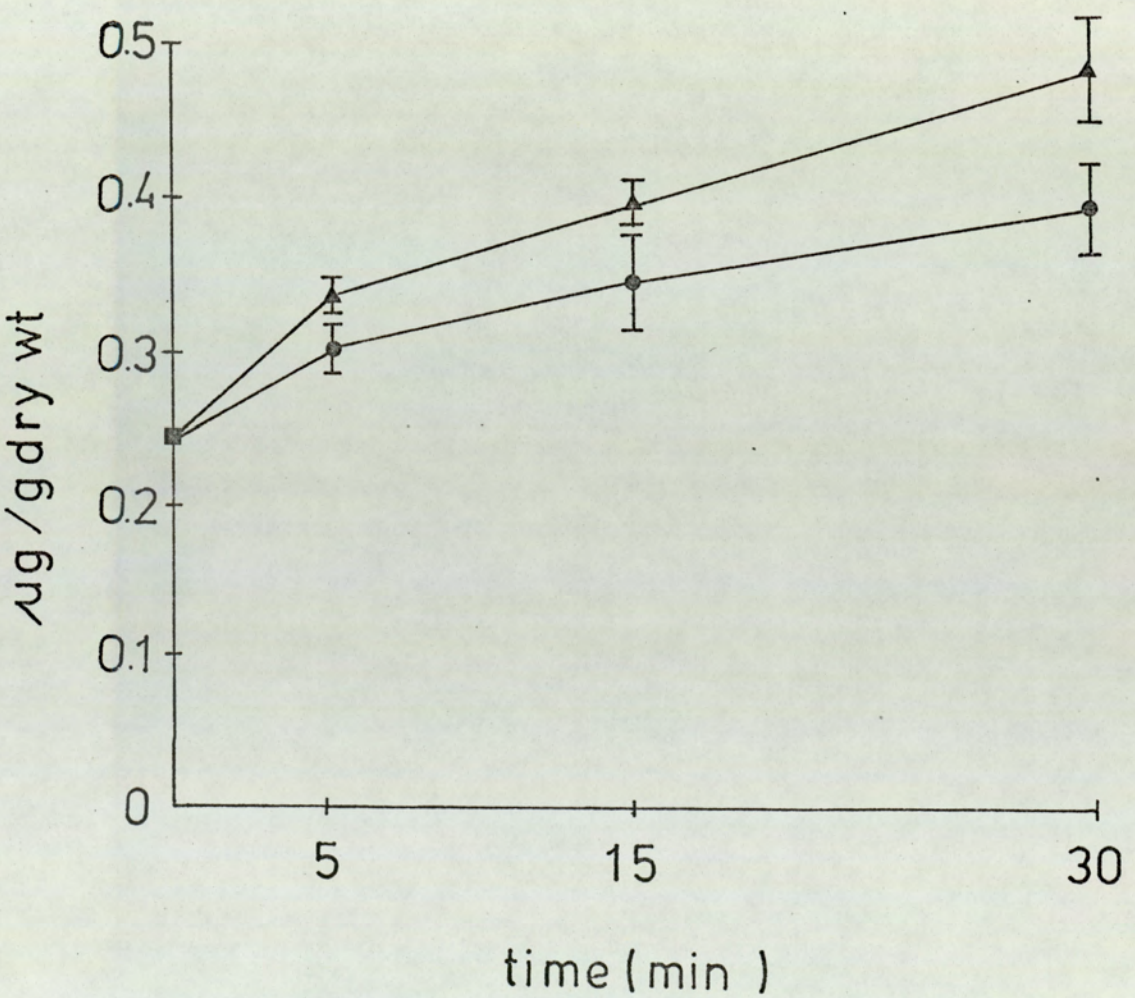


FIGURE 3.19 Serosal transfer of pre-loaded 5-CH_3H_4PteGlu in the presence (● —— ●) and absence (▲ —— ▲) of mucosal 5-CH_3H_4PteGlu. (■) indicates serosal 5-CH_3H_4PteGlu content just after loading.

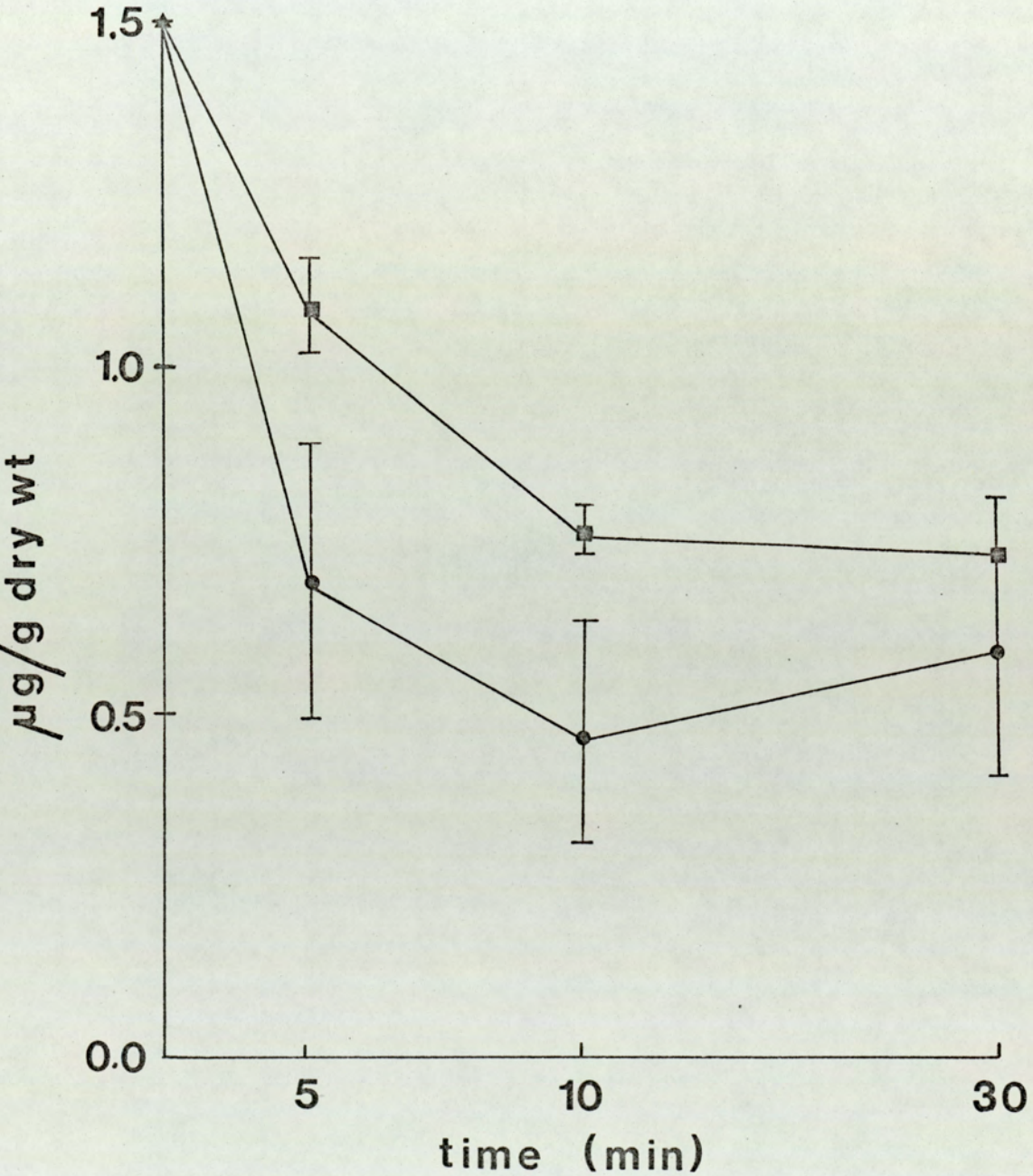


FIGURE 3.20 Tissue content of radioactive compound in the presence (● —— ●) and absence of (■ —— ■) of mucosal 5-CH_3H_4PteGlu. (▲) indicates tissue content just after loading.

TABLE 3.14

Serosal transfer after pre-loading with labelled $5CH_3H_4$PteGlu and subsequent incubation in the presence of mucosal non-labelled 5-CH_3H_4PteGlu.

Time (minutes)	Serosal transfer (µg/g dry wt)		
	5-CH_3H_4PteGlu free	10^{-5}M 5-CH_3H_4PteGlu	p*
5	0.33 ± 0.04 (3)	0.30 ± 0.04 (3)	NS
15	0.39 ± 0.06 (3)	0.34 ± 0.03 (3)	NS
30	0.48 ± 0.06 (3)	0.39 ± 0.07 (3)	NS

Values are given as means with s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates the no. of rats used. One sac was used from each rat.

TABLE 3.15

Tissue content after pre-loading with labelled 5-CH_3H_4PteGlu and subsequent incubation in the presence of mucosal non-labelled 5-CH_3H_4PteGlu.

Time (minutes)	Tissue Content (µg/g dry wt)		
	5-CH_3H_4PteGlu free	10^{-5}M 5-CH_3H_4PteGlu	p*
5	1.09 ± 0.20 (3)	0.70 ± .0.06 (3)	> 10%
15	0.77 ± 0.16 (3)	0.48 ± 0.03 (3)	> 10%
30	0.72 ± 0.19 (3)	0.58 ± 0.09 (3)	> 20%

Values are given as means with s.e.m.

* As indicated by Student's paired "t" test.

( ) indicates the no. of rats used. One sac was used from each rat.

viii) IDENTIFICATION OF THE DISPLACED SPECIES.

Attempts were made to identify the principal radioactive species displaced from the tissue in the "efflux" experiment by means of autoradiography.

A jejunal sac (6 cm. initial length) was prepared and filled as usual. Pre-loading was done as before in 5-^{14}C-CH_3H_4PteGlu (0.5×10^{-6}M) for 20 minutes after which the sac was washed and transferred to 1.0 ml. of incubation medium (Krebs ringer phosphate buffer with 28 mM glucose and 3 mg/ml sodium ascorbate) containing unlabelled 5-CH_3H_4PteGlu at a concentration of 10^{-5}M. Incubation was continued for 30 minutes.

At the end of the incubation the sac was discarded and bands of mucosal solution were applied to thin layer chromatography plates as described in Section 2, Part 3. A marker solution containing 5-^{14}C-CH_3H_4PteGlu was also applied to each plate and the chromatograms were run in two solvent systems (Section 2, Part 3). Autoradiographs were set up as described in Section 2, and exposed for 45 days. The principal radioactive component (Rf 0.28 in solvent system 1 and 0.57 in solvent system 2) in the mucosal solution was not 5-CH_3H_4PteGlu as it did not co-chromatograph with the radioactive 5-CH_3H_4PteGlu (Rf 0.55 in solvent system 1 and 0.85 in solvent system 2). The compound may be an oxidised product of 5-CH_3H_4PteGlu or a complex with protein (cellular). Reference to Rf values (Blair & Dransfield, 1971; Brown et al, 1973) suggest that the displaced compound may be 5-CH_3H_2PteGlu (Rf 0.58 in solvent system 2) or 5,10CH_2H_4PteGlu (Rf 0.30 in solvent system 1) but it was not confirmed.

ix) IDENTIFICATION OF THE SPECIES PRESENT IN THE MUCOSAL, SEROSAL MEDIA AND TISSUE DURING TRANSPORT.

The nature of the species present in the mucosal and serosal solutions and tissue after incubation with 5-^{14}C-CH_3H_4PteGlu was investigated by means of autoradiography.

The sacs after an incubation period of 30 minutes in the presence of radioactive 5-CH_3H_4PteGlu were washed as usual and cut open to drain the serosal content. The sac tissues were then freeze dried and homogenized with 5 ml. of 5% trichloroacetic acid. The material was centrifuged and the supernatant, the final incubation medium (mucosal) and the final serosal medium were run on thin-layer chromatograms in 2 solvent systems as described in Section 2, Part 3. Autoradiographs were prepared as described in Section 2, Part 3, and exposed for 45 days in case of external and serosal medium and for 55 days in case of tissue. It was found that for each solvent system the major band in the incubation and serosal solutions co-chromatographed with the radioactive 5CH_3H_4PteGlu standard. A spot or band of the labelled 5-CH_3H_4PteGlu solution diluted with non-labelled 5-CH_3H_4PteGlu in Krebs ringer phosphate with 28 mM glucose and 3 mg/ml sodium ascorbate was routinely applied as the standard in the same plate along with the experimental ones. But in the case of tissue, although the major component of the solution cochromatographed with 5CH_3H_4PteGlu standard in solvent system 1 (Rf 0.55) it did not co-chromatograph with the standard in solvent system 2 (Rf. 0.60). Comparison to the previously reported Rf values (Blair & Dransfield, 1971; Brown et al, 1973) did not confirm the identity of this compound.

x) ISOLATED CELL STUDIES.

Isolated cells were prepared as described in Section 2, Part 2. The final preparation was found to consist of isolated cells and some "clumps" which had a tendency to reaggregate in association with contaminant mucus. In tryptan blue exclusion test, 85% to 90% of the cells were found to exclude the dye. The appearance of the cells under light microscopy is illustrated in Plates I to V.

5-CH_3H_4PteGlu Uptake.

The absorption of 5-CH_3H_4PteGlu by isolated mucosal cells was studied employing the techniques described in Section 2, Parts 2 and 3. Incubation of isolated cells was done at 37°C and at a starting pH of 7.0 in 10^{-5}M radioactive 5-CH_3H_4PteGlu adequately diluted with non-radioactive 5-CH_3H_4PteGlu. Cells were withdrawn at intervals of 3, 6 and 11 minutes.

Figure 3.21 and Table 3.16 illustrate the results of a typical experiment at two different pH values. Two rats were used for each experimental set. At pH 7.0 all the uptake takes place in 3 minutes and very little or none in the following 3 minutes and 5 minutes after that as Students "t" test indicates $p > 50\%$ between the uptakes at 3 and 6 minute intervals and $p > 50\%$ between uptakes at 6 and 11 minutes intervals. So the difference in uptake at 3 minute and 11 minute intervals is not significant. However, at pH 5.0 there was similar rapid uptake within the first 3 minutes followed by a slower but significant uptake in the next 3 minutes as indicated by Students "t" test ($p > 1\%$).

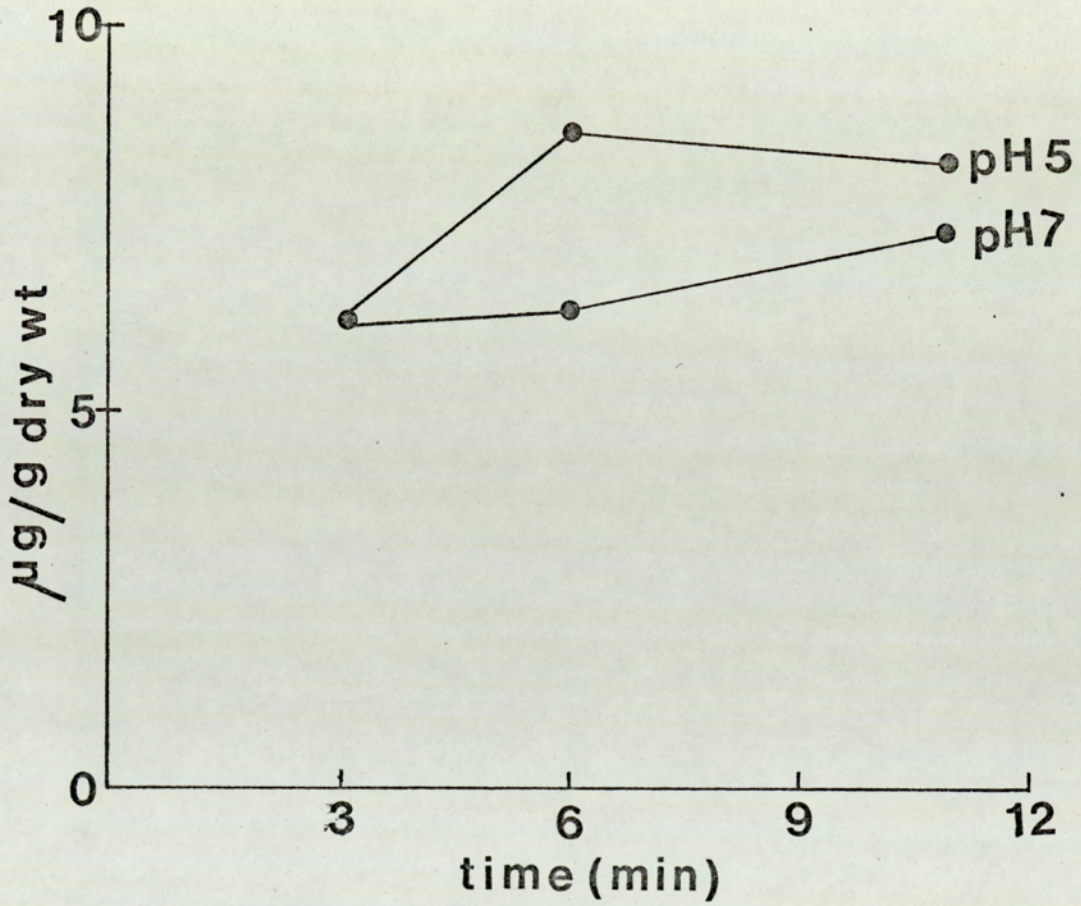


FIGURE 3.21 Uptake of 5-CH_3H_4PteGlu (10^{-5}M) by isolated cells during incubation at two pH values. pH shown are the initial pH of the medium.

TABLE 3.16

Uptake of 5-CH_3H_4PteGlu by Isolated Cells at 37°C.

pH		5-CH_3H_4PteGlu uptake in µg/g dry cells.		
Initial	Final	3 mins. incubation	6 mins. incubation.	11 mins. incubation.
7.0	6.7	6.09 ± 0.74 (5)	6.24 ± 1.16 (5)	7.26 ± 1.04 (4)
5.0	5.2	6.12 ± 0.20 (4)	8.53 ± 0.67 (4)	8.14 ± 1.20 (4)

Results are shown as mean with s.e.m.

( ) indicates the sets of experiments done. Each set consists of two rats and each set value is the mean of duplicate values.

The next 5 minutes incubation failed to show any uptake ($p > 50\%$). So, it appeared that the uptake was saturated at 6 minutes of incubation.

Uptake of 5-CH_3H_4PteGlu by isolated cells was also studied at 27°C at two pH values. Table 3.17 shows the results of such studies. Here also like Table 3.16 there is no significant difference between uptakes of 5-CH_3H_4PteGlu at 3 and 6 minutes ($p > 50\%$) and 6 and 11 minutes ($p > 20\%$) at an initial pH of 7.0. At pH 5.0 a similar trend is also observed but there appears to be an uptake in between 3 and 6 minutes' incubation, although not significant ($p > 10\%$). At both pH values there is initial uptake at 3 minutes.

Figure 3.22 illustrates the "wash out" of 5-CH_3H_4PteGlu from the isolated cell preparation. The supernatant solutions after washing and centrifuging of the cells are counted during the four washes. The detailed method of washing has been described in Section 2, Part 2. This particular figure (3.22) deals with the "wash out" of 5-CH_3H_4PteGlu from the isolated cells which were incubated for 6 minutes in the presence of 10^{-5}M labelled 5-CH_3H_4PteGlu diluted with non-labelled one.

The purpose of the experiment was to observe the loss of 5-CH_3H_4PteGlu by the preparation during dilution and washing process and to see whether the loss was from the inside of the cell or from the cell exterior where 5-CH_3H_4PteGlu was likely to be sticking during incubation.

From Figure 3.22 it appears that the majority of 5-CH_3H_4PteGlu comes out in the first wash. The amounts coming in the 2nd, 3rd and 4th wash are very small in comparison to 1st wash, moreover the amounts washed out in

3rd and 4th washing, are almost same, indicating efflux from cell interior during the later stage of washing.

TABLE 3.17

Uptake of 5-CH_3H_4PteGlu by Isolated Cells at 27°C.

pH		5-CH_3H_4PteGlu uptake in µg/g dry cells.		
Initial	Final	3 mins. incubation.	6 mins. incubation.	11 mins incubation.
7.0	6.7	2.94 ± 0.35 (4)	2.97 ± 0.25 (4)	4.13 ± 0.99 (4)
5.0	5.2	2.34 ± 0.11 (4)	2.68 ± 0.16 (4)	2.48 ± 0.22 (4)

Results are shown as mean with s.e.m.

( ) indicates the sets of experiments done. Each set consists of two rats and each set value is the mean of duplicate values.

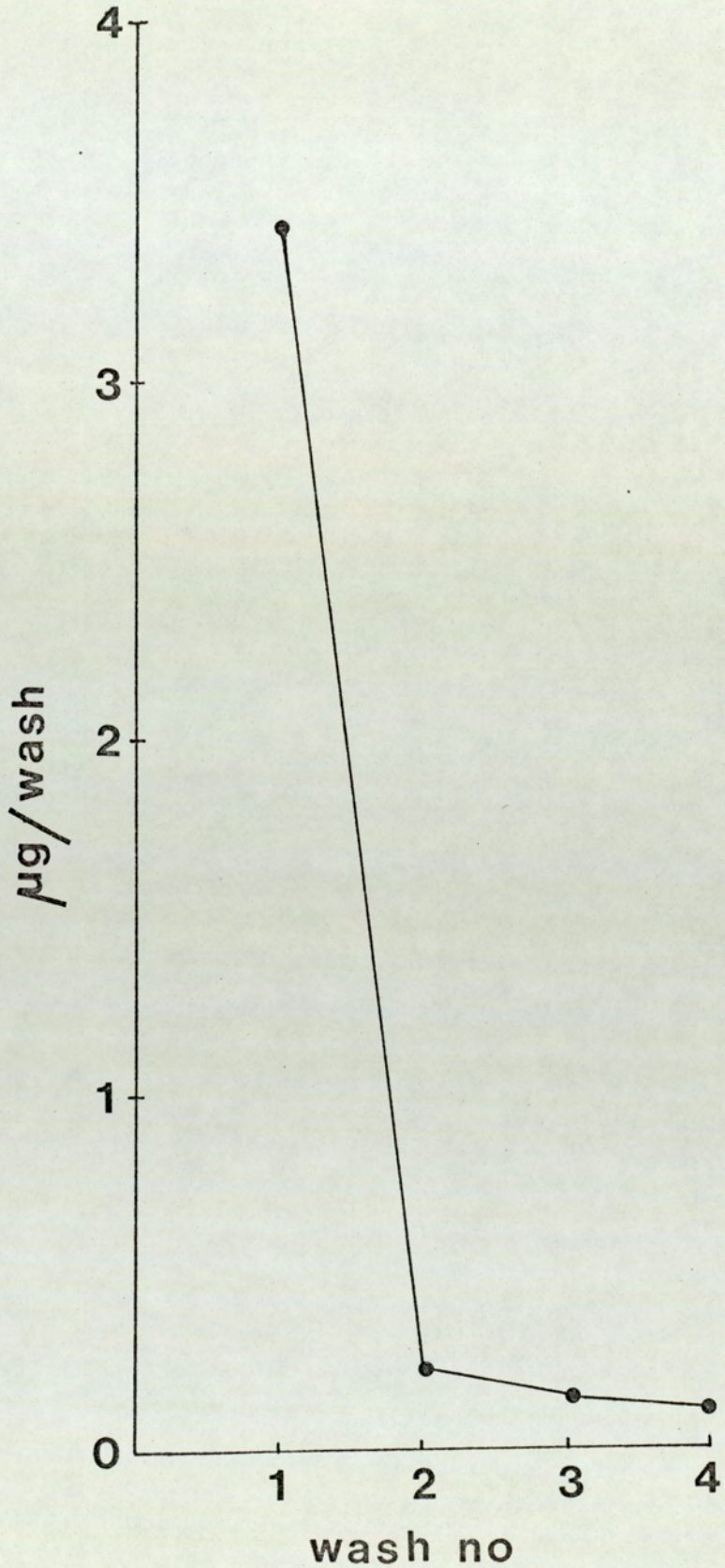


FIGURE 3.22. Wash out of 5-CH_3H_4PteGlu from isolated cells incubated for 6 mins. in the presence of 5-CH_3H_4PteGlu.

PLATES.

