TRANSPORT OF FOLIC ACID AND RELATED COMPOUNDS ACROSS THE SMALL INTESTINE.

A THESIS

submitted for the degree of

Doctor of Philosophy

by

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at

The University of Aston in Birmingham.

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August 1974.

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SUMMARY

The intestinal transport of folic acid (pteroyl-L-glutamic acid) was investigated using *in vitro* preparations of rat jejunum.

It was established that folic acid is accumulated in the gut wall and transported into the serosal compartment by a saturable mechanism. The kinetic parameters and the temperature dependence of the process were calculated, and it was shown that the transport mechanism had a limited dependence on aerobic metabolism.

When the folic acid derivative 10-formylfolic acid was present in the incubation medium at a 10:1 molar ratio with labelled folic acid the tissue accumulation was significantly reduced though the serosal transfer was not significantly changed. It was also shown that the efflux of pre-loaded folic acid from the gut wall was stimulated in the presence of a high mucosal concentration of pteroyl-L-glutamic acid, 10-formylfolic acid and methotrexate. Pteroyl-D-glutamic acid had a less significant effect, and pteroic acid produced no displacement. Folic acid was not present at a high concentration, in free solution, within the gut wall after a loading incubation, and it was concluded that the stimulated efflux arose as the result of displacement of labelled folic acid from binding sites within the tissue. It was shown that the displacement phenomenon was localised in the proximal region of the small intestine.

The uptake of folic acid by a preparation of isolated jejunal mucosal cells was studied, together with some aspects of their morphology.

The interpretation of these results is discussed at some length in the light of the previous literature, and having regard to the known physico-chemical properties of folic acid. A mechanism for folate absorption is proposed in which absorption occurs by passive, non-ionic diffusion. It is suggested that the uptake of folic acid is enhanced in the jejunum by a "microclimate" at the mucosal surface having a pH more acidic than the bulk luminal phase.

This work was carried out from October 1971 to July 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.

Ian Johnson

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ACKNOWLEDGEMENTS.

I would like to express my gratitude to the following people for their assistance during the course of this work.

Dr. J. A. Blair, for his supervision, constant encouragement, and advice.

Professor A. J. Matty of the Department of Biological Sciences.

Professor W. G. S. Parker, in whose Department the work was done.

Mr. R. J. Leeming of the Birmingham General Hospital and Mr. K. Ratanasthien for the microbiological assays.

Mr. W. E. Jensen of the University of Chicago for the isolated cell preparation depicted in Plate IV.

Dr. Yeadon of Lederle Laboratories Division, American Cyanamid, for gifts of pteroic acid and pteroyl-D-glutamic acid.

My mother, Mrs. P. M. Johnson, for patience and excellent typing.

The Wellcome Trust for financial support.

Finally, the many people who have contributed advice, encouragement, and humour over the past three years.

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 (1).

The first step towards an understanding of the folates and their importance as growth factors in man, animals and micro-organisms, was taken in the early 1930s by Wills and her co-workers (Wills, 1931; Wills, Clutterbuck & Evans, 1937). Wills demonstrated that "a new hemopoietic factor" present in autolysed yeast and liver was effective in the treatment of macrocytic anaemia amongst pregnant, poorly nourished women in India. Furthermore, it was shown that a similar condition induced in monkeys by an experimentally restricted diet would also respond to the unknown nutritional factor. (Wills & Bilmoria, 1932). During the same period, Day, Langston and Schukers (1935) also described an experimentally induced deficiency condition in monkeys which they called nutritional cytopenia. This too responded to oral treatment with yeast and liver extracts and the term