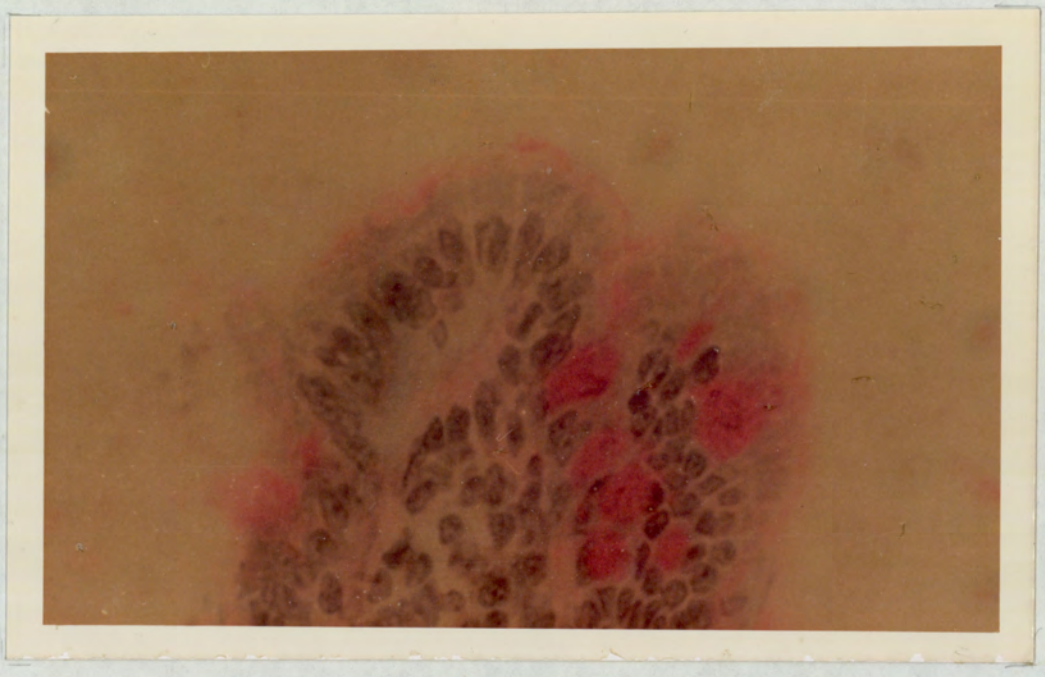


PLATE I

The tips of two adjacent villi of the upper jejunum of the rat. Section prepared from an everted ring maintained at 4°C for 30 minutes after eversion, sectioned in a cryostat, and stained with periodic-acid Schiff stain. The polysaccharide layer at the extremity of the brush border is clearly visible.

X200

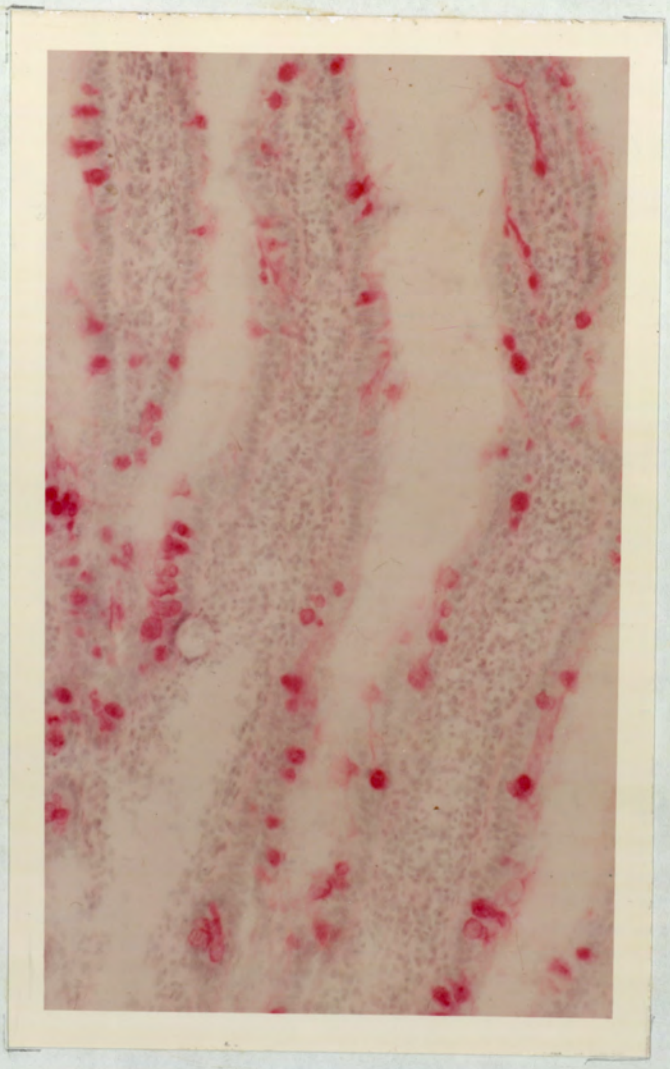


PLATE II

The villi from the jejunum of rat. Section prepared from an everted sac, incubated in Krebs Ringer Phosphate containing 28 mM glucose at 37°C for 30 minutes, sectioned in a cryostat, and stained with periodic-acid Schiff stain. The polysaccharide layer at the extremity of the brush border is visible.

X 100.

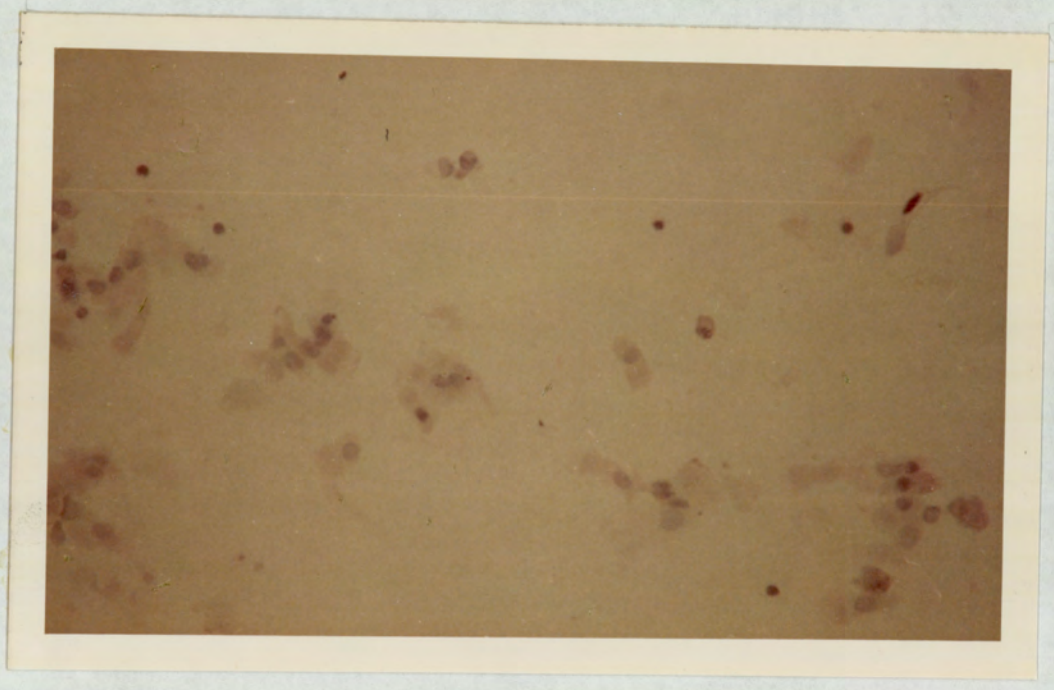


PLATE III

Isolated mucosal cells obtained by hyaluronidase disaggregation and stained with periodic-acid Schiff stain.

X 100.

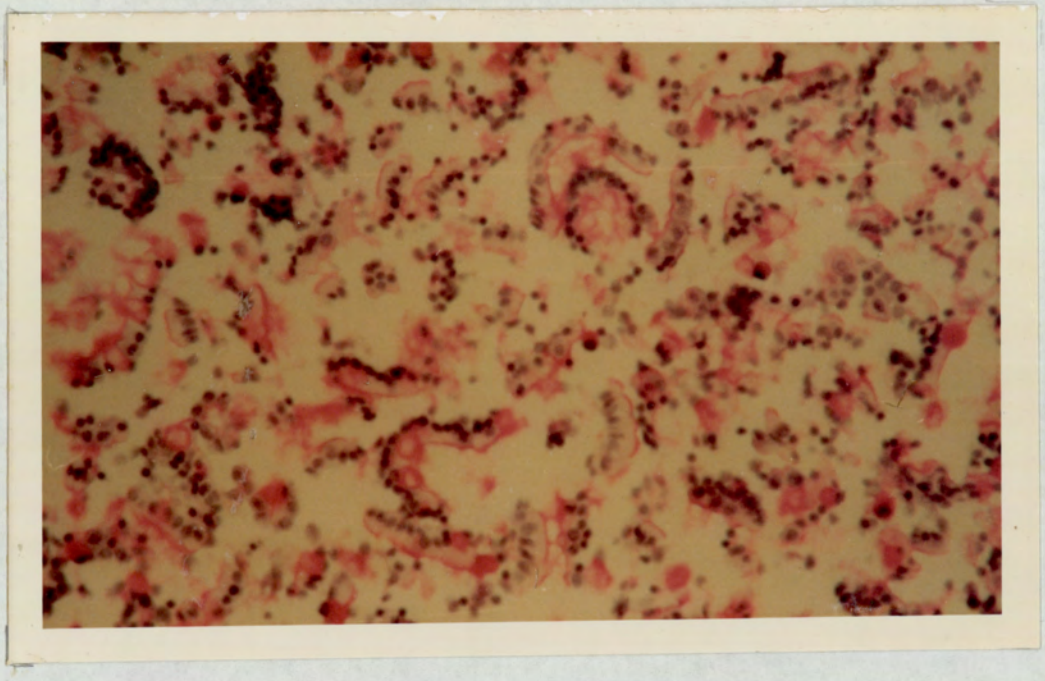


PLATE IV

Isolated mucosal cells obtained by citrate dissociation and stained with periodic-acid Schiff stain. The pink-staining polysaccharide layer at the extremities of the brush borders are clearly visible in this cell preparation (Slide by courtesy of Dr. W. E. Jensen of the University of Chicago).

X 50.

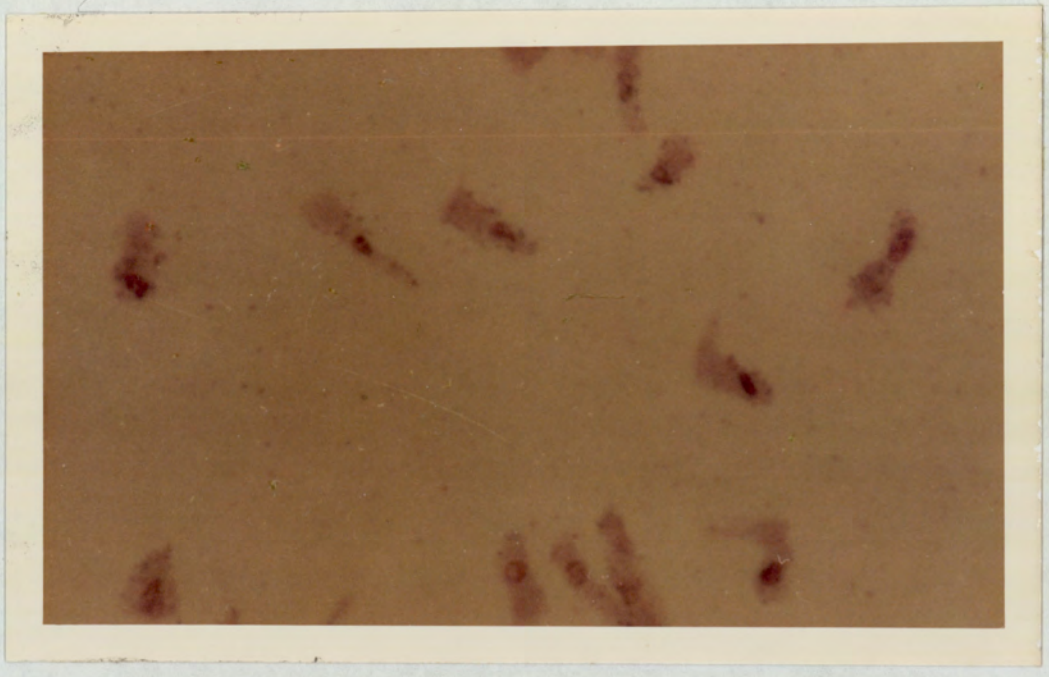


PLATE V.

A group of isolated mucosal cells obtained by hyaluronidase disaggregation and stained with haemotoxylin-eosin.

X 150.

SECTION 4.

DISCUSSION AND CONCLUSION.

The question of how organic compounds cross the membranes of living cells has always fascinated the biological interest and stimulated a vast amount of research. The model of the cell membrane as an uninterrupted bimolecular layer of lipid with proteins at the surface accounts for the ability of lipid soluble material to enter the cell; such material could pass down concentration gradients from the "outside" solution into the lipid layers and then into the "inside" solution. However, it is not obvious that water, inorganic ions or a variety of other substances that are not readily soluble in lipids but enter the cell could easily pass a barrier of continuous lipid layers. Some molecules require enzyme activity to pass the membrane. Many others, however, appear to pass through without enzymatic intervention. To explain this sort of transport of some molecules into the cell and also to explain some restrictions on the sizes of the molecules that can readily enter, it is often hypothetised that special channels pass through the membrane, interrupting the lipid layers. Usually these channels are referred to pores, although one need not think literally in terms of holes. Rather, the channels are presumed to be arrangements of membrane molecules or of portions of molecules, such that there extends from one surface of the membrane to the other as continuous zone of hydrophilic groups and perhaps even of water molecules loosely bound to membrane components. Probably a variety of non-lipid-soluble molecules could diffuse through such channels far more rapidly than through continuous layers of hydrophobic layers.

This concept is inadequate to explain the rapid

cellular transfer of certain relatively large, lipid insoluble molecules like glucose. Hober (1945), noted that the sugars which readily enter cells despite their lipid insolubility are all normal cell substrates, and he used the term physiological permeability to describe the peculiar permeability of cells to these natural substrates. The relatively large lipid insoluble molecules like sugars and amino acids which cannot pass through the simple lipid bilayers or pores are transported via carrier mediated route. The essential feature is that a substance can do so if combined with some substance in the membrane – the carrier. Later, the terms active transport and facilitated diffusion were adopted to denote two different specialized forms of this type of membrane penetration. Schanker et al (1958) and Schanker (1960) reported that drugs which are weak organic acids and bases, exist in solution as a mixture of ionized and unionized molecules. The unionized neutral form is fairly soluble in lipid, and as such is capable of penetrating the lipoidal membrane. The ionized moities are always barred from penetration. Thus there is a relation between the degree of ionization and the rate of absorption of drugs. Many weak acids and bases are readily absorbed, stronger, more highly ionized acids and bases are less readily absorbed and completely ionized compounds diffuse across the membrane with relative difficulty. The change in the intestinal pH increase the absorption of bases and decrease the absorption of acids but compounds which remain essentially undissociated at various pH show no change in their rate of absorption (Hogben et al, 1959). They also suggested that a zone with a pH of 5.3 different from the

intestinal pH of 6.6 was possibly located at the surface of the intestinal epithelial boundary which eventually determined the degree of ionization. Although most drugs and other foreign organic compounds appear to cross the intestinal boundary by process of simple diffusion, there is evidence that a drug can be absorbed by a specialized transport process. Apart from the three transcellular transport routes, e.g. lipid route, aqueous route and carrier mediated route, there is an intercellular route through the tight junction and also a fifth route is envisaged through gaps in the epithelium caused by shedding of epithelial cells.

A brief account of various transport mechanisms will now be furnished here. The term "passive diffusion" will be used to describe the movement of solutes from the solution of higher concentration to that of lower concentration until the concentrations on both sides of the membrane are equal. The only driving force moving the molecule is thermal agitation and the concentration gradient. Passive transport of an uncharged substance gives a linear relationship between the rate of diffusion and the difference in concentration across the membrane. For a charged substance, its rate of diffusion depends upon its chemical and electrical concentration gradients across the membrane. The rate of penetration of a substance through membranes is also related to its lipid solubility so it will be relatively difficult for hydrophilic polar compounds having poor lipid solubility to penetrate the cells and for such materials the rate of transport into the cell is likely to be negligible. Many naturally occurring substances would thus be prevented from rapid incorporation into the cells had it not been for the exis-

tence of specialised transport mechanisms.

The term "active transport" has often been used to designate any process that appeared to be inconsistent with the characteristics of passive diffusion. All active transfer mechanisms must be able to concentrate solutes against their electrochemical gradients. Clearly for this type of activity, work has to be done on the solute transferred, the actual pump directly consuming the energy derived from the metabolic reactions of the biological system. The active transport system is mediated by a mobile "carrier molecule" a substance postulated to be a membrane component which has the following properties:

1. It can combine with the substance to be transported to form a complex.
2. The complex can traverse the permeability barrier with ease.
3. The complex releases the bound solute into the cytoplasm when it reaches the inside aspect of the cell membrane.
4. The unloaded carrier is then thought to move back to the exterior face of the membrane.

This type of "ferryboat" mechanism will allow lipid insoluble substances to traverse the lipid layers of the membrane. It is generally called "carrier mediated transfer". Carrier mediated active transfer of a substance will go on continuously as long as energy and oxygen are supplied to the cell. Few active transfer mechanisms can function anaerobically.

The concept of a membrane carrier has a good deal in common with that of an enzyme, although it has also some important differences. The feature most obviously in common

is the active site or active centre. It is this which gives the specificity to the carrier or enzyme. The big difference comes when the separation of the substance is considered from the enzyme or carrier active centre. In the case of the enzyme the substrate leaves as a different substance or substances and the products are found in the same compartment as the substrate, and the separation is chemical not spatial. In the case of carrier the substrate (absorbate) leaves the carrier in the same form in which it became attached. Furthermore, it leaves not in the same compartment but a spatial separation has been achieved.

Another important difference between enzymes and carriers is the experimental approach to defining their properties. The enzymologist often considers that the first problem is to purify and isolate the enzyme, and this has been achieved in a large number of cases. On the other hand, no carrier molecule has yet been isolated with any certainty, although many attempts have been made. The possible reasons for this may be that (I) the amount of carrier involved is so small that no identification and separation is possible, (II) it is a fundamental part of the cell structure and hence when the cell is broken, the carrier is changed into a form in which it can no longer be identified.

In case of ions the term active transport generally refers to the net movement of ions against a combined electrochemical gradient, whereas passive movement can result from diffusion of ions down such a gradient through a permeable membrane. Other possible mechanisms of ion transport include convection by the bulk movement of water and coupled exchanges of cations or anions.

Sodium and potassium are involved in the transport of a large number of substances across cell membranes.

The term 'facilitated transfer' is defined as a process which results in penetration of a substance through a membrane at a rate more rapid than that expected as the result of free diffusion but which does not require energy and is not directly coupled to metabolic reactions and cannot lead to transport against an electrochemical gradient. "Facilitated transfer", however, has some characteristics similar to those of active transport, such as saturation kinetics, competitive inhibition by related compound, and a high temperature coefficient.

The transport phenomenon associated with the flow of solute under the influence of the flow of a solvent is termed as "solvent drag".

This discussion will be concerned with the possible interpretation of 5-CH_3H_4PteGlu transport in terms of currently accepted transport models.

i) INTESTINAL TRANSPORT OF 5-CH_3H_4PteGlu.

Results as represented by Table 3.1 and Figures 3.5, 3.6 and 3.7 in Section 3 show that the total, serosal and tissue uptake of 5-CH_3H_4PteGlu are linear, at least, for 60 minutes. Tissue uptake was higher than the serosal uptake. A rapid uptake of 5-CH_3H_4PteGlu at the early stage of incubation is visible (Figure 3.5), this may be attributed to some rapid uptake mechanism by tissue like binding at sites within the brush border itself (Leslie and Rowe, 1972) or at intracellular sites accessible via damaged cells at the cut surface of the gut wall.

The total and serosal transfer of 5-CH_3H_4PteGlu in terms of water uptake as appear from Table 3.2 show an upward trend up to 30 minutes, after which the value became constant indicating that up to 30 minutes incubation the 5-CH_3H_4PteGlu uptake is proportionately higher in terms of water transport but after that period both 5-CH_3H_4PteGlu uptake and water uptake continue proportionately at similar rate, i.e. the 5-CH_3H_4PteGlu uptake rate is offset by water uptake rate. In case of tissue just the reverse happens (Table 3.2), i.e. 5-CH_3H_4PteGlu uptake in terms of water uptake remains unchanged up to 30 minutes of incubation and then shows an upward trend indicating that at first the 5-CH_3H_4PteGlu uptake rate is offset by the water uptake and finally 5-CH_3H_4PteGlu uptake gets the upper hand coupled with the saturation of water uptake (Table 3.1).

The ratio of final tissue concentration to final mucosal concentration of 5-CH_3H_4PteGlu seems to be higher than that of final serosal concentration to final mucosal concentration of 5-CH_3H_4PteGlu (Table 3.3) but the ratio in either case is not high enough to advocate flux to tissue or serosal medium against concentration gradient.

At "zero" concentration gradient, i.e. when 5-CH_3H_4PteGlu concentration was equal in both mucosal and serosal media, both total and tissue uptake of 5-CH_3H_4PteGlu were high but the serosal transfer was small (Table 3.4). A ratio of 1.09 $\pm$ 0.19 sem for the concentration of 5-CH_3H_4PteGlu in the final serosal to that of the final mucosal solution was found for an incubation period of 15 minutes. This value was in conformity with the value obtained from Nernst equation suggesting no transport against an electrochemical gradient. There was no accumulation of

5-CH_3H_4PteGlu by tissue with respect to the incubating solution and this was also true for serosal solution as the serosal to mucosal concentration and tissue to mucosal concentration after 15 minutes of incubation was calculated to be 1.09 and 0.81 respectively.

The temperature co-efficient for transport, i.e. Q_{10} value was found to be 1.8.

Transport studies at concentrations of 5-CH_3H_4PteGlu ranging from 10^{-7}M to 10^{-4}M fail to show any saturation of uptake (Table 3.7 and Figures 3.9, 3.10, 3.11). When the serosal transfer and tissue uptakes of 5-CH_3H_4PteGlu were considered separately at the aforesaid concentrations, saturation effect was still absent.

The characteristics of the transport process that have been enumerated so far do not advocate, in any way, carrier mediated mechanism for the 5-CH_3-H_4PteGlu transport.

The tissue uptake of 5-CH_3H_4PteGlu was always found to be higher than that of serosal transfer both on the basis of time and concentration variations (Tables 3.1 and 3.7). This higher tissue uptake value may be due to the binding of 5-CH_3H_4PteGlu onto a protein or proteins within the tissues or onto the cell surfaces. Despite the higher uptake of 5-CH_3H_4PteGlu as exhibited by tissue, no evidence in support of concentration of the compound was arrived at. Non-saturable uptake (Figure 3.11) and low ratio of tissue to final mucosal concentration of the compound (Table 3.3) failed to bear testimony to the phenomenon of carrier-mediated mechanism. Strum *et al* (1971) demonstrated that the transport of 5-CH_3H_4PteGlu into serosal solution was without saturation and there was no transport against a concentration gradient. This finding is in close conformity with

the findings spelled out here as regards serosal transport (Figure 3.10 and Table 3.3).

The transport mechanism of the parent compound, folic acid, shrouds with controversies and contradictions. As yet no molecular mechanism has yet been established but a number of pertinent observations have been made.

Accumulation of PteGlu by rat intestinal tissue has been reported to be observed (Smith et al 1970b; Smith, 1973; Selhub et al, 1973; Blair et al 1974a; Johnson 1974). Smith et al (1970b), Smith (1973), Blair et al (1974a) reported rate limiting uptake of PteGlu by tissue at the mucosal PteGlu concentrations ranging from 1×10^{-6}M to 1×10^{-7}M, 1×10^{-8}M to 1×10^{-7}M and 5×10^{-6}M and above respectively. Baugh et al (1971) using dog intestine in vivo reported that 44% of a given dose of PteGlu remained in the lumen whereas 48% was retained by the tissue which was precipitated with the proteins. In view of the above observations they concluded that there was an accumulation of PteGlu by tissue and also suggested the formation of some sort of protein binding with PteGlu during absorption.

Elsborg (1974) reported accumulation of PteGlu against a concentration gradient in the tissues but there was no evidence of saturation kinetics over a range of 2×10^{-8}M to 2×10^{-4}M in the incubation medium. He concluded that PteGlu might be bound to cellular proteins. Folic acid has a high binding affinity for protein (Elsborg, 1972; Leslie & Rowe, 1972) and in experiments where the buffer solution was exchanged with plasma (Elsborg, 1974) no tissue accumulation had occurred. It is, therefore, most likely

that folic acid is bound to proteins in the intestinal wall, probably by interaction of ionized groups of folic acid with corresponding groups of tissue protein.

Herbert and Shapiro (1962), Cohen et al (1964) and Blair et al (1974a) noted that PteGlu transport into the serosal medium was saturable but Blair and Matty (1974) stated that the effect of pH change could have been significant in producing these results. They also added that the greater mucosal to serosal flux observed by Burgen and Goldberg (1962) might have been influenced by the difference in pH on the two sides, or the effects of folate binding proteins in the serum. Bhantumnavin et al (1974) using everted sacs of rat gut exhibited a saturable uptake of tritiated PteGlu at a mucosal PteGlu concentration ranging from 0.2 μM to 20 μM. Halsted et al (1974) employing in vivo perfusion reported a saturable uptake of PteGlu by rat jejunum between concentrations of 1 μM and 4.6 μM. Turner and Hughes (1962), however, failed to prove accumulation of PteGlu against concentration gradient. Studies followed afterwards (Spencer & Bow, 1964; Smith, 1973; Selhub et al, 1973; Elsborg, 1974) also presented no evidence of accumulation or saturation kinetics.

The following conclusions are being furnished in order to sum up the mechanism of PteGlu transport as a compromise between the passive diffusion and carrier mediated process. Smith et al (1970b) suggested that PteGlu was transported by both passive diffusion and solvent drag with the water flow, when it was initially present on the serosal side of the sac at 1×10^{-6}M concentration. But when concentrations varied between 1×10^{-6}M and 1×10^{-7}M, a

saturable process was shown to exist specially for tissue. Smith (1973) further added that the process whereby PteGlu is accumulated in the tissues is not apparent at 1 x 10^{-6}M and higher concentrations because of the greater contribution by passive diffusion and solvent drag to its total transport. Transport in serosal solution may be largely independent of the special uptake process. Selhub *et al* (1973) also reported that tissue uptake might be saturable below 7 x 10^{-6}M but no saturable serosal transfer was evident either at low or at higher concentration. Elsborg (1974) could confirm no saturable uptake either for tissue or serosal medium within the concentration range 2 x 10^{-8}M to 2 x 10^{-4}M. Blair *et al* (1974a) showed, however, concentrative and saturable uptake of PteGlu both into tissue and serosal medium. But the tissue concentrations of PteGlu that are encountered, for the reason, as stated earlier, need not necessarily involve active transport. The lower temperature coefficients (1.5 to 2.0) observed in everted sacs with 10^{-8}M PteGlu (Smith, Matty & Blair, 1970; unpublished data) and with intestinal segments (Blair, Johnson & Matty, 1974a) would certainly favour this suggestion against that which claims concentration to be the result of active transport. In this study a low temperature coefficient of 1.8 was found for 5-CH_3H_4PteGlu transport into sac which along with other phenomena, e.g. absence of saturation kinetics, low serosal to mucosal and low tissue to mucosal concentrations favour the suggestion that 5-CH_3H_4PteGlu transport follows a passive diffusion with a solvent drag for serosal component, which, as stated before, is in close agreement with the findings of Strum *et al* (1971). The transport mechanism of 5-CH_3H_4PteGlu, is not, like its

parent compound PteGlu, beset with controversies and contradictions, because only a few studies have been undertaken so far. It is still in its beginning and controversies are likely to ensue in future.

Weir et al (1973) after oral administration of both active and inactive diastereoisomers of 5-CH_3H_4PteGlu to human subjects found a greater excretion of the active form (43%) than the inactive (22%) one in urine. The intravenous administration of the active form resulted in 55% urinary excretion of the dose. However, the entire intravenous administered dose (104%) of the inactive form was excreted within 24 hours. Thus, the amount of the inactive form excreted in the urine after absorption represented the total that has been absorbed whereas the corresponding value for the active form was considerably lower than the actual amount absorbed since some was retained by the subject as evidenced by the intravenous studies. On this basis they put forward that in man the intestine preferentially absorbs the active naturally occurring diastereoisomer and a carrier mediated process is involved. Blair and Matty (1974) criticized this carrier mediated concept of 5-CH_3H_4PteGlu transport in human intestine on the basis that in the experiments of Weir et al (1973) the body was presented with a large dose of active diastereoisomer (300 µg orally plus metabolites from 15 mg. parenteral folic acid) but only with a small dose of the inactive diastereoisomer (300 µg orally; no contribution from parenteral folic acid) and consequently this large dose administration resulted in large excretion in urine as Blair and Dransfield (1971) showed that the percentage urinary excretion of folic acid was very much

greater with large doses than with small doses, e.g. 30 per cent against 6 per cent.

So the carrier mediated process as claimed by Weir et al (1973) remains disputed. The data obtained in this study along with the findings of Strum et al (1971), on the other hand go in favour of passive diffusion. However, since between pH 6.0 and 7.0 in aqueous solution 5-CH_3H_4PteGlu is present wholly as a dianion, i.e. 100 per cent ionised (Table 4.1) the transport mechanism must explain how the strong electrostatic repulsion between the dianion and the negative charges on the phospholipid membrane of the intestinal epithelium is overcome.

TABLE 4.1

Approximate percentage fractions of PteGlu and 5-CH_3H_4PteGlu present as the neutral species in water at various pH values*

pH	PteGlu %	CH_3H_4PteGlu %
3.0	75	25
3.5	50	50
4.0	20	65
4.5	5	60
6.0	0	0
7.0	0	0

*PteGlu as undissociated species; $5CH_3H_4$PteGlu as zwitterion. Data calculated using pK values of 3.5 for α-carboxyl group (Kallen and Jencks, 1966); 5.0 and 4.8 for the γ-carboxyl groups of PteGlu and $5CH_3H_4$PteGlu respectively (Pohland et al, 1951; Kallen & Jencks, 1966) and 5.2 for N^5 group of 5-CH_3H_4PteGlu (Whiteley et al, 1969). Table taken from Blair and Matty (1974).

It is impressive that the molecule of 5-CH_3H_4PteGlu, when soluble, contains two parts - the pterin base and the glutamic acid - both of which are highly electrically charged. These are properties which 5-CH_3H_4PteGlu shares with many other weak acids and bases, and it seems reasonable to suggest that the intestine would handle 5-CH_3H_4PteGlu in the same way. An explanation of 5-CH_3H_4PteGlu uptake may, therefore, be developed from the concept of non-ionic diffusion and the possible existence of an "acid microclimate" at the surface of the jejunal mucosa.

The principle of non-ionic diffusion, i.e. the penetration of the cell membrane in a fairly easy way by drugs in their non-ionised neutral form has been described at the beginning of this section. Most drugs are weak organic electrolytes which exist in solution as a mixture of the dissociated and undissociated forms. Schanker et al (1958) have found that epithelial lining of the intestine allows the ready penetration of undissociated drug molecules but impedes the passage of ionised molecules. In experiments with rats, the entire small intestine was perfused with a drug solution, and the extent of absorption estimated from the difference in the concentration entering and leaving the intestine. A relation between the degree of ionisation and rate of absorption was revealed: the weaker acids and bases were readily absorbed; stronger, highly ionised organic electrolytes were slowly absorbed; and the completely ionised quaternary ammonium compounds and sulphonic acid were hardly absorbed at all. As a general rule they suggested that acidic drugs were rapidly absorbed if their pKa's were greater than 3 and basic drugs were rapidly absorbed if their pKa's

were less than 8. The proportion of drug in the undissociated form depends on the dissociation constant of the compound and pH of the medium; consequently, to apply the lipoid membrane hypothesis to the absorption of drugs it is necessary to know the dissociation constant of the drugs as well as the lipid/water partition ratio of the undissociated form. But the ready absorption of a relatively strong acid like salicylic acid at the almost neutral pH, where the ratio of unionised to ionised drug is 1 to 4,000, tends to disqualify the non-ionic diffusion of organic electrolytes as the ratio of the unionised drug is too small to explain rapid absorption.

Hogben et al (1959) studied the effect of intestinal pH on the absorption of a number of weak organic acids and bases in the rat. In varying the pH of the drug solution from 4 to 8, the authors found that the rates of dissociation and consequently the rates of absorption dependent upon the intestinal pH. The steady state distribution of drugs between the intestinal lumen and plasma suggested a "virtual" pH for lumen of 5.3 when the observed pH was 6.6. This slightly acidic zone provided an explanation for the rapid absorption of moderately strong acid such as salicylic acid where the ratio of unionized to ionized drug was 1:200. If the "virtual" pH is an expression of a zone or "microclimate" at the site of absorption, the pH of this zone is to a considerable extent, but not completely, independent of the pH of the bulk solution within the intestinal lumen. This pH "microclimate" was conceived of a fluid compartment about 2 pH units more acidic than the intestinal chyme. (Hogben, 1960).

Goldman et al (1965, 1968a & 1968b) while working

on the behaviour of enzymes in synthetic membrane demonstrated the existence of an "acid microclimate" within the membrane matrix having pH several units more acidic than the bulk phase.

Numerous studies have shown that some mechanism exists in the jejunal epithelium of the rat and man for the maintenance of the pH gradient in the lumen (Ponz & Larralde, 1950; Parsons, 1956; Wilson & Kazyak, 1957; Blair, Lucas & Matty, 1975). The acidification in the small intestine can occur both in the presence and absence of glucose and the increase in acidification due to glucose has been attributed to the glucose metabolism subsequent to transport (Blair & Matty, 1974).

The acidification process is caused by the breakdown of ATP at the jejunal mucosal surface by the adenosine triphosphatase concentrated there and is associated with a mechanism of proton production necessary for the maintenance of an acidic zone at the said surface (Blair, Lucas & Matty, 1972, 1975). This mechanism of proton formation by ATP breakdown is further supported by the fact that metabolic inhibitors and anaerobiosis depress the acidification whereas ATP stimulates it when they are present in the mucosal medium (Lucas, 1974; Blair, Lucas & Matty, 1975).

It will not be irrelevant, at this stage, to point out one special structural feature of the absorptive cell surface. Bennett (1963) has suggested that a polysaccharide-rich component is of widespread and possibly of universal occurrence on all cell surfaces. He proposed the general term glycocalyx for all such cell coats on micro-organisms as well as plant and animal cells. Ito (1965) has shown

that the microvilli of the cat, bat and man are coated with a conspicuous layer composed of fine filaments radiating from the outer dense leaflet of the plasma membrane. In other species the surface coat is poorly developed or inconsistent, but all intestinal microvilli have traces of such coating consisting of acid mucopolysaccharides over the tips and sides of the microvilli. Ito termed this coat as the enteric surface coat.

The glycocalyx or the enteric surface coat on the surface of the epithelial cell occupies an important position in being the compartment through which the substrate must pass during absorption before entering the cell proper and may be described as defining the micro-environment of the microvilli. The environment in the interstices of the glycocalyx must constitute the immediate microenvironment with which mucosal cells exchange. Because of the possibility of structural glyco-proteins bearing fixed charges, the immediate environment of the microvillous membrane may be very different from that in the lumen (Parsons & Boyd, 1972). These authors also suggested the existence of a significant mucosal extracellular fluid compartment interposed between the intestinal lumen proper and the microvillous luminal face. The fluid within this compartment evidently included: (1) that adjacent to the epithelial layer and possibly unstirred and (2) that occupying the interstices between the matrix of the fibres that compose the glycocalyx proper. Parsons and Subjeck (1972) also added evidence to the existence of unstirred layers at all surfaces. It is between this fluid compartment and the cell interior that the functions of membrane transport

operate and this is likely to present an effective diffusion barrier to substances diffusing outward from the glycocalyx. Recently Wilson and Dietschy (1974) reported that the surface area of intestinal unstirred layer varied from 1.02 cm^2 to 14.24 cm^2 per 100 mg. dry weight of rat intestine. In addition to the fluid compartment mentioned above, overlying the true glycocalyx, a region of relatively small volume, there is a larger volume of fluid possibly unstirred. The glycocalyx along with the unstirred layers plays a paramount role in maintaining the microenvironment 'the acid microclimate' for the absorption of weak acids and bases. If the glycocalyx were to be removed from the cell, the microclimate would cease to exist.

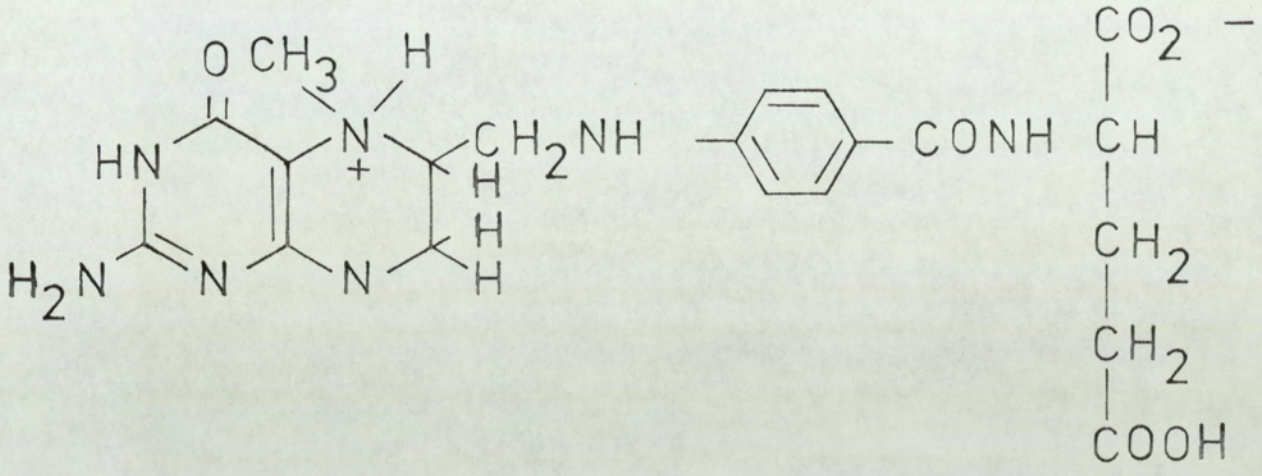
Apart from the enzymatic model of the maintenance of acid microclimate, another theoretical model, i.e. the electrostatic model, may be considered for the acidification process in the jejunum. The negatively charged lipid membrane of the intestinal cell will attract the positively charged ions and repel the negatively charged ions, as a result there will be a concentration of protons at the cell surface. Davison and Danielli (1948) showed that the opposite effects of proton concentration and anion depletion leave the amount of neutral acid the same as in the intestinal phase. Such a microclimate could not account for the transport of 5-CH_3H_4PteGlu in dianion form, i.e. 100 per cent ionised in the intestinal chyme at pH 6 to 7.

So far no evidence could be presented as regards the existence of an acid microclimate in the intestine though Caldwell (1958) presented a useful observation suggesting the occurrence of a microclimate at the surface of a crab muscle fibre.

The enzymatic model of acid microclimate may go a long way in interpreting the processes of intestinal absorption although, at present, its role is not fully recognised. The microclimate in this section will be referred to the fluid compartment consisting of the unstirred fluid adjacent to the epithelial layer and that occupying the interstices between the matrix of the glycocalyx.

If the usual assumption is made that only the neutral species will be capable of crossing the lipoid barrier, passive diffusion of 5-CH_3H_4PteGlu only becomes possible in an aqueous medium with pH less than 5 (Table 4.1). Above pH 5.0 5-CH_3H_4PteGlu exists mainly as dianion, the movement of which across the negatively charged phospholipid membrane would be prevented by the strong electrostatic repulsion. Below pH 5.0, i.e. in acid microclimate pH this repulsion is avoided by conversion to a neutral species. Thus in an acid microclimate it would exist as the effectively neutral zwitterion, that is, an organic compound having one positive charge and one negative charge; thus effectively a neutral compound (Figure 4.1).

FIGURE 4.1



Zwitterion of 5-CH_3H_4PteGlu

At pH 3.5 about 50 per cent of the total species are present in the zwitterion form and this proportion would not alter much in the pH range 3.5 to 4.5 (Table 4.1). The neutral zwitterion could freely diffuse across the lipoid membrane. Table 4.2 exhibits the solubility data for 5-CH_3H_4PteGlu (Blair, J.A. & Robb, A.J., 1973; unpublished observations). From the solubility data it appears 5-CH_3H_4PteGlu transport will not be saturated in the acid microclimate until a concentration of 3.2×10^{-2}M is reached, i.e. a concentration far in excess of the physiological range. As the transport has been studied only at concentrations less than 1×10^{-4}M the saturation effect has not been observed, probably within the concentration range studied.

TABLE 4.2

pH dependent solubility of 5-CH_3H_4PteGlu.

pH	Solubility
6.0	5.6×10^{-2}M
5.0	5.6×10^{-2}M
4.0	3.3×10^{-2}M
3.0	3.2×10^{-2}M
2.0	6.2×10^{-2}M

As regards the parent compound PteGlu, Matty and Blair (1968) and Smith et al (1970a) postulated that it was converted to the neutral unionised form in the microclimate which was more acid than the bulk phase and only the unionised PteGlu then passed through the lipoid membranes. The microclimate would be saturated with PteGlu when the bulk phase PteGlu concentration reaches optimum solubility of the microclimate depending upon the bulk phase pH. Thereafter, raising the bulk concentration will not increase the concentration of diffusable species at the microclimate and no further increase in the rate of transport would be observed or in other words kinetics of saturation would be observed. Hence, initially, the kinetic pattern for transport of PteGlu via this microclimate would be linearly related to concentration, followed by saturation. Using intestinal segments and short incubation periods, Blair et al (1974a) and Johnson (1974) showed saturation kinetics in the PteGlu uptake study and got excellent straight plot from 0.8×10^{-7}M (correlation coefficient 0.97) with saturation at about 5.1×10^{-6}M. A re-analysis (Blair & Matty, 1974) of the kinetic data of Smith et al (1970b) also showed similar pattern of curve. Thus, with both everted sacs (Smith et al, 1970b) and segments (Blair et al, 1974a; Johnson, 1974) the kinetics of PteGlu uptake follow that to be expected for passive diffusion of the neutral species formed in an acid microclimate at the cell surface. The pH of the microclimate was calculated to be slightly greater than 3.

Although it is a universally accepted hypothesis that organic acids and bases penetrate the lipoid membrane

of the cells only in unionised form, Hogben *et al* (1959) on the basis of slow but definite absorption of quaternary ammonium compounds concluded that intestinal mucosa was very slightly permeable to organic ions. Nogami and Matsuzawa (1961) demonstrated that salicylic acid was absorbed both in its unionised and ionised form, the ratio being about 6:1 in the rat small intestin *in vitro*. This has been confirmed in principle, in perfusion studies in the human rectum (Bechgaard, 1971). Kunze *et al* (1972) observed high rate of absorption for salicylic acid in rat jejunum. The high degree of ionisation of salicylic acid at the pH studied (6.5) appeared to be incompatible with predominantly non-ionic absorption at the high rate observed. Ethylenediaminetetraaceticacid increased the absorption of salicylic acid. On the basis of these observations they reached the conclusion that salicylic acid was absorbed mainly in the ionised state.

Ochsenfahrt and Winne (1974) have recently demonstrated that benzoic and salicylic acid crossed the rat jejunum both in the non-ionised as well as ionised form. The diffusion was found to be highly dependent on water flux indicating the operation of a solvent drag mechanism. They explained this solvent drag effect on the basis of an interaction of lipophilic drug and water molecules within the lipid part of the cell membrane.

The transport of amethopterin into sublines of Sarcoma-180 cells *in vitro* was demonstrated to be a passive diffusion process (Hakala, 1965). The influx was found to increase doubly when the incubation was done at pH 5.4 instead of pH 7.4. Ca^{+2} was also found to increase the

influx rate. Both these observations tend to advocate that amethopterin diffusion preferably takes place when it is present in the unionised form. Johnson (1974) argued that at pH 5.4 the proportion of neutral molecule was not likely to be appreciable and as such increased influx at this pH might crop up to a certain degree by the monoanion diffusion. On this basis Johnson (1974) suggested that a certain proportion of PteGlu can be absorbed by rat intestine as a monoanion form in between the bulk phase of 5.0 and 6.0 but he added that the microclimate pH corresponding to this ambient pH would not put a limitation on the solubility of PteGlu to account for the saturation kinetics demonstrated by him. He concluded some other mechanism other than monoanion transport was operative in the intestinal mucosa for the PteGlu absorption.

It has been mentioned in many reports that PteGlu transport in the rat and man is influenced by the pH of the bulk mucosal phase. Smith, Matty and Blair (1970b) using everted sacs in an *in vitro* study demonstrated that PteGlu uptake was stimulated when the bulk phase pH was adjusted to values less than 7.0. A reduction in uptake rate was encountered when the incubation medium pH was changed from 6.0 to 7.0. Blair and Matty (1974) proposed that the reduction in uptake was caused by the microclimate at approximately pH 3.0 becoming more alkaline and thereby reducing the concentration of the neutral form of PteGlu available for transport.

Strum *et al* (1971) did not carry out any study on the effect of pH of the incubation medium on the transport of 5-CH_3H_4PteGlu. In this study, as will be evident from Table 3.6 and Figure 3.8 it has been found

that the pH of the incubation medium influences the transport. The maximum uptake was found at an initial pH of 6.2, and the uptake decreased when the incubation was done at a higher or lower pH. It will also be found in Table 3.6 that the pH of the incubation medium changed in each case to a certain extent during incubation. The variation of uptake of 5-CH_3H_4PteGlu with pH can again be interpreted in the light of the proportion of the zwitterion present in the microclimate. If the pH of the incubation medium, i.e. pH 6.2 and 6.7 correspond to some pH values of the microclimate, ranging from 3.5 to 4.5, as suggested by Blair and Matty (1974) then the uptake rate will not show any appreciable change as the proportion present as the zwitterion is little affected within the microclimate pH by the above change in the bulk phase pH. The uptake at the initial pH of 6.2 and 6.7 did not show, in fact, any appreciable change, so these incubation pH values corresponded to the microclimate pH range from 3.5 to 4.5. The lower uptake, at the initial incubation pH of 7.2 and 5.7 can also be explained by the fact that at an incubation pH of 7.2 the microclimate pH became slightly alkaline, thereby reduced the concentration of zwitterion; similarly at an incubation pH of 5.7 the microclimate pH became more acidic and gave rise to the reduction in the concentration of diffusable zwitterion (Table 4.1).

The postulate of absorption of organic compounds in the monoanion form may make a partial contribution towards the total jejunal uptake of 5-CH_3H_4PteGlu in rat as the uptake at pH 6.2 was higher than that at pH 7.2 but absorption via zwitterion form will remain the main pathway of absorption.

Elsborg (1974) using rats *in vivo* studies reported that the maximum absorption of PteGlu took place at a pH 6.0, and the lower as well as higher intraluminal pH reduced the absorption of PteGlu. He also stressed the importance of non-ionic diffusion both in rats and man.

In a study with human subjects the administration of sodium bicarbonate was found to depress the jejunal PteGlu uptake (Benn *et al*, 1971). In the same study it was also found that the administration of sodium bicarbonate results in an increased jejunal pH, and it was suggested that an increase in alkalinity at the mucosal membrane was responsible for an impairment of the non-ionic diffusion of PteGlu. Some drugs, such as diphenylhydantoin, are strong alkaline agents and may impart a highly alkaline-intraluminal pH with impairment of PteGlu absorption (Benn *et al*, 1971; Elsborg, 1971). Perry and Chanarin (1972) have disputed both the observations that the anti-convulsant phenytoin reduces PteGlu uptake, and the simultaneous administration of sodium bicarbonate and PteGlu causes impaired PteGlu absorption.

The elevated intraluminal jejunal pH associated with the coeliac disease patients (Benn & Cooke, 1971) has been suggested to be the cause of the impaired absorption of PteGlu in these patients (Benn *et al*, 1971). Elevation of intrajejunal pH in this disease may in turn be triggered by the reduction of enzyme adenosine triphosphatase which is responsible for the hydrolysis of ATP for the production of hydrogen ion (Riecken *et al*, 1966).

Investigation of malabsorption relating to $5\text{-}CH_3H_4$PteGlu has not been undertaken so far, so no comments

or review can be made about it.

Contradictions exist regarding the chemical state in which PteGlu is transferred. Baker et al (1965), Cohen (1965), Perry and Chanarin (1970, 1973), Selhub et al (1973) found that PteGlu, at least partially, was reduced and converted to 5-CH_3H_4PteGlu while passing through the intestinal cell. This view has been disputed by the findings of Whitehead and Cooper (1967), Smith et al (1970b), Melikian et al (1971), Pratt and Cooper (1971) and Leeming et al (1972). Olinger et al (1973) reported that the transport of PteGlu can take place both in unmetabolized and metabolized states and metabolism is not a prerequisite of transport. Strum et al (1971) did not find any evidence of metabolism of 5-CH_3H_4PteGlu during its transport through the intestinal wall of rat. In the present study 5-CH_3H_4PteGlu was found to enter the serosal medium unmetabolized but in tissue as per the autoradiographic indication (Section 3, part 2 IX, chromatographic solvent system 1) the majority of 5-CH_3H_4-PteGlu was absorbed as unchanged but a minor portion was found to be different. This minor compound was not any of the other folates, but supposed to be a complex with tissue protein or an oxidised product. The autoradiographic analysis of the chromatographic plate run in solvent 2 showed that the major compound absorbed in tissue was not 5-CH_3H_4-PteGlu. This contradictory picture of the metabolic fate of 5-CH_3H_4PteGlu in tissue stemmed from the autoradiographic analysis of the compound run in two different chromatographic solvent systems is difficult to explain.

So far it could be established that 5-CH_3H_4PteGlu transport through the rat jejunum is a passive diffusion,

the diffusion mainly takes place through the acid micro-climate as a non-charged molecule.

The absence of saturation kinetics has been considered as the main criterion to establish the non-existence of a carrier mediated transport mechanism. Studies on competitive inhibition and counter transport were undertaken to throw more light on the uptake mechanism of 5-CH_3H_4PteGlu. The experiments with isolated cells were, on the other hand, undertaken in an effort to verify the prediction that if passive absorption is main transport mechanism, then reduction of pH of the incubation solution would induce increase in the influx of 5-CH_3H_4PteGlu. Metabolic inhibition was also studied to record the effects on its uptake.

ii) METABOLIC AND COMPETITIVE INHIBITION.

2,4 dinitrophenol was reported to inhibit the synthesis of ATP by mitochondrial preparations without affecting respiration, i.e. oxidation unaccompanied by phosphorylation (Loomis & Lippman, 1948; Cross et al, 1949). It is also reported to induce the reduction of the intracellular levels of ATP (Fridhandler & Quastel, 1955). Literature gives evidence of wide application of DNP as a metabolic inhibitor.

In the present study DNP was found to induce decrease in the tissue uptake and the serosal transfer of 5-CH_3H_4PteGlu relative to the control sacs from the same animals incubated under same conditions (Table 3.8). When the uptake was considered in terms of water uptake (Table 3.9) the reduction in total and tissue uptake appeared significant compared to the values of the control sacs but the serosal transfer of 5-CH_3H_4PteGlu in terms of water transfer was not significant. Higher 5-CH_3H_4PteGlu transfer in terms of water transfer in presence of DNP, i.e. low reduction in the ratio of folate to water transfer in comparison to control ones accrued from the higher reduction in the serosal transfer of water. In case of tissue the reduction of water uptake was comparatively low and that was also reflected in the ratio of total 5-CH_3H_4PteGlu transfer to total water transfer. Here a unique phenomenon has been represented, e.g. significant reduction in the absolute uptake of 5-CH_3H_4PteGlu by both tissue and serosal medium and significant reduction also in terms of folate to water uptake in tissue but insignificant reduction in terms of folate to water in

serosal medium due to higher reduction in the uptake of water.

Strum et al (1971) in their studies on rats did not find any significant effect of DNP on the transport of 5-CH_3H_4PteGlu. They considered only the serosal transport. In case of the PteGlu, Turner and Hughes (1962) could not prove any appreciable effect of DNP on its transport. Yoshino (1968b) also concluded that DNP was without effect on the absorption of PteGlu from perfused in vivo loops. In a more recent investigation, Johnson (1974) demonstrated that DNP reduced both tissue-accumulation and serosal transfer of PteGlu through the intestine of rat. The fluid transfer to serosal medium was highly reduced in this case.

In the present study as DNP was found to inhibit the transport of 5-CH_3H_4PteGlu it may be suggested that some aspect of the intestinal absorption of the compound is accomplished by the energy accrued from the aerobic metabolism. But the fact that there was still effective transport of 5-CH_3H_4PteGlu in presence of DNP favours the suggestion that transport of 5-CH_3H_4PteGlu may be effected by the energy derived from anaerobic metabolism. Johnson (1974) suggested that carrier mediated transport can operate in the absence of metabolic energy.

The concept of acid microclimate can also interpret the results obtained with DNP inhibition. As the acidity of this microclimate depends on the protons formed by hydrolysis of ATP by mucosal adenosine triphosphatase (Blair, Lucas & Matty, 1972) and as DNP has been reported to be the inhibitor of jejunal acidification mechanism (Lucas, 1974; Blair, Lucas & Matty, 1975) and the addition of ATP

offsets the inhibitory effect of DNP, it appears that DNP by creating conditions of inhibiting the proton formation, elevates the pH of the surface layer with the consequent disruption of the microclimate. This impairment of microclimate decreases the ratio of the available zwitterion and eventually causes the inhibition of 5-CH_3H_4PteGlu uptake.

Passive PteGlu transport has been reported to be increased as the result of solvent drag (Smith et al, 1970b) and it is also true in case of 5-CH_3H_4PteGlu transport. In the test sacs in this study the normal water transfer was inhibited and perhaps it was inhibited to an extent where the solvent drag component has been removed and as such resulted in the depression of 5-CH_3H_4PteGlu transfer. A recent report of Ochsenfahrt and Winne (1974) on the role of solvent drag to the uptake of benzoic acid and salicylic acid suggests that a proportion of the ionised molecules may be conveyed across the intestinal barrier through the lipid phase. A parallel role of solvent drag is presumably operative in case of 5-CH_3H_4PteGlu.

The material used to study competitive inhibition of 5-CH_3H_4PteGlu transport was its analogue 5-CHO H_4PteGlu. Baker et al (1965) reported on the fate of orally and parenterally administered 5-CHO H_4PteGlu in normal subjects. In case of orally administered compound, 5-CH_3H_4PteGlu was found to be the main compound in serum testifying to the fact that 5-CHO H_4PteGlu was converted to 5 CH_3H_4PteGlu during transport. Perry and Chanarin (1973) presented evidence that 5CHO-H_4PteGlu in addition to 10CHO PteGlu, 10-CHO H_4PteGlu and 5-CH_3H_4PteGlu was recovered from the serosal fluid when PteGlu, H_2PteGlu and H_4PteGlu were present

on the mucosal side of everted gut of rat. There should be no doubt, therefore, about its absorption in intestine or its having no toxic effect on the subject. It was also reported to be one of the main components of dietary folates (Butterworth et al, 1963).

In the present study 5-CHO H_4PteGlu was found to inhibit tissue uptake and serosal transport of 5-CH_3H_4PteGlu significantly (Table 3.8). 5-CH_3H_4PteGlu transport in terms of water transport in the presence of 5-CHO H_4PteGlu is also very low in comparison to that in control sacs (Table 3.9). These observations strongly indicate the possible existence of a carrier in the epithelial membrane for which both 5-CH_3H_4PteGlu and 5CHO H_4PteGlu compete. On the contrary, the absence of saturation kinetics as observed in earlier studies surely discard any argument in favour of carrier transport system.

Leslie and Rowe (1972) showed the existence of a brush border protein with an affinity for PteGlu and some of its derivatives. It was also found that 5-CHO H_4PteGlu had higher affinity for the brush border protein in comparison to 5-CH_3H_4PteGlu. The brush border protein may be considered as a membrane carrier protein at the mucosal surface. The depression of both tissue uptake and serosal transfer of 5-CH_3H_4PteGlu in the presence of 5-CHO H_4PteGlu may, therefore, be attributed to the lesser availability of the carrier sites to 5CH_3H_4PteGlu due to competition from 5-CHO H_4PteGlu for the same sites. But brush border protein has been shown to participate in binding of folate instead of its transport, so it may also be envisaged that 5-CH_3H_4PteGlu after entering the cell passively most

probably binds itself to brush border and intracellular protein or alternatively accumulates to intracellular sites by energy dependent process. The diminished tissue uptake observed in this study may then arise from the competition for binding sites or competitive inhibition of specific transfer mechanism at the intracellular sites. The reduced serosal transfer of 5-CH_3H_4PteGlu in presence of 5-CHO H_4Pte-Glu strongly indicates a carrier mediated serosal transfer for 5-CH_3H_4PteGlu. Alternatively if it is assumed that the 5-CH_3H_4PteGlu bound to intracellular and brush border proteins is released into the serosal medium after a regular interval of time, then less binding of 5-CH_3H_4PteGlu to cellular protein in the presence of 5-CHO H_4PteGlu will be reflected in less flux into the serosal medium.

Johnson (1974) using everted segments of rat jejunum also demonstrated a reduction in the tissue accumulation of PteGlu in presence of 10CHOPteGlu. Selhub et al (1973) and Olinger et al (1973) using everted sacs from rat jejunum observed reduction in the tissue accumulation of PteGlu in presence of higher concentration of methotrexate. All these three reports either show no change or increase in the serosal transfer of PteGlu in presence of 10CHOPteGlu and methotrexate. The unchanged or higher serosal transfer of PteGlu in presence of methotrexate can easily be explained by the fact that the presence of methotrexate affects the dihydrofolate reductase activity and consequently inhibits the formation of 5-CH_3H_4PteGlu resulting in uninterrupted or increased flux of PteGlu to the serosal side as PteGlu is reported to be less retained by the jejunal mucosa with respect to 5-CH_3H_4PteGlu (Olinger et al, 1973). Johnson

(1974) ascribed the uninterrupted serosal transfer of PteGlu in presence of 10-CHO PteGlu to a passive process which is proportional to the mucosal concentration. Strum et al (1971) demonstrated a significant increase in the serosal transfer of 5-CH_3H_4PteGlu in the presence of 5-CHO H_4PteGlu but it remained unexplained. This is quite contradictory to the present finding that serosal transfer of 5CH_3H_4PteGlu is also significantly reduced in the presence of 5CHO H_4PteGlu.

iii) STIMULATED EFFLUX STUDIES.

Stimulated efflux studies were undertaken in Section 3, part 2 vi) to investigate the possible existence of a carrier mediated step in the transport of 5-CH_3H_4PteGlu across the mucosal surface by means of an exchange diffusion test. The term exchange diffusion has been introduced to cover the phenomenon that an ion species crosses a membrane by combining with some carrier molecule which is part of the membrane (Ussing, 1947). Due to the thermal movements the carrier-ion complex may alternatively come into contact with either mucosal or serosal solution. When in contact with one of the solutions the complex may exchange the bound ion against one from that solution; but if the carrier-ion affinity is high, the carrier will always be saturated with the ion in question and the same number of ions will be carried from the left to right as from the right to left. The flux of ion, would be nearly the same in both directions even if there were a considerable difference in electrochemical potential for the ion across the membrane. The exchange diffusion thus stimulates active transport and as a matter of fact both processes are mediated through carrier systems. They differ in that exchange diffusion does not require work and that it cannot bring about a net transport (Ussing, 1949). Wilbrandt and Rosenberg (1961), however, pointed out that exchange diffusion does not require a system in which the carrier is necessarily capable of moving in the substrate-complex form. In any carrier system, under conditions of appreciable saturation of the carrier, part of the movement of the substrate molecules will occur in exchange for others.

Rosenberg and Wilbrandt (1957) showed that if a carrier transported substrate is equilibrated across a membrane, counter transport which is an uphill transport of the substrate may be elicited by the energy derived from the downhill transport of a second substrate using the same carrier.

In a qualitative way the phenomenon of counter-transport can be derived from a consideration of the gradients in the membrane. A first substrate, R, is equilibrated on the two sides of a carrier membrane. A second substrate, S, then is added to the external medium. This creates an inwardly directed gradient for the complex, CS, resulting in an outwardly directed gradient for the free carrier, C. The substrate R, originally equilibrated, then will meet unequal concentrations of free carrier, C, on the two sides of the membrane. Therefore, more of the complex, CR, will be formed on the inside and less on the outside of the membrane. A gradient of CR will thus arise, producing an outward movement of CR and the release of R on the outside, thus establishing an uphill transport for R. The results of this type of experiment were theoretically predicted on the basis of the carrier model for membrane transport and obtained in practice using the glucose transport system of the erythrocyte (Rosenberg & Wilbrandt, 1957).

In the present studies, sacs from rat jejunum were preloaded with labelled 5-CH_3H_4PteGlu and then reincubated in 5-CH_3H_4PteGlu free Krebs ringer phosphate medium containing 28 mM glucose and sodium ascorbate (3 mg/ml) and also in the same media containing higher concentration of unlabelled 5-CH_3H_4PteGlu, PteGlu, methotrexate and

5-$CHOH_4$PteGlu. Sacs incubated in 5CH_3H_4PteGlu free medium were used as controls against which the "stimulated efflux" from the test sacs was measured. Unlabelled 5-CH_3H_4PteGlu, PteGlu, methotrexate and 5-$CHOH_4$PteGlu were found to stimulate significantly the rate of efflux of the labelled compound.

The results obtained from these studies can be interpreted in terms of two transport mechanisms. There may be a carrier mediated transport system at the mucosal surface having a high affinity for PteGlu, methotrexate, 5CH_3H_4PteGlu and 5-$CHOH_4$PteGlu. On the other hand if absorbed 5-CH_3H_4PteGlu is accumulated by some means within the cell, then it is possible that the intracellular labelled compound is displaced from the accumulation sites by a high concentration of unlabelled compound which has entered the cell by passive diffusion. This displacement may be caused either by the competition for the binding sites offered by unlabelled 5-CH_3H_4PteGlu, PteGlu, methotrexate and 5-$CHOH_4$PteGlu or by a counter-transport effect at an intracellular site.

By autoradiography it was demonstrated that the displaced species in the mucosal solution and the species present in tissue during efflux incubation were the same compounds although neither one could be confirmed as 5-CH_3H_4PteGlu. The new compound may be an oxidised product of 5-CH_3H_4PteGlu or a complex with protein, the identity of which was not confirmed. Since the identity of the compound could not be confirmed, the displaced compound will be described as "X" compound. Similarity of the compounds indicates that species arises in the

bathing medium after efflux has come as a result of displacement from binding proteins of cells. The serosal compound was found to be the labelled 5-CH_3H_4PteGlu from autoradiographic study.

In another study, control and test jejunal sacs were prepared and preloaded as usual with a view to comparing the serosal transfer and tissue content of labelled compounds of the sacs reincubated in the presence and absence of unlabelled 5-CH_3H_4PteGlu. Figures 3.19 and 3.20 and Tables 3.14 and 3.15 exhibit that both the tissue content and serosal transfer are lower in the presence of unlabelled 5-CH_3H_4PteGlu than in its absence at each incubation interval. The difference is not, of course, significant. The lower tissue content of labelled 'X' compound resulting from incubation in the presence of unlabelled 5-CH_3H_4PteGlu can easily be explained in terms of intracellular accumulation through binding sites. Unlabelled 5-CH_3H_4PteGlu displaces the labelled 'X' compound from the binding sites and causes higher efflux of the labelled compound into the mucosal medium with a simultaneous fall in the tissue concentration. The gradual decrease in the tissue content of labelled 'X' compound against time, when incubated in the presence and absence of unlabelled 5CH_3H_4PteGlu is reflected in the gradual increase in the time dependent efflux of labelled 'X' compound in the mucosal medium. The findings from the autoradiographic studies and the studies on the tissue content of labelled 'X' compound incubated in higher concentration of unlabelled 5-CH_3H_4PteGlu envisage a transport process for 5-CH_3H_4PteGlu which involves passive influx at the mucosal pole of epithelial cells followed by a process of intracellular sequestration at

binding sites rather than a carrier mediated transport mechanism.

In such a case the displaced labelled 'X' compound should provide more of it for higher serosal transfer but on the contrary an absence of labelled 'X' compound and a decrease in the serosal transfer of labelled 5-CH_3H_4PteGlu was observed. The absence of labelled 'X' compound in the serosal medium can be accounted for by the fact that all the labelled 'X' compound are solely effluxed in the mucosal medium by being displaced from the binding sites by higher concentration of unlabelled 5-CH_3H_4PteGlu. The decrease in the serosal transfer of labelled 5-CH_3H_4PteGlu in presence of unlabelled 5-CH_3H_4PteGlu in the mucosal medium can be explained if the transfer of labelled 5-CH_3H_4PteGlu into the serosal compartment may be mediated by specific transport step, evidence for which has also been found in the uptake studies with competitive inhibitor. If it is assumed that a carrier is located at the serosal pole of the mucosal epithelial cells then the reduced serosal transfer of labelled 5-CH_3H_4PteGlu in presence of competitive inhibitor can be explained as 5-CHO H_4PteGlu competes with labelled 5-CH_3H_4-PteGlu for the carrier site and consequently reduces the serosal transfer. The reduced serosal transfer of labelled 5-CH_3H_4PteGlu in presence of unlabelled 5-CH_3H_4PteGlu can also be explained in terms of serosal pole carrier. The unlabelled 5-CH_3H_4PteGlu competes for this carrier site with free intracellular labelled 5-CH_3H_4PteGlu and thereby decreases the serosal transfer whereas the free intracellular labelled 5-CH_3H_4PteGlu does not encounter any such competition when unlabelled 5-CH_3H_4PteGlu is absent in the mucosal medium

resulting in uninterrupted serosal transfer. But the absence of saturation kinetics for serosal transfer of 5-CH_3H_4PteGlu and absence of labelled 5-CH_3H_4PteGlu in tissue as indicated by autoradiography do not favour this carrier transport hypothesis. This hypothesis of transport via serosal pole carrier remains conjectural. Johns and Plenderleith (1963) comparing a range of folate derivatives and related compounds for their ability to displace an absorbed dose of labelled folic acid, concluded that compounds having an intact unsubstituted pteridine moiety were effective displacers. They reported competition for binding sites or counter transport as the possible mechanisms of displacement and concluded that counter transport was the more likely mechanism of the two on the basis that a carrier system presumably exists in order to account for the rapid saturable transport of folate into cells.

Johnson (1974) and Blair et al (1974b) also studied the rate of efflux of intracellular folic acid in the presence of high external concentrations of folic acid and its derivatives. Folic acid, methotrexate and 10-formylfolic acid were found to stimulate the efflux of labelled folic acid but pteroic acid was without effect. Two possible mechanisms to account for this stimulated efflux have been suggested. The first is the counter transport if the movement of folate molecules across the membrane is mediated by a specific carrier system having a relatively high affinity for folic acid, 10 formylfolic acid and methotrexate. No direct evidence for the presence of such a carrier system has yet been obtained. Contradictory to this hypothesis, however, is the repeated observation that methotrexate has no inhibitory effect on the influx of folic acid (Olinger et al,

1973; Selhub et al, 1973). The second mechanism is the displacement of labelled folate from intracellular binding sites by the influx of unlabelled species having a strong affinity for the binding sites. Johnson (1974) also observed stimulated efflux when sacs preloaded with labelled PteGlu were reincubated in mucosal medium in the presence of a high serosal concentration of unlabelled PteGlu and offered the explanation that the stimulated efflux was the result of competitive displacement of labelled PteGlu by unlabelled PteGlu at the same site or sites within the gut wall. Blair et al (1974b) drew a line of difference between the brush border protein of Leslie and Rowe (1972) and the PteGlu binding site of theirs because the former has got appreciable affinity for pteroic acid but not for the methotrexate and the reverse is true for the latter.

In the present study methotrexate was found to be an effective displacer of labelled 'X' compound resulted from the preloaded 5-CH_3H_4PteGlu in the tissue. So both PteGlu and 5 CH_3H_4PteGlu are likely to have the same binding site. 5-CHO H_4PteGlu was also found to be an effective displacer of labelled 'X' compound resulted from the preloaded 5-CH_3H_4PteGlu at the tissue binding sites, the competitive inhibition induced by 5CHO H_4PteGlu on 5-CH_3H_4PteGlu uptake by sac arises from its affinity to binding sites. Johnson (1974) suggests that the benzoylglutamate moiety is of considerable importance in the binding process. Dihydrofolate reductase was thought to be one of the possible binding sites, presumably because reduction of PteGlu prior to methylation during transport occurred at this site (Olinger et al, 1973). Dihydrofolate reductase was reported to be localised in the apical cytoplasm of the mucosal cells (Darzynkiewicz et al, 1966). This

is an obvious site of accumulation for newly transported PteGlu, but in view of the relatively small amounts of PteGlu which are methylated during transport (Olinger et al, 1973) and the need for smaller concentration of methotrexate to inhibit the above conversion (Selhub et al, 1973) the candidacy of dihydrofolate reductase as the major site of tissue storage is liable to be ruled out. The suggestion that the jejunal mucosa has got higher ability to retain $5\text{-}CH_3H_4$PteGlu than PteGlu (Olinger et al, 1973) leads to the assumption that $5\text{-}CH_3H_4$PteGlu may remain bound with dihydrofolate reductase which is absorbed in tissue or it may be bound to some other tissue protein.

A recent interesting communication (Corrocher et al, 1974) suggests that the cytoplasmic Y and Z proteins involved in hepatic transfer of bilirubin and other organic ions (Levi et al, 1969; Reyes et al, 1971) or closely related proteins may be involved in transfer of folates from plasma to liver. The studies in vitro showed that PteGlu was mainly bound to 'Y' and 'Z' proteins but in vivo studies indicated the main binding to X and Z proteins for shorter period and to X and Y at longer periods endowing the major role of binding on 'X', 'Y' proteins and minor role on 'Z' protein. Fleischner et al (1972) reported that Y protein is the major determinant of organic anion flux across the plasma membrane of the liver cell. Evidence for the presence of Y protein and Z protein in the intestinal mucosa of rat has appeared (Levi et al, 1969; Fleischner et al, 1972). Some studies (Mishkin et al, 1972; Mishkin et al, 1973) established 'Z' as the predominant free fatty acid binding protein in liver, kidney and intestine, but Mishkin

et al (1973) did not rule out the importance of 'Z' protein in hepatic uptake of other organic anions.

As 'Y' and 'Z' proteins have been demonstrated to bind folates *in vitro* hepatic folate uptake a parallel role for these proteins to bind folates in intestine can also be visualized where 'Y' may play the major role. This hypothesis may further be strengthened by the fact that phenobarbitone and oestrogens affect 'Y' and 'Z' proteins as well as folate metabolism.

iv) ISOLATED CELL STUDIES.

In these studies the hyaluronidase disaggregation method of cell isolation has been used. For the sake of interest a brief review of the other methods of the isolated cell preparations will be furnished here.

The first attempt to separate the absorptive units from the remainder of the rat intestine was made by Dickens and Weil-Malherbe (1941). They scraped the mucosa from the underlying muscle layers with a glass microscope slide and used the scrapes to investigate the *in vitro* aerobic metabolism of the intestinal mucosa. This method was also used to study the uptake and incorporation of formate into the purines of the epithelial cells by Paterson and Zbarsky (1955, 1958). Crane and Mandelstam (1960) reported that both the mucosal scrapes, and the "isolated villi" preparation obtained by further mechanical fragmentation of the scrapes were capable of carrying out the transport of sugars. The mucosal scrapes of Dickens and Weil-Malherbe (1941) have been shown to consist of the epithelial lining and the underlying lamina propria (Schultz *et al*, 1966). Another simple and rapid method of obtaining suspension of morphologically intact, viable individual intestinal cells (based on application of controlled mechanical stress to the intestinal mucosa) has been reported (Harrison & Webster, 1969; Levine & Weintraub, 1970). Their metabolic characteristics were also reported to be comparable to those of intact intestine (Iemhoff *et al*, 1970).

Digestion of cells by chemicals is the most common method currently used for the isolation and preparation of mucosal cells. Harrer *et al* (1964) reported the isolation

of surviving epithelial cells from rat intestine free from contamination by mesenchymal elements and intestinal flora. They digested the cells from the underlying basement membrane with a solution of trypsin and pancreatin. Cells obtained by sodium citrate method of isolation were shown to be capable of energy dependent glucose accumulation (Stern & Jensen, 1966). Successful glucose and L-leucine uptake studies were performed by the citrate method of isolation of cells (Reiser & Christiansen, 1971; Murer et al, 1974).

Huang (1965) has described a method of isolation of the intestinal epithelial cells of the rabbit using lysozyme but this has been criticised on the grounds that the long period of incubation (55 minutes) involved seemed drastic and led to possible cell damage (Stern & Jensen, 1966; Perris, 1966).

Hyaluronidase digestion method described and employed by Perris (1966) and Kimmich (1970) yielded isolated cells having normal morphology and satisfactory metabolism and transport characteristics. The use of 1% serum albumin appeared to assist in cellular maintenance. In its presence oxygen uptakes were enhanced. Nakamura et al (1959) showed how homogenates of rat intestinal mucosa liberated fatty acids which could inhibit succinooxidase. Cell damage during the preparative procedure could have a similar effect, and the protective action of the serum albumin may be related to its ability to bind these free fatty acids. The use of hyaluronidase technique by Leslie and Rowe (1972) for the study of PteGlu transport and PteGlu and its derivatives binding to cellular protein

suggested its further investigation in the 5-CH_3H_4PteGlu transport and binding.

A technique for isolating cells from guinea pig small intestine employing ethylenediaminetetra-acetate (EDTA) which by virtue of its chelation to metal ions disaggregate the cells from the basement membrane; has been described by Evans et al (1971). The cells produced by this technique are of good functional and morphological appearance (Evans, 1974; personal communication).

In the present studies the uptake of 5-CH_3H_4PteGlu by isolated cells was undertaken against a mucosal concentration of 10^{-5}M 5-CH_3H_4PteGlu at 37°C and at a starting pH of 7.0. Figure 3.21 and Table 3.16 illustrate the result of such experiments. It can be seen that all the uptake of 5-CH_3H_4PteGlu is complete within 3 minutes and at each subsequent time interval there is none or very insignificant uptake as $P > 50\%$ for the uptake between 3 and 6 minutes of incubation and again $P > 50\%$ for the uptake between 6 and 11 minutes incubation. Johnson (1974) also reported that the PteGlu uptake by isolated cells at mucosal concentration of 10^{-5}M PteGlu and at starting pH of 7.0 was complete within 2 minutes followed by a gradual decrease in folate uptake with increasing time. Leslie and Rowe (1972) demonstrated that in similar experiments there was no increase in the amount of folate bound to cells as a function of time over the nanomolar to the millimolar concentration range. The present results (about 13.0 p moles/mg dry weight) for 3 minutes is about half the value (23.0 p moles/mg dry weight) for 2 minutes obtained by Johnson. Therefore it appears that the cell uptake capacity for 5-CH_3H_4PteGlu

is lower than that for PteGlu which is in agreement with the findings of Leslie and Rowe (1972) who demonstrated insignificant displacement of PteGlu from binding sites by 5-CH_3H_4-PteGlu.

Figure 3.22 illustrates the "washout" of 5-CH_3H_4PteGlu from the isolated cells. It shows a similar trend of "washout" of radioactivity as exhibited by PteGlu (Johnson, 1974). Johnson also demonstrated that the washout of radioactivity from the cells were very similar for PteGlu and labelled inulin, a compound usually assumed to be incapable of penetrating viable cell membranes and concluded that the association of PteGlu with the cells was primarily extracellular. Association of 5-CH_3H_4PteGlu with the cells, on the basis of similarity in washout trend, can also be termed as extracellular.

The hypothesis of PteGlu absorption mediated through extracellular pores and intercellular spaces (Johnson, 1974) is susceptible to challenge by the findings of Momtazi and Herbert (1973). They isolated the cells by vibration technique (Harrison & Webster, 1969) and reported uptake of PteGlu by the suspensions of cells, which was pH dependent, linear with increased concentration, sensitive to glucose and diphenylhydantoin. In view of accumulation of PteGlu by the cells obtained by vibration methods it can be stated that the cells obtained by those methods exhibit true intracellular accumulation, and the failure to do so by the cells prepared by the hyaluronidase may be ascribed to some sort of functional lesion undergone during the isolation procedure.

Johnson (1974) by comparing the behaviour of uptake of folate by mucosal scrape with that of isolated

cells has confirmed that the isolation technique used for cell preparation has a detrimental effect on the folate absorptive capacity of the cells. He observed that the isolated cell preparation exhibited a rapid initial accumulation of PteGlu with no measurable time dependence, the mucosal scrapes, on the other hand, accumulated PteGlu over the first ten minutes or so of the incubation, and then reached a plateau value. Strum et al (1970) has reported increased uptake with increased 5-CH_3H_4PteGlu concentration using mucosal scrapes of rat small bowel. In this study no gradual increase in uptake by cells was found, except the initial accumulation at 3 minutes. So again it leads to the fact that there is certain functional damage affecting 5-CH_3H_4PteGlu transport in these isolated cells wrought during the isolation procedure.

The question now crops up as to the nature of the functional lesion affecting folate transport in these isolated cells. Cells of the mucosal epithelium are likely to suffer some degree of physiological damage when exposed to drastic procedures involved in any isolation technique. Stewart and Zbarsky (1963) reported that isolated cells prepared from mucosal scrapes, were capable of complex metabolism for useful periods of time but showed a rapid loss of RNA (75%) and DNA (99%) during incubation. This colossal loss of RNA and DNA simply reflects the poor integrity of the cells caused by the incubation method, and the disintegration of most of the cells within two hours of incubation as revealed by microscopic study further confirmed cell lesion.

The enzyme trypsin commonly employed in the preparation of isolated mucosal cells has long been known

to disturb the integrity of cell membranes, for example when tissue culture cells were trypsinized, they lost their adhesiveness and dissociated. This result was attributed to removal by the enzyme of an extraneous coat (Moscona, 1952).

Hyaluronidase treated cells are capable of carrying out energy dependent sugar, electrolyte and amino acid transport and as such it can be safely said that, at least, some of the specific transport systems of the membrane remain operative (Kimmich, 1970; Reiser & Christiansen, 1971; Leslie & Rowe, 1972). Leslie and Rowe (1972) failed to detect glycoproteins or lipoproteins in the analysis of the brush borders isolated from their cell preparations involving hyaluronidase technique. This loss of glycoproteins and lipoproteins resulting in the possible structural derangement has been ascribed to the hyaluronidase technique.

The loss of glycoproteins and lipoproteins from the hyaluronidase treated cells is likely to result from the removal of the glycocalyx or enteric cell coat structure associated with the microvilli of the brush border. The histochemical stains for carbohydrates, such as periodic acid-Schiff, which specifically reveals 1,2 glycol groups, and colloidal iron, which reveals acidic groups, have demonstrated that the surface coat was composed of carbohydrate side chains of acid glycoproteins and it constitutes a morphological as well as a physiological barrier at the cell surface (Ito, 1965; Rambourg *et al*, 1966; Ito, 1969; Leblond & Bennett, 1974). It is not clear to what extent this coat is distinct from the mucus secretions of the goblet cells.

The elaboration of cell coat glycoproteins was examined by autoradiography and electron microscopy of the intestinal epithelium after injection of labelled precursors e.g. labelled proline, mannose, galactose and fucose. The evidence suggests that the protein moiety is synthesized on ribosomes and migrates through endoplasmic reticulum, where mannose is collected, and through the Golgi apparatus, where galactose and fucose are collected. The completed glycoprotein is then transported to the cell surface, apparently by vescicles which donate their wall and its glycoprotein lining to the plasma membrane (Neutra & Leblond, 1966; Bennett & Leblond, 1970; Leblond & Bennett, 1974). These observations advocated that each cell was the source of its own cell coat material.

Bennett et al (1974) also demonstrated that even during the short life span of these renewing intestinal epithelial cells, some turnover of cell coat component occurs. Ito (1965, 1969) and Mukherjee and Williams (1967) have also emphasized that the surface coat is a product of the individual cell to which it is clung, rather than an absorbed layer covering the epithelium as a whole. Parsons and Subjeck (1972) reported the existence of an unusually thick second layer of the intestine, which may be originated from free polysaccharide of the epithelial mucus secretions and distinct from the cell secreted glycoprotein coat. Kennedy (1973) advocated that hyaluronidase was liable to disrupt the intrinsic glycoprotein coat of the cell membrane.

Ito (1965) failed to find any damaging effect of trypsin and hyaluronidase on the surface coat of everted sacs and pieces of minced mucosa from cat intestine and concluded that the surface layer remained unusually stable

while the cell was viable but the procedures like isolation of brush borders, or the natural process of cell extrusion from the tips of the villi resulted in its loss.

It appears logical, however, that the long period of hyaluronidase digestion used in this study accompanied with the disruption of the epithelial structure and the subsequent saline washing procedure is likely to bring about some derangement of the surface layer. The histological studies described in Section 3 were carried out in an effort to investigate this point.

Plate I shows a group of cells *in situ* at the tip of a jejunal villus prepared from an everted ring. Periodic acid Schiff (PAS) staining has been employed to selectively stain muco-polysaccharide structures and the red staining layer attached to the brush borders of the cells is quite prominent, as are the distinct mucus red goblet cells. From the picture obtained it can be said that a relatively thick polysaccharide layer is a feature of the functioning absorptive cells.

Plate II shows the presence of PAS stained polysaccharide layer of the jejunal villi obtained from the sections of everted sac incubated in Krebs Ringer Phosphate medium containing 28 mM glucose for 30 minutes. Distict mucus rich goblet cells are also visible. Recently Bhantumnavin *et al* (1974) presented evidence that distortion of villus pattern from the everted sac of 6 week rat after 30 minutes of incubation in presence of glucose is at minimum, whereas there is a marked mucosal change after 30 minutes of incubation in the absence of glucose.

Plate III is a cell preparation obtained by the hyaluronidase disaggregation technique and stained with

PAS as before. No stainable polysaccharide material is visible at the brush border and it is obvious that the isolation technique has brought about a marked loss of surface layer of polysaccharides and glycoproteins, and this is in agreement with the findings of Leslie and Rowe (1972) on the absence of glycoproteins or lipoproteins of the brush border attached layers obtained from similar cells.

This loss of the enteric surface coat implemented by the enzyme digestion contradicts Ito's (1965) observations that the surface coat is resistant to the action of proteolytic and lipolytic agents. The difference may be due to the fact that the present observations have been carried out on truly isolated cells rather than tissue fragments or whole preparations of gut wall. Comparison of the cells of Plate III with those of Plate IV which were prepared by the citrate dissociation method (a preparation kindly provided by Dr. W.E. Jensen, University of Chicago) and stained with PAS exhibits the marked difference in the brush border of these cells. In Plate IV the pink staining polysaccharide layer at the extremities of the brush borders are clearly visible indicating that the surface coat has not been deranged by the citrate dissociation method. The cells in plate occur as small fragments of epithelium rather than individual cells. It seems feasible that the stability of the surface layer in the cells isolated by citrate techniques is due in part to the partial preservation of the epithelial integrity.

Uptake studies of 5-CH_3H_4PteGlu by isolated cells were also done at pH 5.0. Figure 3.21 and Table 3.16

exhibit that at pH 5.0 there is similar rapid uptake as that at pH 7.0 within the first 3 minutes followed by a slower but significant uptake in the next 3 minutes ($P > 1\%$). This later uptake represents transport into the cell. The next 5 minute's incubation fails to show any uptake. So it seems that the uptake becomes saturated at 6 minutes. Thus as judged either by total uptake at six minutes or by uptake between three and six minutes the uptake of 5-CH_3H_4PteGlu by isolated cells is greater at pH 5.0 than pH 7.0.

Uptake of 5 CH_3H_4PteGlu by isolated cells at 27°C shows that there is no significant difference of uptakes between 3 and 6 minutes ($P > 50\%$) and between 6 and 11 minutes ($P > 20\%$) at pH 7.0. At pH 5.0 a similar trend is also observed but there appears an uptake although not significant ($P > 10\%$) between 3 and 6 minutes (Table 3.17). Uptake at pH 7.0 was a little higher (not significant) than that at pH 5.0. Perhaps, at 27°C only binding to cellular protein takes place at both pH 5.0 and 7.0, and therefore there is no significant difference between the uptake values.

Failure of the isolated cells to transport 5 CH_3H_4PteGlu at 37°C and at initial pH 7.0 can be explained by the fact that the "glycocalyx" a general term for all polysaccharide cell surface coats (Bennett, 1963) being lost during isolation procedure gave rise to higher micro-climate pH and thereby restricted the permeable 5-CH_3H_4Pte-Glu zwitterion available for diffusion. At pH 5.0 about 15% of 5CH_3H_4PteGlu is present as zwitterion and this is sufficient to allow transport into the cell to occur without glycocalyx. Sacs were found capable of 5-CH_3H_4PteGlu transport because the glycocalyx was not damaged in this

case as evident from histological studies.

So these observations confirm the suggestion made earlier in this discussion that the passage of $5\text{-}CH_3H_4PteGlu$ into the mucosal cell occurs by passive diffusion, and the permeability depends on the percentage of zwitterion, which in turn depends on the pH of the acid microclimate, the aqueous phase in contact with the brush-border membrane which is again influenced by the glyco-protein surface coat, glycocalyx. One of the functions of glycocalyx is to maintain a low pH within the brush border. The absence of enteric surface coat would mean that the fluid layer in contact with the brush border membrane would tend toward the conditions of the bulk phase, and thereby reduce the availability of zwitterion for transport.

v) GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK.

From the discussion of the results obtained in this study it can be said that 5-CH_3H_4PteGlu transport across rat jejunum is a linear process with increasing incubation time at the 5-CH_3H_4PteGlu concentration of 10^{-5}M in the incubation medium. Tissue uptake is much higher than serosal uptake. Tissue uptake is appreciable whether there is any concentration gradient between the mucosal and serosal media or not.

On the basis of various studies undertaken it has been proposed that 5-CH_3H_4PteGlu is transported as the neutral zwitterion and to some extent as ionized and the transport process is a passive diffusion one. Diffusion across the mucosal surface is followed, in the jejunum, by binding or sequestration with the gut wall, and is dependent upon the availability of the zwitterion controlled by the pH of the microclimate which in turn is influenced to a certain extent by the bulk phase. Absence of saturation kinetics has emerged from the fact that the concentrations of 5-CH_3H_4PteGlu used in incubation medium was well within the limit of solubility of the acid microclimate pH range. Johnson (1974) and Blair *et al* (1974a) observed saturation kinetics in case of PteGlu uptake because the "microclimate" imposes an upper limit on the rate of transport of this compound because of the low solubility at low pH.

pH characteristic of the "microclimate" can be investigated by using 5-CH_3H_4PteGlu, as its transport is influenced, as reported here by the existence of acid microclimate.

It appears from the current studies relating to

isolated cells that uptake of $5CH_3H_4$PteGlu is closely related to the stability of the polysaccharide layer at the brush border the "glycocalyx". The inter-relation of 5-CH_3H_4PteGlu uptake and persistence of "glycocalyx" can be meticulously investigated and verified if 5-CH_3H_4PteGlu uptake studies are undertaken with cells isolated and prepared by various other available methods.

Such indirect studies must of course be accompanied by a direct measurement of the pH in the micro-climate; such studies, utilising pH micro electrodes, are currently being carried out in this department (J.A. Blair & M. L. Lucas, 1974, personal communication).

In this study no firm evidence has been obtained for the presence of carrier-mediated step at the brush border membrane. Non-saturable uptake, low temperature coefficient, low ratios of serosal and tissue to final mucosal concentration contributed to the phenomenon of passive diffusion by which 5-CH_3H_4PteGlu is proposed to be transported through the rat jejunum. The reduced serosal and tissue uptake in presence of DNP has been explained satisfactorily in terms of solvent drag and acid micro-climate theory. Lower tissue uptake in presence of competitive inhibitor like $5CHOH_4$PteGlu and the stimulated mucosal efflux of labelled 'X' compound in presence of unlabelled 5-CH_3H_4PteGlu and other structural analogues have been explained in terms of intracellular binding sites for which such compounds compete and bring about the reduction in the tissue uptake and stimulated mucosal efflux. The findings from the autoradiographic studies and from the studies on the tissue content of labelled

'X' compound incubated in higher concentration of unlabelled $5CH_3H_4PteGlu$ have further subscribed to the postulate of binding $5CH_3H_4PteGlu$ to intracellular sites. So, from above a transport process for $5\text{-}CH_3H_4PteGlu$ involving passive influx at the mucosal pole of the epithelial cell followed by a process of intracellular sequestration at binding or accumulation sites can be envisaged. Nevertheless, the observation that the serosal transfer of $5\text{-}CH_3H_4PteGlu$ is depressed prominently in presence of $5\text{-}CHOH_4PteGlu$ indicate the presence of a carrier-mediated process, possibly at the serosal poles of the absorptive cells as appears to be the case for thiamine (Rindi & Ventura, 1972). The fact that the serosal transfer of preloaded labelled $5\text{-}CH_3H_4PteGlu$ is inhibited by a high mucosal concentration of unlabelled $5\text{-}CH_3H_4PteGlu$ also contributes to the hypothesis that serosal transfer is a carrier mediated one. But the absence of saturation kinetics in serosal transfer of $5\text{-}CH_3H_4PteGlu$ contradicts the carrier-mediated active transport. On the basis of the results of competitive inhibition and efflux studies two hypotheses for the serosal transfer can be proposed. The first one is the carrier-mediated transfer, into the serosal compartment, the carrier being located at the serosal pole of the intestinal mucosa. The alternative one is that if it is assumed that $5\text{-}CH_3H_4PteGlu$ bound to cellular proteins is released into the serosal compartment at a regular interval of time, the less binding of $5\text{-}CH_3H_4PteGlu$ to cellular protein in presence of $5\text{-}CHOH_4PteGlu$ will be reflected in less flux into the serosal compartment. The absence of labelled $5\text{-}CH_3H_4PteGlu$ in the tissue and labelled 'X' compound in the serosal medium tends to discard both the hypotheses, so they remain conjectural.

The stimulation of 5-CH_3H_4PteGlu by 5-$CHOH_4$PteGlu as reported by Strum et al (1971) has been unexplained. In order to resolve all these controversies regarding serosal transfer of 5-CH_3H_4PteGlu it would be interesting to employ a new approach to the problem, possibly using a new preparation.

Weir et al (1973) has shown that active diastereoisomer of 5-CH_3H_4PteGlu is transported across the intestine of normal subject at a higher rate. Blair and Matty (1974) ascribed this observation to the higher parenteral dose of active diastereoisomer of 5-CH_3H_4PteGlu in comparison to no parenteral contribution from the inactive diastereoisomer. So another interesting study of 5-CH_3H_4PteGlu can be undertaken in view of equal parenteral administration of both active and inactive diastereoisomers.

The identification of the bound species to cellular proteins and also the species effluxed in efflux medium could not be established as 5-CH_3H_4PteGlu. It has been proposed that those may be protein bound complexes of 5-CH_3H_4PteGlu or its oxidised product. Attempts may be directed to identify the nature of this compound by using some other solvent systems and modified extraction methods.

Fox and Hogben (1974) have recently reported the evidence of active transport for nicotinic acid across sheets of bullfrog intestine in vitro. The reported failure of exhibiting active transport mechanism in the transfer of PteGlu has been ascribed by these authors to the fact that isotope equilibrium cannot be achieved in mammalian preparation in the relatively short incubation periods undertaken by several workers. Since their preparation

is capable of transporting nicotinic acid for as long as 9 hours, full equilibrium is attained and small differences in opposing flux rate become detectable. This prolonged incubation time is likely to tell upon the physiological and morphological integrity of the cells under study. Whether or not their argument holds water remains to be further investigated but it would be advisable to examine 5-CH_3H_4PteGlu transport in similar preparation. The behaviour of 5-CH_3H_4PteGlu transport can also be investigated by employing preparations from hamster and guinea pig jejunum.

Application of high resolution autoradiography at the microscopic level by using sections of gut wall and isolated cell at incubation periods from a few seconds upwards can throw more light on the cellular binding of $5CH_3H_4$PteGlu. Such an approach has already been undertaken by Darzynkiewicz *et al* (1966) who demonstrated the localisation of labelled methotrexate in the apical cytoplasm of mouse intestinal cells.

These studies are likely to confirm the proposed role of glycocalyx in maintaining a healthy microclimate and its influence on the folate transport by pH control. Any physiological disturbance on the glycocalyx integrity caused by disease state is likely to have a bearing on folate transport. In fact, it has been observed that in coeliac disease the microclimate is less acidic because of reduced mucosal ATPases (Riecken *et al*, 1966). Weir *et al* (1973) observed a similar reduction of 5-CH_3H_4PteGlu absorption in coeliac patients.

Folate malabsorption occurs in coeliac disease

(Benn et al, 1971; Hoffbrand, 1971), ulcerative colitis (Franklin & Rosenburg, 1973) and regional enteritis (Cox et al, 1958). It may be drug induced (Reynolds et al, 1965; Elsborg, 1971) or alcohol (Halsted et al,1971) induced. The effect on the absorption of 5-CH_3H_4PteGlu can also be studied in all the diseases and in presence of alcohol and drugs, as a matter of interest. PteGlu has been found to have therapeutic effects on the symptoms of coeliac disease, ulcerative colitis and drug and alcohol induced PteGlu deficiency. The role of 5-CH_3H_4PteGlu, the major component of dietary folate can also be put to test with respect to its therapeutic effects on the above mentioned abnormalities.

The above studies, if undertaken, may further strengthen the role played by glycocalyx in maintaining the pH of the microclimate and as such in overall regulation of 5-CH_3H_4PteGlu and other folates transport. Any interesting findings arising out of the future investigation on 5-CH_3H_4PteGlu transport is likely to enrich the present knowledge on the physiology of the intestinal mucosal cells.

BIBLIOGRAPHY.

Anderson, B., Belcher, E.H., Chanarin, I. and Mollin, D.L., (1960).
The urinary and faecal excretion of radioactivity after oral doses of ^{3}H - folic acid. Brit. J. Haemat 6, 439 - 455.

Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H., Semb, J., Stokstad, E.L.R., Subbarow, Y., Waller, C.W., Consulich, D.B., Fahrenbach, M.J., Hultquist, M.E., Kuh, E., Northey, E.H., Seeger, D.R., Sickels, J.P. and Smith, J.M. Jr. (1946).
The structure and synthesis of the liver L. casei factor.
Science 103, 667 - 669.

Arakawa, T., Honda, Y. and Yoshida, T., (1972)
Increase in serum folate following an oral histidine load.
Tohoku J. Exp. Med., 108, 239 - 243.

Baker, H., Frank, O., Feingold, S., Ziffer, H., Gellene, R.A., Leevy, C.M. and Sobotka, H. (1965).
The fate of orally and parenterally administered folates.
Am. J. Clin. Nutr. 17, 88 - 95.

Baker, H., Thomson, A.D., Feingold, S. and Frank, O. (1969)
Role of the jejunum in the absorption of folic acid and its polyglutamates.
Am. J. Clin. Nutr. 22, 124 - 132.

Banerjee, D.K. and Chatterjea, J.B. (1966)
Observations on the presence of pteroyl polyglutamates in human serum.
Blood 28, 913 - 917.

Barry, R.J.C., Dikstein, S., Matthews, J., Smyth, D.H., and Wright, E.M. (1964)
Electrical potentials associated with intestinal sugar transfer.
J. Physiol. 171, 316 - 338.

Barry, B.A., Matthews, J. and Smyth, D.H. (1961).
Transfer of glucose and fluid by different parts of the small intestine of the rat.
J. Physiol. 157, 279 - 288.

Baugh, C.M. and Krumdieck, C.L. (1969).
Effects of phenytoin on folic acid conjugases in man.
Lancet 2, 519 - 521.

Baugh, C.M., Krumdieck, C.L., Baker, H.J. and Butterworth, C.E. Jr. (1970).
The intestinal absorption of polyglutamates of folic acid.
Fed. Proc. 29, 634 (abstr.).

Baugh, C.M., Krumdieck, C.L., Baker, H.J. and Butterworth, C.E. Jr. (1971).
Studies on the absorption and metabolism of folic acid.
1. Folate absorption in the dog after exposure of isolated intestinal segments to synthetic pteroylpolyglutamates of various chain lengths.
J. Clin. Invest. 50, 2009 - 2021.

Beavon, J.R.G. (1973).
Aspects of folate and tetrahydrofolate chemistry and metabolism in the rat.
Ph.D. Thesis, University of Aston in Birmingham.

Bechgaard, E. (1971).
Absorption of salicylic acid from the perfused human rectum.
Acta. Pharmacol. Toxicol. 29 (Suppl. 4), 4 (abstr).

Benn, A. and Cooke, W.T. (1971).
Intraluminal pH of duodenum and jejunum in fasting subjects with normal and abnormal gastric or pancreatic function.
Scand. J. Gastroenterol. 6, 313 - 317.

Benn, A., Swan, C.H.J., Cooke, W.T., Blair, J.A., Matty, A.J., Smith, M.E. (1971).
Effect of intraluminal pH on the absorption of pteroyl-monoglutamic acid.
Brit. Med. J. 1, 148 - 150.

Bennett, G. and Leblond, C.P. (1970).
Formation of cell coat material for the whole surface of columnar cells in the rat small intestine, as visualized by radioautography with L-fucose-^{3}H.
J. Cell. Biol. 46, 409 - 416.

Bennett, G., Leblond, C.P. and Haddad, A. (1974).
Migration of glycoprotein from the golgi apparatus to the surface of various cell types as shown by radioautography after labelled fucose injection into rats.
J. Cell. Biol. 60, 258 - 284.

Bennett, H.S., (1963).
Morphological aspects of extracellular polysaccharides.
J. Histochem. Cytochem. 11, 14 - 23.

Bennett, M.C., Berry, V., Chanarin, I. and Ardeman, S.(1964).
The assay of folic-acid activity of liver.
J. Clin. Path. 17, 27 - 30.

Bernstein, L.H., Gutstein, S. and Weiner, S. (1970b)
Gamma glutamyl carboxypeptidase (conjugase), the folic acid-releasing enzyme of intestinal mucosa.
Am. J. Clin. Nutr. 23, 919 - 925.

Bernstein, L.H., Gutstein, S., Weiner, S. and Efron, G. (1970a).
Absorption and malabsorption of folic acid and its polyglutamates. Am. J. Med. 48, 570 - 579.

Bethell, F.H., Meyers, M.C., Andrews, G.A., Swendseid, M.E., Bird, O.D. and Brown, R.A. (1947).
Metabolic function of pteroylglutamic acid and its hexaglutamyl conjugate (hematologic and urinary excretion studies on patients with macrocytic anaemia).
J. Lab. Clin. Med. 32, 3 - 22.

Bhantumnavin, K., Wright, J.R. and Halsted, C.H. (1974).
Intestinal transport of tritiated folic acid (^{3}H-PGA) in the everted gut sac of different aged rats.
Hopkins Med. J. 135, 152 - 160.

Bianchi, A., Chipman, D.W., Dreskin, A. and Rosensweig, N.S. (1970).
Nutritional folic acid deficiency with megaloblastic changes in the small-bowel epithelium.
New. Engl. J. Med. 282, 859 - 861.

Binkley, S.B., Bird, O.D., Bloom, E.S., Brown, R.A., Calkins, D.G., Campbell, C.J., Emmett, A.D. and Pfiffner, J.J. (1944).
On the vitamin B_c conjugate in yeast.
Science 100, 36 - 37.

Bird, O.D., Binkley, S.B., Bloom, E.S., Emmett, A.D. and Pfiffner, J.J. (1945).
On the enzymic formation of vitamin B_c from its conjugate.
J. Biol. Chem. 157, 413 - 414.

Bird, O.D., Bressler, B., Brown, R.A., Campbell, C.J. and Emmett, A.D. (1945).
The microbiological assay of Vitamin B_c conjugate.
J. Biol. Chem. 159, 631 - 636.

Bird, O.D., McGlohon, V.M. and Vaitkus, J.W. (1965).
Naturally occurring folates in the blood and liver of the rat.
Analyt. Biochem. 12, 18 - 35.

Blair, J.A. (1958).
Isolation of iso-xanthopterin from human urine.
Biochem. J. 68, 385 - 387.

Blair, J.A. (1969).
Utilization of folate in man.
Brit. Med. J. 1, 510 - 511.

Blair, J.A. (1970).
Toxicity of folic acid.
Lancet 1, 360.

Blair, J.A. and Dransfield, E. (1971).
The urinary excretion of orally administered pteroyl-L-glutamic acid by the rat.
Biochem. J. 123, 907 - 914.

Blair, J.A., Johnson, I.T., and Matty, A.J. (1974a).
Absorption of folic acid by everted segments of rat jejunum.
J. Physiol. 236, 653 - 661.

Blair, J.A., Johnson, I.T. and Matty, A.J. (1974b).
Stimulated efflux of folic acid from the mucosal epithelium of the rat *in vitro*.
Life Sci.15, 819 - 824.

Blair, J.A., Lucas, M.L. and Matty, A.J. (1972).
The acidification process of the jejunum.
Gut 13, 321.

Blair, J.A., Lucas, M.L. and Matty, A.J. (1975).
Acidification in the rat proximal jejunum.
J. Physiol. 245, 333 - 350.

Blair, J.A. and Matty, A.J. (1974).
Acid microclimate in intestinal absorption. Intestinal absorption of folic acid and 5-methyltetrahydrofolic acid.
Clin. Gastroenterol. 3, 183 - 197.

Blair, J.A. and Pearson, A.J. (1974).
Kinetics and mechanism of the autoxidation of the 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines.
J. Chem. Soc. Perkin Transactions II. No. 1, 80 - 88.

Blair, J.A., Pearson, A.J. and Robb, A.J. (1974).
The autoxidation of 5-methyltetrahydrofolic acid.
J. Chem. Soc. Perkin Transactions II. In Press.

Blair, J.A. and Saunders, K.J. (1970).
A convenient method for the preparation of dl-5-methyl-tetrahydrofolic acid (dl-5-methyl-5,6,7,8,-tetrahydro-pteroyl-L-monoglutamic acid).
Analyt. Biochem. 34, 376 - 381.

Blakley, R.L. (1957).
The interconversion of serine and glycine: some further properties of the enzyme system.
Biochem. J. 65, 342 - 348.

Blakley, R.L. (1959).
The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde.
Biochem. J. 72, 707 - 715.

Blakley, R.L. (1960).
Spectrophotometric studies on the combination of formaldehyde with tetrahydropteroyl-glutamic acid and other hydropteridines.
Biochem. J. 74, 71 - 82.

Blakley, R.L. (1969).
The Biochemistry of folic acid and related pteridines.
Amsterdam and London. North-Holland Publishing Co.
569 pages.

Bobzien, W.F. and Goldman, I.D. (1972).
The mechanism of folate transport in rabbit reticulocytes.
J. Clin. Invest. 51, 1688 - 1696.

Bond, T.J., Bardos, T.J., Sibley, M. and Shive, W. (1949).
The folinic acid group. A series of new vitamins related to folic acid.
J. Am. Chem. Soc. 71, 3852 - 3853.

Booth, C.C. (1967).
Sites of absorption in the small intestine.
Fed. Proc. 26, 1583 - 1588.

Broquist, H.P. and Albrecht, A.M. (1955).
Pteridines and the nutrition of the protozoon Crithidia fasciculata.
Proc. Soc. Exptl. Biol. Med. 89, 178 - 180.

Brown, J.P., Davidson, G.E. and Scott, J.M. (1974).
The identification of the forms of folate found in the liver, kidney and intestine of the monkey and their biosynthesis from exogenous pteroylglutamate (folic acid).
Biochim. Biophys. Acta. 343, 78 - 88.

Brown, S.S., Neal, G.E. and Williams, D.C. (1965).
Subcellular distribution of some folic acid linked enzymes in rat liver.
Biochem. J. 97, 34c - 36c.

Brown, J.P., Scott, J.M., Foster, F.G. and Weir, D.G. (1973)
Ingestion and absorption of naturally occurring pteroyl-monoglutamates (folates) in man.
Gastroenterol. 64, 223 - 232.

Burchall, J.G. (1973).
Mechanism of action of trimethoprim-sulfamethoxazole - II.
J. Infect. Dis. 128 (Suppl), S 437 - S 441.

Burgen, A.S.V. and Goldberg, N.J. (1962).
Absorption of folic acid from the small intestine of the rat.
Brit. J. Pharmacol. 19, 313 - 320.

Butterworth, C.E. Jr. (1968).
Absorption and malabsorption of dietary folate.
Am. J. Clin. Nutr. 21, 1121 - 1127.

Butterworth, C.E. Jr., Baugh, C.M. and Krumdieck, C. (1969).
A study of folate absorption and metabolism in man utilizing carbon-14-labelled polyglutamates synthesized by solid phase method.
J. Clin. Invest. 48, 1131 - 1142.

Butterworth, C.E. Jr., Santini, R. Jr. and Frommeyer, W.B. Jr. (1963).
The pteroylglutamate components of American diets as determined by chromatographic fractionation.
J. Clin. Invest. 42, 1929 - 1939.

Caldwell, P.C. (1958).
Studies on the internal pH of large muscle and nerve fibres.
J. Physiol. 142, 22 - 62.

Chanarin, I. (1969).
The megaloblastic anaemias.
Blackwell, Oxford and Edinburgh 700 pages.

Chanarin, I., Anderson, B.B. and Mollin, D.L. (1958).
The absorption of folic acid.
Brit. J. Haemat. 4, 156 - 166.

Chanarin, I. and Bennett, M.C. (1962).
Absorption of folic acid and D-Xylose as tests of intestinal function.
Brit. Med. J. 1, 985 - 989.

Chanarin, I. and Perry, J. (1969).
Evidence for reduction and methylation of folate in the intestine during normal absorption.
Lancet 2, 776 - 778.

Chanarin, I., Perry, J. and Reynolds, E.H. (1974).
Transport of 5-methyltetrahydrofolic acid into the cerebrospinal fluid in man.
Clin. Sci. and Mol. Med. 46, 369 - 373.

Chanarin, I., Rothman, D., Perry, J. and Stratfull, D.(1968).
Normal dietary folate, iron, and protein intake, with particular reference to pregnancy.
Brit. Med. J. 2, 394 - 397.

Chello, P.L. and Bertino, J.R. (1973).
Dependence of 5-methyltetrahydrofolate utilization by L5178Y Murine leukemia cells *in vitro* in the presence of hydroxycobalamin and transcobalamin II.
Cancer Res. 33, 1898 - 1904.

Chen, Chi-Po and Wagner, C. (1974).
Transport of folic acid (FA) and 5-methyltetrahydrofolic acid (5MTHF) in the isolated choroid plexus.
Fed. Proc. 33, 716 (abstr).

Chida, N., Hirono, H. and Arakawa, T. (1972).
Effects of dietary folate deficiency on fatty acid composition of myelin cerebroside in growing rats.
Tohoku, J. Exp. Med. 108, 219 - 224.

Clark, S.L., Jr. (1953)
Oral folic acid tolerance test in normal human subjects and patients with pernicious anaemia.
Proc. Soc. Exptl. Biol. Med. 82, 25 - 28.

Clarke, H.C. (1973).
In pregnancy: Effect of iron and folic acid on riboflavin status.
Internat. J. Vit. Nutr. Res. 43, 438 - 441.

Cohen, N. (1965).
Differential microbiological assay in study of folic acid absorption *in vitro* by everted intestinal sacs.
Clin. Res. 13, 242 (Abstr).

Cohen, N., Gelb, A. and Sobotka, H. (1964).
Intestinal absorption of folic acid *in vitro*.
Clin. Res. 12, 206.

Cooke, W.T., Cox, E.V., Fone, D.J., Meynell, M.J. and Gaddie, R. (1963).
The clinical and metabolic significance of jejunal diverticula.
Gut. 4, 115 - 131.

Cooke, W.T., Cox, E.V., Gaddie, R., Matthews, D.M., and Meynell, M.J. (1959).
The vitamin B_{12}, folic acid and ascorbic acid content of human bone marrow cells.
J. Physiol. 145, 35P - 36P.

Cooper, B.A. (1970).
Studies of 3H-folic acid uptake by Lactobaccilus casei.
Biochim. Biophys. Acta. 208, 99 - 109.

Cooperman, J.M. and Luhby, A.L. (1965).
The physiological fate in man of some naturally occurring polyglutamates of folic acid.
Israel. J. Med. Sci. 1, 704 - 707.

Corcino, J.J., Waxman, S. and Herbert, V. (1971).
Uptake of tritiated folates by human bone marrow cells in vitro.
Brit. J. Haemat. 20, 503 - 509.

Corrocher, R., Bhuyan, B.K. and Hoffbrand, A.V. (1972).
Composition of pteroylpolyglutamates (conjugated folates) in guinea-pig liver, and their formation from folic acid.
Clin. Sci. 43, 799 - 813.

Corrocher, R., De Sandre, G., Pacor, M.L. and Hoffbrand, A.V. (1974).
Hepatic protein binding of folate.
Clin. Sci. Mol. Med. 46, 551 - 554.

Cowperthwaite, J., Weber, M.M., Packer, L. and Hutner, S.H. (1953).
Nutrition of Herpetomonas (Strigomonas) culicidarum.
Ann. N.Y. Acad. Sci. 56, 972 - 981.

Cox, E.V., Meynell, M.J., Cooke, W.T. and Gaddie, R., (1958).
The folic acid excretion test in the steatorrhoea syndrome.
Gastroenterol. 35, 390 - 397.

Crane, R.K., and Mandelstam, P. (1960).
The active transport of sugars by various preparations of hamster intestine.
Biochim. Biophys. Acta 45, 460 - 476.

Cross, R.J., Taggart, J.V., Covo, G.A. and Green, D.E. (1949).
Studies on the cyclophorase system. VI. The coupling of oxidation and phosphorylation.
J. Biol. Chem. 177, 655 - 678.

Darzynkiewicz, Z., Rogers, A.W., Barnard, E.A., Wang, Dah-Hsi and Werkheiser, W.C. (1966).
Autoradiography with tritiated methotrexate and the cellular distribution of folate reductase.
Science 151, 1528 - 1530.

Das, K.C. and Hoffbrand, A.V. (1969).
Folate uptake by lymphocytes.
Brit. J. Haemat. 17, 613 - 614.

Davis, R.E. and Jackson, J.M. (1973).
Trimethoprim/Sulphamethoxazole and folate metabolism.
Pathology 5, 23 - 29.

Davis, B.D. and Mingioli, E.S. (1950).
Mutants of Escherichia coli requiring methionine or vitamin B_{12}.
J. Bacteriol. 60, 17 - 28.

Davison, H. and Danielli, J.F. (1948).
The permeability of natural membranes PP313-314.
Cambridge; Cambridge University Press.

Day, P.L., Langston, W.C. and Darby, W.J. (1938)
Failure of nicotinic acid to prevent nutritional cytopenia in the monkey.
Proc. Soc. Exptl. Biol. Med. 38, 860 - 863.

DeGrazia, J.A., Fish, M.B., Pollycove, M., Wallerstein, R.O. and Hollander, L. (1972).
The oxidation of the beta carbon of serine in human folate and vitamin B_{12} deficiency.
J. Lab. Clin. Med. 80, 395 - 404.

Denko, C.W. (1951).
Pteroylglutamic acid clearance in normal adults.
J. Appl. Physiol. 3, 559 - 562.

Denko, C.W., Grundy, W.E., Wheeler, N.C., Henderson, C.R., Berryman, G.H., Friedemann, T.E. and Youmans, J.B. (1946).
The excretion of B-complex vitamins by normal adults on a restricted intake.
Arch. Biochem. 11, 109 - 117.

Dickens, F. and Weil Malherbe, H. (1941).
Metabolism of normal and tumour tissue.
The metabolism of intestinal mucous membrane.
Biochem. J. 35, 7 - 15.

Diez-Ewald, M. and Molina, R.A. (1972).
Iron and folic acid deficiency during pregnancy in Western Venezuela.
Am. J. Trop. Med. Hyg. 21, 587 - 591.

Dinda, P.K. and Beck, I.T. (1969).
The effect of local anaesthetics on the transmural potential difference of the small intestine of the rat.
Canad. J. Physiol. Pharmacol. 47, 445 - 451.

Doig, A. and Girdwood, R.H. (1960).
The absorption of folic acid and labelled cyanocobalamin in intestinal malabsorption.
Quart. J. Med. 29, 333 - 374.

Donaldson, K.O. and Keresztesy, J.C. (1959a).
Naturally occurring forms of folic acid - I "Prefolic A": Preparation of concentrate and enzymatic conversion to citrovorum factor.
J. Biol. Chem. 234,3235-3240.

Donaldson, K.O. and Keresztesy, J.C. (1959b).
Enzymatic formation of citrovorum factor in autolyzing liver.
Fed. Proc. 18, 216 (abstr).

Donaldson, K.O. and Keresztesy, J.C. (1961a).
Further evidence on the nature of prefolic A.
Biochem. Biophys. Res. Comm. 5, 289 - 292.

Donaldson, K.O. and Keresztesy, J.C. (1961b)
The interconversion of prefolic acid A and tetrahydrofolic acid.
Fed. Proc. 20, 453 (abstr).

Donaldson, K.O. and Keresztesy, J.C. (1962).
Naturally occurring forms of folic acid II Enzymatic conversion of methylenetetrahydrofolic acid to prefolic A-methyltetrahydrofolate.
J. Biol. Chem. 237, 1298 - 1304.

Ellegaard, J. and Esmann, V. (1973).
Folate activity of human lymphocytes determined by measurement of serine synthesis.
Scand. J. Clin. Lab. Invest. 31, 9 - 19.

Elsborg, L. (1971).
Jejunal pH and folic acid.
Brit. Med. J. 2, 340.

Elsborg, L. (1972).
Binding of folic acid to human plasma proteins.
Acta Haemat 48, 207 - 212.

Elsborg, L. (1974)
Folic acid: A new approach to the mechanism of its intestinal absorption.
Dan. Med. Bull 21, 1 - 11.

Evans, E.M., Wrigglesworth, J.M., Burdett, K. and Pover, W.F.R. (1971).
Studies on epithelial cells isolated from guinea pig small intestine.
J. Cell Biol. 51, 452 - 464.

Fehling, C., Jägerstad, M., Lindstrand, K. and Westesson, A. (1973).
The effect of anti-convulsant therapy upon the absorption of folates.
Clin. Sci. 44, 595 - 600.

Fisher, R.B. and Parsons, D.S. (1949)
A preparation of surviving rat small intestine for the study of absorption.
J. Physiol. 110, 36 - 46.

Fleischner, G., Robbins, J. and Arias, I.M. (1972)
Immunological studies of Y protein: A major cytoplasmic organic anion-binding protein in rat liver.
J. Clin. Invest. 51, 677 - 684.

Ford, J.E., Knaggs, G.S., Salter, D.N. and Scott, K.J. (1972).
Folate nutrition in the kid.
Brit. J. Nutr. 27, 571 - 583.

Forshaw, J. (1969).
Effect of vitamin B_{12} and folic acid deficiency on small intestinal absorption.
J. Clin. Path. 22, 551 - 553.

Fox, K.R. and Hogben, C.A.M. (1974).
Nicotinic acid active transport by *in vitro* bullfrog small intestine.
Biochim. Biophys. Acta 332, 336 - 340.

Foxall, C.D. (1967).
Ph.D. Thesis. University of Aston in Birmingham.
Part 1. The metabolism of folic acid.
(Pteroyl-L-glutamic acid).
Part 2. The synthesis and tumour inhibitory properties of simple hydrazines.

Frank, O., Baker H. and Sobotka, H. (1963).
Blood and serum levels of water soluble vitamins in man and animals.
Nature 197, 490 - 491.

Franklin, J.L. and Rosenberg, I.H. (1973).
Impaired folic acid absorption in inflammatory bowel disease: effects of salicylazosulfapyridine (azulfidine).
Gastroenterol. 64, 517 - 525.

Fridhandler, L. and Quastel J.H. (1955).
Absorption of sugars from isolated surviving intestine.
Arch. Biochem. Biophys. 56, 412 - 423.

Fukushima, T. and Shiota, T. (1972).
Pterins in human urine.
J. Biol. Chem. 247, 4549 - 4556.

Gawthorne, J.M. and Smith, R.M. (1973).
The synthesis of pteroylpolyglutamates by sheep liver enzymes *in vitro*.
Biochem. J. 136, 295 - 301.

Ghitis, J., Mandelbaum-Shavit, F. and Grossowicz, N. (1969).
Binding of folic acid and derivatives by milk.
Am. J. Clin. Nutr. 22, 156 - 162.

Gilvarg, C. and Katchalski, E. (1965).
Peptide utilization in *Escherichia coli*.
J. Biol. Chem. 240, 3093 - 3098.

Girardi, A.J., McMichael, H. and Henle, W. (1956).
The use of HeLa cells in suspension for the quantitative study of virus propagation.
Virology 2, 532 - 544.

Goldman, I.D. (1971).
A model system for the study of heteroexchange diffusion: Methotrexate-folate interactions in L_{1210} leukemia and Ehrlich ascites tumour cells.
Biochim. Biophys. Acta 233, 624 - 634.

Goldman, I.D., Lichtenstein, N.S., and Oliverio, V.T. (1968)
Carrier-mediated transport of the folic acid analogue, methotrexate, in the L_{1210} leukemia cell.
J. Biol. Chem. 243, 5007 - 5017.

Goldman, R., Kedem, O. and Katchalski, E. (1968b).
Papain-collodion membrane II. Analysis of the kinetic behaviour of enzymes immobilised in artificial membranes.
Biochemistry 7, 4518 - 4532.

Goldman, R., Kedem, O., Silman, I.H., Caplan, S.R. and Katchalski, E. (1968a).
Papain-collodion membranes I. Preparation and properties.
Biochemistry 7, 486 - 500.

Goldman, R., Silman, H.I., Caplan, S.R., Kedem, O. and Katchalski, E. (1965).
Papain membrane on the collodion matrix:
Preparation and enzymic behaviour.
Science 150, 758 - 760.

Goresky, C.A., Watanabe, H. and Johns, D.G. (1963).
The renal excretion of folic acid.
J. Clin. Invest. 42, 1841 - 1849.

Gough, K.R., Read, A.E., McCarthy, C.F. and Waters, A.H. (1963).
Megaloblastic anaemia due to nutritional deficiency of folic acid.
Quart. J. Med. 32, 243 - 256.

Grossowicz, N., Izak, G. and Rachmilewitz, M. (1964).
Folate activity in tissues of rats on folate-free diets.
Proc. Soc. Exptl. Biol. Med. 115, 953 - 957.

Grossowicz, N., Rachmilewitz, M. and Izak, G. (1963).
Folate activity in rat tissue before and after pteroylglutamic acid load.
Proc. Soc. Exptl. Biol. Med. 112, 486 - 489.

Grossowicz, N., Rachmilewitz, M. and Izak, G. (1972).
Absorption of pteroylglutamate and dietary folates in man.
Am. J. Clin. Nutr. 25, 1135 - 1139.

Grossowicz, N., Rachmilewitz, M., Izak, G. and Galewski, K. (1973).
Absorption of liver folates from small intestinal segments of the rat.
Proc. Soc. Exptl. Biol. Med. 142, 387 - 389.

Gutstein, S., Bernstein, L.H., Levy, L. and Wagner, G.(1973).
Failure of response to N^5-methyltetrahydrofolate in combined folate and B_{12} deficiency.
Evidence in support of the "Folate Trap" hypothesis.
Am. J. Dig. Dis. 18, 142 - 146.

Hakala, M.T. (1965).
On the nature of permeability of sarcoma-180 cells to amethopterin *in vitro*.
Biochim. Biophys. Acta 102, 210 - 225.

Halsted, C.H., Bhantumnavin, K. and Mezey, E. (1974).
Jejunal uptake of tritiated folic acid in the rat studied by *in vivo* perfusion.
J. Nutr. 104, 1674 - 1680.

Halsted, C.H., Griggs, R.C. and Harris, J.W. (1967).
The effect of alcoholism on the absorption of folic acid (^{3}H-PGA) evaluated by plasma levels and urine excretion.
J. Lab. Clin. Med. 69, 116 - 131.

Halsted, C.H., and Mezey, E. (1972)
Mechanism of absorption of labelled folic acid (^{3}H-PGA) *in vivo* studies in the rat.
Clin. Res. 20, 455 (Abstr.)

Halsted, C.H., Robles, E.A. and Mezey, E. (1971).
Decreased jejunal uptake of labelled folic acid (^{3}H-PGA) in alcoholic patients: Roles of alcohol and nutrition.
New Engl. J. Med. 285, 701 - 706.

Halsted, C.H., Robles, E.A. and Mezey, E. (1973).
Intestinal malabsorption in folate-deficient alcoholics.
Gastroenterol. 64, 526 - 532.

Halsted, C.H., Sourial, N., Guindi, S., Mourad, K.A.H., Kattab, A.K., Carter, J.P. and Patwardhan, V.N. (1969).
Anaemia of Kwashiorkor in Cairo: Deficiencies of protein, iron and folic acid.
Am. J. Clin. Nutr. 22, 1371 - 1382.

Harrer, D.S., Stern, B.K. and Reilly, R.W. (1964).
Removal and dissociation of epithelial cells from the rodent gastrointestinal tract.
Nature 203, 319 - 320.

Harrison, D.D. and Webster, H.L. (1969).
The preparation of isolated intestinal crypt cells.
Exptl. Cell. Res. 55, 257 - 260.

Hatch, F.T., Larrabee, A.R., Cathou, R.E. and Buchanan, J.M. (1961).
Enzymatic synthesis of the methyl group of methionine I. Identification of the enzymes and cofactors involved in the system isolated from Escherichia coli.
J. Biol. Chem. 236, 1095 - 1101.

Helbock, H.J. (1968).
Folic acid absorption in man.
Lancet 2, 775 - 776.

Henderson, G.B. and Huennekens, F.M. (1974).
Transport of folate compounds into Lactobacillus casei.
Arch. Biochem. Biophys. 164, 722 - 728.

Hepner, G.W. (1969).
The absorption of pteroylglutamic (folic) acid in rats.
Brit. J. Haemat. 16, 241 - 249.

Hepner, G.W., Aledort, L.M., Gerson, C.D., Cohen, N., Herbert, V. and Janowitz, H.D. (1970).
Inhibition of intestinal ATPase by diphenylhydantoin and acetazolamide.
Clin. Res. 18, 382 (abstr).

Hepner, G.W., Booth, C.C., Cowan, J., Hoffbrand, A.V. and Mollin, D.L. (1968).
Absorption of crystalline folic acid in man.
Lancet, 2, 302 - 306.

Hepner, G.W. and Herbert, V. (1969).
In vitro studies with suspensions of intact intestinal cells: Uptake of pteroylglutamic acid by guinea pig mucosa.
Clin. Res. 17, 112 (abstr).

Herbert, V. (1968).
Nutritional requirements for vitamin B_{12} and folic acid.
Am. J. Clin. Nutr. 21, 743 - 752.

Herbert, V., Larrabee, A.R. and Buchanan, J.M. (1962).
Studies on the identification of a folate compound of human serum.
J. Clin. Invest. 41, 1134 - 1138.

Herbert, V. and Shapiro, S.S. (1962).
The site of absorption of folic acid in rat in vitro.
Fed. Proc. 21, 260 (abstr).

Herbert, V. and Zalusky, R. (1961).
Selective concentration of folic acid activity in cerebro-spinal fluid.
Fed. Proc. 20, 453 (abstr).

Herbert, V. and Zalusky, R. (1962).
Interrelations of vitamin B_{12} and folic acid and folic acid metabolism: folic acid clearance studies.
J. Clin. Invest. 41, 1263 - 1276.

Hibbard, B.M. and Hibbard, E.D. (1968).
Folate metabolism and reproduction.
Brit. Med. Bull. 24, 10 - 14.

Hitchings, G.H. (1973).
Mechanism of action of trimethoprim-sulfamethoxazole - I.
J. Infect. Dis. 128 (suppl) S433 - S436.

Höber, R. (1945).
Physical Chemistry of Cells and Tissues.
The Blakistan Co. Philadelphia, Pa. 676 pages.

Hoffbrand, A.V. (1971).
Folate absorption.
J. Clin. Path. 24, Suppl. (Royal Coll. Path.) 5, 66 - 76.

Hoffbrand, A.V., Ganeshaguru, K., Lavoie, A., Tattersall, M.H.N. and Tripp, E. (1974).
Thymidylate concentration in megaloblastic anaemia.
Nature 248, 602 - 604.

Hoffbrand, A.V. and Necheles, T.F. (1968).
Mechanism of folate deficiency in patients receiving phenytoin.
Lancet. 2, 528 - 530.

Hoffbrand, A.V., Necheles, T.F., Maldonado, N., Horta, E. and Santini, R. (1969).
Malabsorption of folate polyglutamates in tropical sprue.
Brit. Med. J. 2, 543 - 547.

Hoffbrand, A.V. and Peters, T.J. (1969).
The subcellular localisation of pteroyl polyglutamate hydrolase and folate in guinea pig intestinal mucosa.
Biochim. Biophys. Acta 192, 479 - 485.

Hoffbrand, A.V. and Peters, T.J. (1970).
Recent advances in knowledge of chemical and biochemical aspects of folate.
Schweiz. Med. Wschr. 100, 1954 - 1960.

Hoffbrand, A.V., Stewart, J.S., Booth, C.C. and Mollin, D.L. (1968).
Folate deficiency in Crohn's disease: Incidence, pthogenesis, and treatment.
Brit. Med. J. 2, 71 - 75.

Hoffbrand, A.V., Tabaqchali, S., Booth, C.C. and Mollin, D.L. (1971).
Small intestinal bacterial flora and folate status in gastrointestinal disease. Gut. 12, 27 - 33.

Hoffbrand, A.V. and Tripp, E. (1972).
Unbalanced deoxyribonucleotide synthesis caused by methotrexate.
Brit. Med. J. 2, 140 - 142.

Hoffbrand, A.V., Tripp, E., Houlihan, C.M. and Scott, J.M. (1973).
Studies on the uptake of synthetic conjugated folates by human marrow cells.
Blood 42, 141 - 146.

Hogan, A.G. and Parrott, E.M. (1940).
Anaemia in chicks caused by a vitamin deficiency.
J. Biol. Chem. 132, 507 - 517.

Hogben, C.A.M. (1960).
The first common pathway.
Fed. Proc. 19, 864 - 869.

Hogben, C.A.M., Tocco, D.J., Brodie, B.B. and Schanker, L.S. (1959).
On the mechanism of intestinal absorption of drugs.
J. Pharmacol. Exptl. Therap. 125, 275 - 282.

Hopkins, F.G. (1889).
Note on a yellow pigment in butterflies.
Nature 40, 335.

Houlihan, C.M. and Scott, J.M. (1972).
The identification of pteroylpentaglutamate as the major derivative in rat liver and the demonstration of its biosynthesis from exogenous 3H-pteroylglutamate.
Biochem. Biophys. Res. Comm. 48, 1675 - 1681.

Houlihan, C.M., Scott, J.M., Boyle, P.H. and Weir, D.G. (1972).
The effect of phenytoin on the absorption of synthetic folic acid polyglutamate.
Gut 13, 189 - 190.

Huang, K.C. (1965).
Uptake of L-tyrosine and 3-O-methylglucose by isolated intestinal epithelial cells.
Life-Sci. 4, 1201 - 1206.

Hunter, R., Barnes, J., Oakeley, H.F. and Matthews, D.M. (1970).
Toxicity of folic acid given in pharmacological doses to healthy volunteers.
Lancet 1, 61 - 63.

Hurdle, A.D.F., Barton, D., and Searles, I.H. (1968).
A method for measuring folate in food and its application to a hospital diet.
Am. J. Clin. Nutr. 21, 1202 - 1207.

Hutchings, B.L., Bohonos, N. and Peterson, W.H. (1941).
Growth factors for bacteria: XIII Purification and properties of an eluate factor required by certain lactic acid bacteria.
J. Biol. Chem. 141, 521 - 528.

Iemhoff, W.G.J., Van Den Berg, J.W.O., De Pijper, A.M. and Hulsmann, W.C. (1970).
Metabolic aspects of isolated cells from rat small intestinal epithelium.
Biochim. Biophys. Acta 215, 229 - 241.

Ito, S. (1965).
The enteric surface coat on cat intestinal microvilli.
J. Cell. Biol. 27, 475 - 491.

Ito, S. (1969).
Structure and function of the glycocalyx.
Fed. Proc. 28, 12 - 25.

Izak, G., Galevski, K., Grossowicz, N., Jablonska, M. and Rachmilewitz, M. (1972).
Studies on folic acid absorption in the rat. II. The absorption of crystalline pteroylglutamic acid from selected small intestine segments.
Am. J. Dig. Dis. 17, 599 - 602.

Izak, G., Levy, S.H., Rachmilewitz, M. and Grossowicz, N. (1973).
The effect of iron and folic acid therapy on combined iron and folate deficiency anaemia: The results of a clinical trial.
Scand. J. Haemat. 11, 236 - 240.

Jacob, E. and Herbert, V. (1974).
Evidence against transferrin as a binder of either vitamin B_{12} or folic acid.
Blood 43, 767 - 768.

Jacquez, J.A. (1966).
Permeability of Ehrlich ascites cells to folic acid, aminopterin and amethopterin.
Cancer Res. 26, 1616 - 1624.

Jandl, J.H. and Gabuzda, C.J. Jr. (1953).
Potentiation of pteroylglutamic acid by ascorbic acid in anaemia of scurvy.
Proc. Soc. Exptl. Biol. Med. 84, 452 - 455.

Jandl, J.H. and Lear, A.A. (1956).
The metabolism of folic acid in cirrhosis.
Ann. Int. Med. 45, 1027 - 1044.

Jeejeebhoy, K.N., Desai, H.G., Borkar, A.V., Deshpande, V. and Pathare, S.M. (1968).
Tropical malabsorption syndrome in West Indians.
Am. J. Clin. Nutr. 21, 994 - 1006.

Johns, D.G., and Plenderleith, I.H. (1963).
Folic acid displacement in man.
Biochem. Pharmacol. 12, 1071 - 1074.

Johns, D.G., Sperti, S. and Burgen, A.S.V. (1961).
The metabolism of tritiated folic acid in man.
J. Clin. Invest. 40, 1684 - 1695.

Johnson, I.T. (1974).
Transport of folic acid and related compounds across the small intestine.
Ph.D. Thesis. University of Aston in Birmingham.

Jukes, T.H. (1955)
Assay of compounds with folic acid activity.
Meth. Biochem. Anal. 2, 121 - 151.

Kalberer, F. and Rutschmann, J. (1961).
Eine Schnellmethode zur Bestimmung von Tritium, Radiokohlenstoff und Radioschwefel in beliebigem organischem probenmaterial mettels des Flüssigkeits-Scintillations-Zählers.
Helv. Chim. Acta. 44, 1956 - 1966.

Kallen, R.G. and Jencks, W.P. (1966).
The dissociation constants of tetrahydrofolic acid.
J. Biol. Chem. 241, 5845 - 5850.

Katz, M. (1973)
Potential danger of self medication with folic acid.
New Engl. J. Med. 289, 1095.

Kaufman, S. (1958a).
A new cofactor required for the enzymatic conversion of phenylalanine to tyrosine.
J. Biol. Chem. 230, 931 - 939.

Kaufman, S. (1958b).
The participation of tetrahydrofolic acid in the enzymic conversion of phenylalanine to tyrosine.
Biochim. Biophys. Acta. 27, 428 - 429.

Kaufman, S. (1963).
The structure of the phenyalanine hydroxylation cofactor.
Proc. Nat. Acad. Sci. U.S. 50, 1085 - 1093.

Kaufman, S. (1967).
Pteridine cofactors.
Ann. Rev. Biochem. 36, 171 - 184.

Kennedy, J.F. (1973).
Chemical aspects of glycoproteins, proteoglycans, and carbohydrate-protein complexes of human tissues.
Chem. Soc. Rev. 2, 355 - 395.

Keresztesy, J.C. and Donaldson, K.O. (1961).
Synthetic prefolic A.
Biochem. Biophys. Res. Comm. 5, 286 - 288.

Keresztesy, J.C. and Silverman, M. (1951).
Crystalline citrovorum factor from liver.
J. Am. Chem. Soc. 73, 5510.

Khalil, M., Tanios, A., Moghazy, M., Aref, M.K., Mahmoud, S. and ElLozy, M. (1973).
Serum and red cell folates, and serum vitamin B_{12} in protein calorie malnutrition.
Arch. Dis. Childh. 48, 366 - 369.

Kidder, G.W., Dewey, V.C. and Rembold, H. (1967).
The origin of unconjugated pteridines in Crithidia fasciculata.
Arch. Microbiol. 59, 180 - 184.

Kidder, G.W., and Dutta, B.N. (1958).
The growth and nutrition of Crithidia fasciculata.
J. Gen. Microbiol. 18, 621 - 638.

Kimmich, G.A. (1970).
Preparation and properties of mucosal epithelial cells isolated from small intestine of the chicken.
Biochemistry 9, 3659 - 3668.

King, C.G. (1968).
Present knowledge of ascorbic acid (Vitamin C).
Nutr. Rev. 26, 33 - 36.

Kinnear, D.G., Johns, D.G., MacIntosh, P.C., Burgen, A.S.V. and Cameron, D.G. (1963).
Intestinal absorption of tritium labelled folic acid in idiopathic steatorrhea. Effect of a gluten-free diet.
Canad. Med. Ass. J. 89, 975 - 979.

Klipstein, F.A. (1963).
The urinary excretion of orally administered tritium labelled folic acid as a test of folic acid absorption.
Blood 21, 626 - 639.

Klipstein, F.A. (1967).
Intestinal folate conjugase activity in tropical sprue.
Am. J. Clin. Nutr. 20, 1004 - 1009.

Klipstein, F.A. (1968).
Tropical Sprue.
Gastroenterol. 54, 275 - 293.

Klipsten, F.A. (1972).
Folate in tropical sprue.
Brit. J. Haemat. 23 (Suppl) 119 - 133.

Klipstein, F.A., Lipton, S.D. and Schenk, E.A. (1973).
Folate deficiency of the intestinal mucosa.
Am. J. Clin. Nutr. 26, 728 - 737.

Koschara, W. (1936).
Isolierung eines gelben Farbstoffs (Uropterin) aus Menschenharn.
Zeitschrift fur Physiolog. Chemie. 240, 127 - 151.

Krebs, H.A. and Henseleit, K. (1932).
Untersuchungen über die harnstoffbildung im tierkörper.
Zeitschrift fur Physiolog Chemie. 210, 33 - 66.

Kunze, H., Rehbock, G. and Vogt, W. (1972).
Absorption of salicylic acid and its isomers from the rat jejunum.
Naunyn-Schmiedegberg's Arch. Pharmacol 273, 331 - 340.

Landon, M.J. and Hey, E.N. (1974).
Renal loss of folate in the newborn infant.
Arch. Dis. Childh. 49, 292 - 296.

Langston, W.C., Darby, W.J., Shukers, C.F. and Day, P.L. (1938).
Nutritional cytopenia (Vitamin M deficiency) in the monkey.
J. Exptl. Med. 68, 923 - 940.

Lanzkowsky, P., Erlandson, M.E. and Bezan, A.I. (1969).
Isolated defect of folic acid absorption associated with mental retardation and cerebral calcification.
Blood 34, 452 - 465.

Larrabee, A.R. and Buchanan, J.M. (1961).
A new intermediate of methionine biosynthesis.
Fed. Proc. 20, 9 (abstr).

Larrabee, A.R., Rosenthal, S., Cathou, R.E. and Buchanan, J.M. (1961).
A methylated derivative of tetrahydrofolate as an intermediate of methionine biosynthesis.
J. Am. Chem. Soc. 83, 4094 - 4095.

Lavoie, A. and Cooper, B.A. (1974).
Rapid transfer of folic acid from blood to bile in man, and its conversion into folate coenzymes and into a pteroylglutamate with little biological activity.
Clin. Sci. Mol. Med. 46, 729 - 741.

Leblond, C.P. and Bennett, G. (1974).
Elaboration and turnover of cell coat glycoproteins.
The cell surface in development (Chapter 3).
Edited by Moscona, A.A. New York, London, Sydney, Toronto.
334 pages.

Leeming, R.J. and Blair, J.A. (1974).
Crithidia factors in human urine.
Biochem. Med. 11, 122 - 128.

Leeming, R.J., Portman-Graham, H. and Blair, J.A. (1972).
The occurrence of folic acid (pteroyl-L-monoglutamic acid) in human blood serum after small oral doses.
J. Clin. Path. 25, 491 - 493.

Leslie, G.I. and Rowe, P.B. (1972).
Folate binding by the brush border membrane proteins of small intestinal epithelial cells.
Biochemistry 11, 1696 - 1703.

Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1969).
Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein and other anions. J. Clin. Invest. 48, 2156 - 2167.

Levine, P.H. and Weintraub, L.R. (1970).
Preparations of suspensions of small bowel mucosal epithelial cells.
J. Lab. Clin. Med. 75, 1026 - 1029.

Levine, R.R., McNary, W.F., Kornguth, P.J. and Leblanc, R. (1970).
Histological reevaluation of everted gut technique for studying intestinal absorption.
Europ. J. Pharmacol 9, 211 - 219.

Levitt, M., Nixon, P.F., Pincus, J.H. and Bertino, J.R. (1971).
Transport characteristics of folate in cerebrospinal fluid; a study utilizing doubly labelled 5-methyltetrahydrofolate and 5-formyltetrahydrofolate.
J. Clin. Invest. 50, 1301 - 1308.

Lichtenstein, N.S., Oliverio, V.T. and Goldman, I.D. (1969).
Characteristics of folic acid transport in the L1210 leukemia cell.
Biochim. Biophys. Acta 193, 456 - 467.

Liu, Y.K. and Augusta, Ga (1974).
Folate deficiency in children with sickle cell anaemia.
Am. J. Dis. Child. 127, 389 - 393.

Lloyd, T., Mori, T. and Kaufman, S. (1971).
6-methyltetrahydropterin. Isolation and identification as the highly active hydroxylase cofactor from tetrahydrofolate.
Biochemistry 10, 2330 - 2336.

Loh, H.S. and Dempsey, P.M. (1974).
Ascorbic acid, folic acid inter-relationships in geriatric subjects.
J. Irish. Med. Assoc. 67, 247 - 250.

Loomis, W.F. and Lipmann, F. (1948).
Reversible inhibition of the coupling between phosphorylation and oxidation.
J. Biol. Chem. 173, 807 - 808.

Lopez, R., Shimizu, N. and Cooperman, J.M. (1973).
Recurrent folic acid deficiency in sickle cell disease.
Am. J. Dis. Child. 125, 544 - 548.

Lucas, M.L. (1974).
Acidification in the rat proximal jejunum.
Ph.D. Thesis. University of Aston in Birmingham.

Luhby, A.L., Eagle, F.J., Roth, E. and Cooperman, J.M. (1961).
Relapsing megaloblastic anaemia in an infant due to a specific defect in gastro-intestinal absorption of folic acid.
Am. J. Dis. Child. 102, 482 - 483.

Luther, L., Santini, R., Brewster, C., Perez-Santiago, E. and Butterworth, C.E. Jr. (1965).
Folate binding by insoluble components of American and Puerto-Rican diets.
Ala. J. Med. Sci. 2, 389 - 393.

Mandelbaum-Shavit, F. and Grossowicz, N. (1970).
Transport of folinate and related compounds in
Pediococcus cerevisiae.
J. Bacteriol. 104, 1 - 7.

Mandelbaum-Shavit, F. and Grossowicz, N. (1973).
Carrier-mediated transport of folate in a mutant of
Pediococcus cerevisiae.
J. Bacteriol. 114, 485 - 490.

Mansouri, A., Decter, J.B. and Silber, R. (1972).
Studies on the regulation of one carbon metabolism:
Repression-derepression of serine hydroxymethyltransferase
by methionine in Escherichia Coli.
J. Biol. Chem. 247, 348 - 352.

Mantzos, J.D., Alevizou-Terzaki, V. and Gyftaki, E. (1974).
Folate binding in animal plasma.
Acta. Haemat. 51, 204 - 210.

Marchetti, M., Landi, L. and Pasquali, P. (1965).
Interrelationships between biotin and folic acid.
The distribution of folic acid-active substances in the
liver of biotin deficient rats.
Biochim. Biophys. Acta. 97, 356 - 358.

Markkanen, T. (1968).
Pteroylglutamic acid (PGA) activity of serum in gel
filtration.
Life. Sci. 7, 887 - 895.

Markkanen, T., Himanen, P. and Pajula, R.L. (1974a).
Binding of folic acid to serum proteins: The effect of pernicious anaemia.
Acta. Haemat. 51, 193 - 203.

Markkanen, T., Himanen, P., Pajula, R.L. and Molnar, G. (1973b).
Binding of folic acid to serum proteins: The effect of diphenylhydantoin treatment and of various diseases.
Acta. Haemat. 50, 284 - 292.

Markkanen, T., Himanen, P., Pajula, R.L., Ruponen, S. and Castren, O. (1973a).
Binding of folic acid to serum proteins: The effect of pregnancy.
Acta. Haemat. 50, 85 - 91.

Markkanen, T., Pajula, R.L., Virtanen, S. and Himanen, P. (1972a).
New carrier protein(s) of folic acid in human serum.
Acta. Haemat. 48, 145 - 150.

Markkanen, T. and Peltola, O. (1971).
Carrier proteins of folic acid activity in human serum.
Acta. Haemat. 45, 106 - 111.

Markkanen, T., Virtanen, S., Himanen, P. and Pajula, R.L. (1972b).
Transferrin, the third carrier protein of folic acid activity in human serum.
Acta. Haemat. 48, 213 - 217.

Markkanen, T., Virtanen, S., Pajula, R-L. and Himanen, P. (1974b).
Hormonal dependence of folic acid protein binding in human serum.
Internat. J. Vit. Nutr. Res. 44, 81 - 94.

Matoth, Y., Pinkas, A. and Sroka, Ch. (1965).
Studies on folic acid in infancy; III Folates in breast fed infants and their mothers.
Am. J. Clin. Nutr. 16, 356 - 359.

Matthews, D.M. (1966).
Effects of phenobarbitone and folic acid on D-xylose absorption.
J. Physiol. 182, 46p - 47p.

Mattson, R.H., Gallagher, B.B., Reynolds, E.H. and Glass, D. (1973).
Folate therapy in epilepsy.
Arch. Neurol. 29, 78 - 81.

Matty, A.J. and Blair, J.A. (1968).
Folic acid absorption in man.
Lancet 2, 977 - 978.

McClain, R.M. and SieKierka, J.J. (1974).
Methotrexate inhibition of placental folate transport after the administration of folic acid.
Fed. Proc. 33, 565 (abstr).

McLean, F.W., Heine, M.W., Held, B. and Streiff, R.R. (1969).
Relationship between the oral contraceptives and folic acid metabolism. Serum folate concentrations.
Am. J. Obstet. Gynec. 104, 745 - 747.

McLean, F.W., Heine, M.W., Held, B. and Streiff, R.R. (1970).
Folic acid absorption in pregnancy: Comparison of the pteroylpolyglutamate and pteroylmonoglutamate.
Blood 36, 628 - 631.

Melikian, V., Paton, A., Leeming, R.J. and Portman-Graham, H. (1971).
Site of reduction and methylation of folic acid in man.
Lancet 2, 955 - 957.

Metz, J. and Herbert, V. (1967).
Folate binders in milk and human serum; their use in coated charcoal assay of folic acid; their possible physiologic role.
J. Clin. Invest. 46, 1096.

Metz, J., Zalusky, R. and Herbert V. (1968).
Folic acid binding by serum and milk.
Am. J. Clin. Nutr. 21, 289 - 297.

Meynell, M.J. (1966).
Megaloblastic anaemia in anti-convulsant therapy.
Lancet 1, 487.

Mims, V. and Bird, O.D. (1950).
Proteolytic Enzymes. Quoted by Laskowski, M. (1950).
Ann. Rev. Biochem. 19, 21 - 42.

Mishkin, S., Stein, L., Fleischner, G., Gatmaitan, Z. and Arias, I.M. (1973).
On the function of Z protein in the liver.
Gastroenterol. 64, 154 (abstr).

Mishkin, S., Stein, L., Gatmaitan, Z. and Arias, I.M. (1972)
The binding of fatty acids to cytoplasmic proteins:
Binding of Z protein in liver and other tissues of the rat.
Biochem. Biophys. Res. Comm. 47, 997 - 1003.

Mitchell, H.K., Snell, E.E. and Williams, R.J. (1941).
The concentration of 'folic acid'.
J. Am. Chem. Soc. 63, 2284.

Mitchell, H.K., Snell, E.E. and Williams, R.J. (1944).
Folic acid: 1. Concentration from spinach.
J. Am. Chem. Soc. 66, 267 - 268.

Mohiuddin, A. and Olubi, G.O. (1970).
Continuation of biological activity in excised small intestine of the adult white rat.
Acta. Anat. 75, 79 - 91.

Momtazi, S. and Herbert, V. (1973).
Intestinal absorption using vibration-obtained individual small bowel epithelial cells of the rat: folate absorption.
Am. J. Clin. Nutr. 26, 23 - 29.

Moscona, A. (1952).
Cell suspensions from organ rudiments of chick embryos.
Exptl. Cell. Res. 3, 535 - 539.

Mukherjee, T.M. and Williams, A.W. (1967).
A comparative study of the ultrastructure of microvilli in the epithelium of small and large intestine of mice.
J. Cell. Biol. 34, 447 - 461.

Murer, H., Hopfer, U., Kinne-Saffran, E. and Kinne, R. (1974).
Glucose transport in isolated brush-border and lateral-basal plasma-membrane vesicles from intestinal epithelial cells.
Biochim. Biophys. Acta. 345, 170 - 179.

Nahas, A., Nixon, P.F. and Bertino, J.R. (1972).
Uptake and metabolism of N^5-formyltetrahydrofolate by L1210 leukemia cells.
Cancer Res. 32, 1416 - 1421.

Nakamura, M., Pichette, P., Broitman, S., Bezman, A.L., Zamcheck, N. and Vitale, J.J. (1959).
Studies on the inhibitory effect of intestinal mucosa on succinoxidase activity.
J. Biol. Chem. 234, 206 - 210.

Neal, G.E. and Williams, D.C. (1965).
The fate of intravenously injected folate in rats.
Biochem. Pharmacol. 14, 903 - 914.

Neutra, M. and Leblond, C.P. (1966).
Radioautographic comparison of the uptake of galactose-^{3}H and glucose ^{3}H in the golgi region of various cells secreting glycoproteins or mucopolysaccharides.
J. Cell. Biol. 30, 137 - 150.

Newey, H., Smyth, D.H. and Whaler, B.C. (1955).
The absorption of glucose by the *in vitro* intestinal preparation.
J. Physiol. 129, 1 - 11.

Nichol, C.A. (1959).
Selection of bacterial mutants of increased sensitivity to 'aminopterin'.
Nature 183, 550 - 551.

Nichol, C.A. (1968).
Studies on dihydrofolate reductase related to the drug sensitivity of microbial and neoplastic cells.
Advances in enzyme regulation. Pergamon Press Ltd. London. 6, 305 - 322.

Nichol, C.A., and Welch, A.D. (1950).
Synthesis of citrovorum factor from folic acid by liver slices; Augmentation by ascorbic acid.
Proc. Soc. Exptl. Biol. Med. 74, 52 - 55.

Nixon, P.F. and Bertino, J.R. (1972a).
Impaired utilization of serum folate in pernicious anaemia. A study with radiolabelled 5-methyltetrahydrofolate.
J. Clin. Invest. 51, 1431 - 1439.

Nixon, P.F. and Bertino, J.R. (1972b).
Effective absorption and utilization of oral formyltetrahydrofolate in man.
New. Engl. J. Med. 286, 175 - 179.

Nixon, P.F., Slutsky, G., Nahas, A. and Bertino, J.R. (1973).
The turnover of folate coenzymes in Murine lymphoma cells.
J. Biol. Chem. 248, 5932 - 5936.

Nogami, H. and Matsuzawa, T. (1961).
Studies on absorption and excretion of drugs.
I. Kinetics of penetration of acidic drug salicylic acid through the intestinal barrier in vitro.
Chem. Pharm. Bull. 9, 532 - 540.

Noronha, J.M. and Silverman, M. (1961).
Second European symposium on vitamin B_{12} and Intrinsic Factor.
728 - 736, Ferdinand Enke.

Noronha, J.M. and Silverman, M. (1962).
Distribution of folic acid derivatives in natural material. 1. Chicken liver folates.
J. Biol. Chem. 237, 3299 - 3302.

Noronha, J.M. and Sreenivasan, A. (1959).
Metabolism of folic acid in folic acid and biotin deficient rat.
Proc. Soc. Exptl. Biol. Med. 101, 803 - 807.

Ochsenfahrt, H. and Winne, D. (1974).
The contribution of solvent drag to the intestinal absorption of the acidic drugs benzoic acid and salicylic acid from the jejunum of the rat.
Naunyn-Schmiedeberg's Arch. Pharmacol. 281, 197 - 217.

Olinger, E.J., Bertino, J.R. and Binder, H.J. (1973).
Intestinal folate absorption. 11. Conversion and retention of pteroylmonoglutamate by jejunum.
J. Clin. Invest. 52, 2138 - 2145.

Osborne-White, W.S. and Smith, R.M. (1973).
Identification and measurement of the folates in sheep liver.
Biochem. J. 136, 265 - 278.

Pabst, W. and Rembold, H. (1966).
Über das verhalten des biopterins in säugetier-organismus II.
Zeits. Physiol. Chemie. 344, 107 - 112.

Parsons, D.S. (1956).
The absorption of bicarbonate-saline solutions by the small intestine and colon of the white rat.
Quart. J. Exptl. Physiol. 41, 410 - 420.

Parsons, D.S. (1968).
Methods for investigation of intestinal absorption.
Handbook of Physiology, Section 6, Vol. 3, Page 1177-1216.

Parsons, D.S. and Boyd, C.A.R. (1972).
Transport across the intestinal mucosal cell: Hierarchies of function.
Int. Rev. Cytol. 32, 209 - 255.

Parsons, D.F. and Subjeck, J.R. (1972).
The morphology of the polysaccharide coat of mammalian cells.
Biochim. Biophys. Acta 265, 85 - 113.

Pasquali, P., Landi, L. and Marchetti, M. (1968).
Interrelationships between folic acid and biotin: Effect of biotin on the biosynthesis of folate coenzymes.
Biochim. Biophys. Acta 165, 561 - 563.

Paterson, A.R.P. and Zbarsky, S.H. (1955).
In vitro synthesis of purines by rat intestinal mucosa.
Biochim. Biophys. Acta 18, 441 - 442.

Paterson, A.R.P. and Zbarsky, S.H. (1958).
Purine synthesis in suspensions of mucosa from the small intestine of the rat.
Canad. J. Biochem. Physiol. 36, 755 - 770.

Patterson, E.L., Von-Saltza, M.H. and Stokstad, E.L.R.(1956)
The isolation and characterization of a pteridine required for the growth of Crithidia fasciculata.
J. Am. Chem. Soc. 78, 5871 - 5873.

Perris, A.D. (1966).
Isolation of the epithelial cells of the rat small intestine.
Canad. J. Biochem. 44, 687 - 693.

Perry, J. and Chanarin, I. (1968).
Absorption and utilisation of polyglutamyl forms of folate in man.
Brit. Med. J. 4, 546 - 549.

Perry, J. and Chanarin, I. (1970).
Intestinal absorption of reduced folate compounds in man.
Brit. J. Haemat. 18, 329 - 339.

Perry, J. and Chanarin, I. (1972).
Observations on folate absorption with particular reference to folate polyglutamate and possible inhibitors to its absorption.
Gut. 13, 544 - 550.

Perry, J. and Chanarin, I. (1973).
Formylation of folate as step in physiological folate absorption.
Brit. Med. J. 2, 588 - 589.

Pfiffner, J.J., Binkley, S.B., Bloom, E.S., Brown, R.A., Bird, O.D., Emmett, A.D., Hogan, A.G. and O'Dell, B.L. (1943).
Isolation of the anti-anaemia factor (Vitamin B_c) in crystalline form from liver.
Science 97, 404 - 405.

Pfiffner, J.J., Calkins, D.G., O'Dell, B.L., Bloom, E.S., Brown, R.A., Cambell, C.J., and Bird, O.D. (1945).
Isolation of an anti-anaemia factor (Vitamin B_c conjugate) in crystalline form from yeast.
Science 102, 228 - 230.

Pohland, A., Flynn, E.H., Jones, R.G. and Shive, W. (1951).
A proposed structure for folinic acid -SF, a growth factor derived from pteroylglutamic acid.
J. Am. Chem. Soc. 73, 3247 - 3252.

Ponz, F. and Larralde, J. (1950).
La absorcion de azucares en funcion del pH intestinal.
Revta. Esp. Fisiol. 6, 255 - 269.

Pratt, R.F., and Cooper, B.A. (1971).
Folates in plasma and bile of man after feeding folic acid -^{3}H and 5-formyltetrahydrofolate (folinic acid).
J. Clin. Invest. 50, 455 - 462.

Purrmann, R. (1940).
"Über die flügelpigmente der Schmetterlinge VII Synthese des leukopterins und Natur des Guanopterins.
Ann. Chem. 544, 182 - 190.

Purrmann, R. (1941a).
Die synthese des zanthopterins.
"Über die flügelpigmente der schmetterlinge X^{3}.
Ann. Chem. 546, 98 - 102.

Purrmann, R. (1941b).
Konstitution und synthese des sogenannten anhydroleucopterins.
"Über die flügelpigmente der schmetterlinge XII.
Ann. Chem. 548, 284 - 292.

Rader, J.I., Niethammer, D. and Huennekens, F.M. (1974).
Effects of sulfhydryl inhibitors upon transport of folate compounds into L1210 cells.
Biochem. Pharmacol. 23, 2057 - 2059.

Ramasastri, B.V. (1965).
Folate activity in human milk.
Brit. J. Nutr. 19, 581 - 586.

Rambourg, A., Neutra, M. and Leblond, C.P. (1966).
Presence of a "cell coat" rich in carbohydrate at the surface of cells in the rat.
Anat. Rec. 154, 41 - 71.

Rusmussen, H., Waldorf, A., Dziewiatkowski, D.D., and DeLuca, H.F. (1963).
Calcium exchange in isolated intestinal villi.
Biochim. Biophys. Acta. 75, 250 - 256.

Reiser, S. and Christiansen, P.A. (1971).
The properties of the preferential uptake of L-Leucine by isolated intestinal epithelial cells.
Biochim. Biophys. Acta. 225, 123 - 139.

Reizenstein, P. and Lund, L. (1973).
Effect of anticonvulsive drugs on folate absorption and the cerebrospinal folate pump.
Scand. J. Haemat. 11, 158 - 165.

Rembold, H., Chandrashekar, V. and Sundershan, P. (1971).
Catabolism of pteridine cofactors.
In vivo catabolism of reduced pterins in rats.
Biochim. Biophys. Acta. 237, 365 - 368.

Rembold, H. and Metzger, H. (1967).
Aktivierung von biopterin in der ratte.
Z. Naturforsch. 22B, 827 - 830.

Retief, F.P. (1969).
Urinary folate excretion after ingestion of pteroyl-monoglutamic acid and food folate.
Am. J. Clin. Nutr. 22, 352 - 355.

Retief, F.P. and Huskisson, Y.J. (1969).
Serum and urinary folate in liver disease.
Brit. Med. J. 2, 150 - 153.

Retief, F.P. and Huskisson, Y.J. (1970).
Folate binders in body fluids.
J. Clin. Path. 23, 703 - 707.

Reyes, H., Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1971).
Studies of Y and Z, two hepatic cytoplasmic organic anion-binding proteins: Effect of drugs, chemicals, hormones, and cholestasis.
J. Clin. Invest. 50, 2242 - 2252.

Reynolds, E.H. (1968).
Mental effects of anti-convulsants and folic acid metabolism.
Brain 91, 197 - 214.

Reynolds, E.H. (1974).
Folate metabolism and anti-convulsant therapy.
Proc. Roy. Soc. Med. 67, 68.

Reynolds, E.H., Gallagher, B.B., Mattson, R.H., Bowers, M. and Johnson, A.L. (1972a).
Relationship between serum and cerebrospinal fluid folate.
Nature 240, 155 - 157.

Reynolds, E.H., Hallpike, J.F., Phillips, B.M. and Matthews, D.M. (1965).
Reversible absorptive defects in anti-convulsant megaloblastic anaemia.
J. Clin. Path. 18, 593 - 598.

Reynolds, E.H., Mattson, R.H., and Gallagher, B.B. (1972b).
Relationships between serum and cerebrospinal fluid, anti-convulsant drug and folic acid concentrations in epileptic patients.
Neurology 22, 841 - 844.

Riecken, E.O., Stewart, J.S., Booth, C.C. and Pearse, A.G.E. (1966).
A histochemical study on the role of lysosomal enzymes in idiopathic steatorrhoea before and during a gluten-free diet.
Gut 7, 317 - 332.

Rindi, G. and Ventura, V. (1972).
Thiamine intestinal transport.
Phys. Rev. 52, 821 - 827.

Roberts, P.M., Arrowsmith, D.E., Rau, S.M. and Monk-Jones, M.E. (1969).
Folate state of premature infants.
Arch. Dis. Childh. 44, 637 - 642.

Roos, A.J., Spronk, A.M. and Cossins, E.A. (1968).
5-methyltetrahydrofolic acid and other folate derivatives in germinating pea seedlings.
Canad. J. Biochem. 46, 1533 - 1536.

Rosenberg, I.H. and Godwin, H.A. (1970).
Study of intestinal metabolism of folate using synthetic tritium labelled pteroylheptaglutamic acid.
Gastroenterol. 58, 990 (abstr).

Rosenberg, I.H. and Godwin, H.A. (1971).
The digestion and absorption of dietary folate.
Gastroenterol. 60, 445 - 463.

Rosenberg, I.H., Godwin, H.A., Streiff, R.R. and Castle, W.B. (1968).
Impairment of intestinal deconjugation of dietary folate: a possible explanation of megaloblastic anaemia associated with phenytoin therapy.
Lancet 2, 530 - 532.

Rosenberg, I.H., Streiff, R.R., Godwin, H.A. and Castle, W.B. (1969).
Absorption of polyglutamic folate: participation of deconjugating enzymes of the intestinal mucosa.
New. Engl. J. Med. 280, 985 - 988.

Rosenberg, T. and Wilbrandt, W. (1957).
Uphill transport induced by counterflow.
J. Gen. Physiol. 41, 289 - 296.

Rothenberg, S.P., da Costa, M., Lawson, J. and Rosenberg, Z. (1974).
The determination of erythrocyte folate concentration using a two phase ligand binding radioassay.
Blood 43, 437 - 443.

Rowe, P.B. and Lewis, G.P. (1973).
Mammalian folate metabolism.
Regulation of folate interconversion enzymes.
Biochemistry 12, 1962 - 1969.

Sakami, W. and Ukstins, I. (1961).
Enzymatic methylation of homocysteine by a synthetic tetrahydrofolate derivative.
J. Biol. Chem. 236, PC 50 - PC 51.

Salter, D.N. and Ford, J.E. (1968).
Properties of a folate-protein complex in milk.
Rep. Natn. Inst. Res. Dairy p.127.

Santini, R., Berger, F.M., Berdasco, G., Sheehy, T.W., Aviles, J. and Davila, I. (1962).
Folic acid activity in Puerto Rican foods.
J. Am. Diet. Ass. 41, 562 - 567.

Saraya, A.K. Choudhry, V.P. and Ghai, O.P. (1973).
Inter-relationships of vitamin B_{12}, folic acid, and iron in anaemia of infancy and childhood: effect of vitamin B_{12} and iron therapy on folate metabolism.
Am. J. Clin. Nutr. 26, 640 - 646.

Sauberlich, H.E. (1952).
Comparative studies with the natural and synthetic citrovorum factor.
J. Biol. Chem. 195, 337 - 349.

Sauberlich, H.E. and Baumann, C.A. (1948).
A factor required for the growth of Leuconostoc citrovorum.
J. Biol. Chem. 176, 165 - 173.

Schanker, L.S. (1960).
On the mechanism of absorption of drugs from the gastro-intestinal tract.
J. Med. Pharm. Chem. 2, 343 - 359.

Schanker, L.S. (1962).
Passage of drugs across body membranes.
Pharmacol. Rev. 14, 501 - 530.

Schanker, L.S., Tocco, D.J., Brodie, B.B. and Hogben, C.A.M. (1958).
Absorption of drugs from the rat small intestine.
J. Pharmac. Exptl. Ther. 123, 81 - 88.

Schmidt, U., Dubach, U.C. and Torhorst, J. (1969).
Behaviour of sodium-potassium-activated adenosine triphosphatase in rat kidney tissue by folic acid.
Experientia 25, 1288 - 1290.

Schmidt, U., Torhorst, J., Huguenin, M. and Dubach, U.C. (1973).
Acute renal failure after folate: NaK ATPase in isolated rat renal tubule.
Europ. J. Clin. Invest. 3, 169 - 178.

Schöpf, C. and Wieland, H. (1926).
"Über das leukopterin das weiBe flügelpigment der kohlweiblinge (Pierisbrassicae und P. napi).
Ber. Deut. Chem. Ges. 59, 2067 - 2072.

Schultz, S.G., Fuisz, R.E. and Curran, P.F. (1966).
Amino acid and sugar transport in rabbit ileum.
J. Gen. Physiol. 49, 849 - 866.

Schumacher, A.E., Heuser, G.F. and Norris, L.C. (1940).
The complex nature of the alcohol precipitate factor required by the chick.
J. Biol. Chem. 135, 313 - 320.

Scott, D.E., Whalley, P.J. and Pritchard, J.A. (1970).
Maternal folate deficiency and pregnancy wastage II fetal malformation.
Obstetrics and Gynecology 36, 26 - 28.

Scott, J.M. and Orsi, B.A. (1968).
Folic absorption in man.
Lancet 2, 775.

Searle, C.E. and Blair, J.A. (1973).
The renal toxicity of folic acid in mice.
Fd. Cosmet. Toxicol. 11, 277 - 381.

Selhub, J., Brin, H. and Grossowicz, N. (1973).
Uptake and reduction of radioactive folate by everted sacs of rat small intestine.
Europ. J. Biochem. 33, 433 - 438.

Sheehy, T.W., Rubini, M.E., Perez-Santiago, E., Santini, R. Jr., and Haddock, J. (1961).
The effect of "minute" and "titrated" amounts of folic acid on the megaloblastic anaemia of tropical sprue.
Blood 18, 623 - 636.

Sheehy, T.W., Santini, R. Jr., Guerra, R., Angel, R. and Plough, I.C. (1963).
Tritiated folic acid as a diagnostic aid in folic acid deficiency.
J. Lab. Clin. Med. 61, 650 - 659.

Shin, Y.S., Williams, M.A. and Stokstad, E.L.R. (1972).
Identification of folic acid compounds in rat liver.
Biochem. Biophys. Res. Comm. 47 35 - 43.

Shojania, A.M., Hornady, G. and Barnes, P.H. (1968).
Oral contraceptives and serum-folate level.
Lancet 1, 1376 - 1377.

Shojania, A.M., Hornady, G. and Barnes, P.H. (1969).
Oral contraceptives and folate metabolism.
Lancet 1, 886.

Sirotnak, F.M., Kurita, S. and Hutchison, D.G. (1968).
On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia.
Cancer Res. 28, 75 - 80.

Smith, M.E. (1973).
The uptake of pteroylglutamic acid by the rat jejunum.
Biochim. Biophys. Acta. 298, 124 - 129.

Smith, M.E., Matty, A.J., and Blair, J.A. (1970a).
The transport of folic acid across the small intestine of the rat.
Gut. 11, 368.

Smith, M.E., Matty, A.J., and Blair, J.A. (1970b).
The transport of pteroylglutamic acid across the small intestine of the rat.
Biochim. Biophys. Acta. 219, 37 - 46.

Smyth, D.H. (1966).
Electric potentials and fluid transfer in the rat intestine.
Excerpta Medica Monographs on Nuclear Medicine and Biology, 1, 195 - 205.

Snell, E.E. and Peterson, W.H. (1940).
Growth factors for bacteria: X. Additional factors required by certain lactic acid bacteria.
J. Bacteriol. 39, 273 - 285.

Sols, A. (1956).
The hexokinase activity of the intestinal mucosa.
Biochim. Biophys. Acta. 19, 144 - 152.

Spaans, F. (1970).
No effect of folic acid supplement on CSF folate and serum vitamin B_{12} in patients on anticonvulsants.
Epilepsia 11, 403 - 411.

Speidel, B.D. (1973).
Folic acid deficiency and congenital malformation.
Dev. Med. and Child Neurol. 15, 81 - 83.

Spencer, R.P. and Bow, T.M. (1964).
In vitro transport of radiolabelled vitamins by the small intestine.
J. Nucl. Med. 5, 251 - 258.

Spray, G.H. (1952).
The Utilisation of folic acid from natural sources.
Clin. Sci. 11, 425 - 428.

Spray, G.H. (1968).
Oral contraceptives and serum-folate levels.
Lancet 2, 110 - 111.

Spray, G.H. and Witts, L.J. (1952).
The utilisation of folic acid given by mouth.
Clin. Sci. 11, 273 - 281.

Stephens, M.E.M., Craft, I., Peters, T.J. and Hoffbrand, A.V. (1972).
Oral contraceptives and folate metabolism.
Clin. Sci. 42, 405 - 414.

Stern, B.K. and Jensen, W.E. (1966).
Active transport of glucose by suspensions of isolated rat intestinal epithelial cells.
Nature, 209, 789 - 790.

Stewart, B.E. and Zbarsky, S.H. (1963).
Nucleic acid metabolism in intestinal mucosa: III. Loss of nucleic acids from intestinal mucosa of the rat during incubation in vitro.
Canad. J. Biochem. Physiol. 41, 1483 - 1486.

Stokstad, E.L.R. (1943).
Some properties of a growth factor for Lactobacillus casei.
J. Biol. Chem. 149, 573 - 574.

Stokstad, E.L.R. and Koch, J. (1967).
Folic acid metabolism.
Physiol. Rev. 47, 83 - 116.

Stokstad, E.L.R. and Manning, P.D.V. (1938).
Evidence of a new growth factor required by chicks.
J. Biol. Chem. 125, 687 - 696.

Streiff, R.R. and Greene, B. (1970).
Drug inhibition of folate conjugase.
In proceedings XIIIth International Congress of Haematology. Munich. P. 377.

Strelling, M.K., Blackledge, G.D., Goodall, H.B. and Walker, C.H.M. (1966).
Megaloblastic anaemia and whole-blood folate levels in premature infants.
Lancet. 1. 898 - 900.

Strum, W.B., Nixon, P.F., Bertino, J.B. and Binder, H.J. (1971).
Intestinal folate absorption 1. 5-methyltetrahydrofolic acid.
J. Clin. Invest. 50, 1910 - 1916.

Strum, W.B., Nixon, P.F., Binder, H.J. and Bertino, J.R. (1970).
Intestinal absorption of 5-methyltetrahydrofolate (MTHF).
Clin. Res. 18, 389 (abstr).

Swendseid, M.E., Bird, O.D., Brown, R.A. and Bethell, F.H. (1947).
Metabolic function of pteroylglutamic acid and its hexaglutamyl conjugate. 11. Urinary excretion studies on normal persons. Effect of a conjugase inhibitor.
J. Lab. Clin. Med. 32, 23 - 27.

Tamura, T. and Stokstad, E.L.R. (1973).
The availability of food folate in man.
Brit. J. Haemat. 25, 513 - 532.

Taylor, C.B. (1963).
The effect of cetyltrimethylammonium bromide and some related compounds on transport and metabolism in the intestine of the rat in vitro.
J. Physiol. 165, 199 - 218.

Terroine, T. (1960).
The vitamin interrelations of ascorbic acid.
Wld. Rev. Nutr. Diet. 2, 101 - 130.

Thenen, S.W., Shin, Y.S. and Stokstad, E.L.R. (1973).
The turnover of rat liver folate pools.
Proc. Soc. Exptl. Biol. Med. 142, 638 - 641.

Thiry, L. (1864).
Über eine neue Methode, den Dünnderm zu isolieren.
Sitzber. Akad. Wiss. Wein. Math.-Naturw. KL. 1. 50, 77 - 96.

Thomson, A.D., Baker, H. and Leevy, C.M. (1971).
Folate-induced malabsorption of thiamin .
Gastroenterol. 60, 756.

Thomson, A.D., Frank, O., De Angelis, B. and Baker H.(1972)
Thiamin depletion induced by dietary folate deficiency in rats.
Nutr. Rep. Inter. 6, 107 - 110.

Tisman, G. and Herbert, V. (1973).
B_{12} dependence of cell uptake of serum folate: An explanation for high serum folate and cell folate depletion in B_{12} deficiency.
Blood 41, 465 - 469.

Trubowitz, S., Frank, O. and Baker, H. (1974).
Survey of vitamin B_{12} and folate in the serum and marrow tissue of hospitalized patients.
Am. J. Clin. Nutr. 27, 580 - 583.

Turner, J.B. and Hughes, D.E. (1962).
The absorption of some B group vitamins by surviving rat intestine preparations.
Quart. J. Exptl. Physiol. 47, 107 - 123.

Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1951).
Manometric techniques and tissue metabolism.
2nd Edition, 1951. Burgess, Minneapolis.

Ussing, H.H. (1947).
Interpretation of the exchange of radio-sodium in isolated muscle.
Nature, 160, 262 - 263.

Ussing, H.H. (1949).
Transport of ions across cellular membranes.
Physiol. Revs. 29, 127 - 155.

Watanabe, H. (1962).
Tissue uptake and distribution of tritiated folic acid.
M.Sc. thesis. McGill University, Montreal, Canada.

Watson-Williams, E.J. (1962).
Folic acid deficiency in sickle-cell anaemia.
East Afr. Med. J. 39, 213 - 221.

Webster, H.L. and Harrison, D.D. (1969).
Enzymic activities during the transformation of crypt to columnar intestinal cells.
Exptl. Cell. Res. 56, 245 - 253.

Weir, D.G. (1974).
The pathogenesis of folic acid deficiency in man.
Irish. J. Med. Sci. 143, 3 - 20.

Weir, D.G., Brown, J.P., Freedman, D.S. and Scott, J.M. (1973).
The absorption of the diastereoisomers of 5-methyltetrahydropteroylglutamate in man: A carrier mediated process.
Clin. Sci. Mol. Med. 45, 625 - 631.

Wells, D.G. and Casey, H.J. (1967).
Lactobaccilus casei C.S.F. folate activity.
Brit. Med. J. 3, 834 - 836.

Whitehead, V.M. and Cooper, B.A. (1967).
Absorption of unaltered folic acid from the gastro-intestinal tract in man.
Brit. J. Haemat. 13, 679 - 686.

Whitehead, V.M., Pratt, R., Viallet, A. and Cooper, B.A. (1972).
Intestinal conversion of folinic acid to 5-methyltetra-hydrofolate in man.
Brit. J. Haemat. 22, 63 - 72.

Whiteley, J.M., Drais, J.H. and Huennekens, F.M. (1969).
Synthesis and properties of 2-amino-4-hydroxy-5,6-dimethyl-5,6,7,8-tetrahydropteridine and the 5,7-dimethylisomer.
Arch. Biochem. Biophys. 133, 436 - 441.

Wieland, H. and Schöpf, C. (1925).
Über den gelben flügelfarbstoff des citronenfalters (Gonepteryxrhamni) Ber. Deut. Chem. Ges. 58, 2178 - 2183.

Wilbrandt, W. and Laszt, L. (1933).
Investigations into the cause of selective sugar absorption.
Biochem. Z. 259, 398 - 417.

Wilbrandt, W. and Rosenberg, T. (1961).
The concept of carrier transport and its corollaries in pharmacology.
Pharmacol. Rev. 13, 109 - 183.

Wilcken, B. and Turner, B. (1973).
Homocystinuria: Reduced folate levels during pyridoxine treatment.
Arch. Dis. Childh. 48, 58 - 62.

Williams, R.B. and Mills, C.F. (1973).
Relationships between zinc deficiency and folic acid status of the rat.
Proc. Nutr. Soc. 32, 2A - 3A.

Wills, L. (1931).
Treatment of pernicious anaemia of "pregnancy" and "tropical anaemia" with special reference to yeast extract as a curative agent.
Brit. Med. J. 1, 1059 - 1064.

Wills, L. (1933).
The nature of the haemopoietic factor in marmite.
Lancet. 1, 1283 - 1286.

Wills, L., Clutterbuck, P.W. and Evans, B.D.F. (1937a).
A new factor in the production and cure of certain macrocytic anaemias.
Lancet 1, 311 - 314.

Wills, L., Clutterbuck, P.W. and Evans, B.D.F. (1937b).
A new factor in the production and cure of macrocytic anaemias and its relation to other haemopoietic principles curative in pernicious anaemia.
Biochem. J. 31, 2136 - 2147.

Wills, L. and Stewart, A. (1935).
Experimental anaemia in monkeys, with special reference to macrocytic nutritional anaemia.
Brit. J. Exptl. Pathol. 16, 444 - 453.

Wilson, F.A. and Dietschy, J.M. (1974).
The intestinal unstirred layer: its surface area and effect on active transport kinetics.
Biochim. Biophys. Acta. 363, 112 - 126.

Wilson, T.H. (1954).
Concentration gradients of lactate, hydrogen and some other ions across the intestine *in vitro*.
Biochem. J. 56, 521 - 527.

Wilson, T.H. (1962).
Intestinal absorption.
Saunders, London. P. 263.

Wilson, T.H. and Kazyak, L. (1957).
Acid-base changes across the wall of hamster and rat intestine.
Biochim. Biophys. Acta. 24, 124 - 132.

Wilson, T.H. and Wiseman, G. (1954).
The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface.
J. Physiol. 123 116 - 125.

Winawer, S.J., Sullivan, L.W., Herbert V. and Zamcheck, N. (1965).
The jejunal mucosa in patients with nutritional folate deficiency and megaloblastic anaemia.
New Engl. J. Med. 272, 892 - 895.

Wolff, R., Drouet, L. and Karlin, R. (1949).
Occurrence of vitamin B_c conjugase in human plasma.
Science 109, 612 - 613.

Yamamoto, K., Habu, S. and Ueda, J. (1972).
Effect of folic acid on plasma renin activity in rats.
Arch. Pharmacol. 275, 343 - 346.

Yoshino, T. (1968a).
The clinical and experimental studies on the metabolism of folic acid using tritiated folic acid. 1. Absorption tests of tritiated folic acid in man.
J. Vitaminol. 14, 21 - 34.

Yoshino, T. (1968b).
The clinical and experimental studies on the metabolism of folic acid using tritiated folic acid. II. The experimental studies on the absorption site and mechanism of tritiated folic acid in rats.
J. Vitaminol 14, 35 - 48.

Yoshino, T. (1968c).
The clinical and experimental studies of the metabolism of folic acid using tritiated folic acid. III. Plasma clearance in man and organ distribution in rat following intravenous administration of tritiated folic acid.
J. Vitaminol 14, 49 - 66.

Zakrzewski, S.F. and Grzelakowska-Sztabert, B. (1973). Uptake and metabolism of folate by a folate-utilizing mutant of Pediococcus cerevisiae (8081/S) and the parent strain (8081).

J. Biol. Chem. 248, 2684 - 2690.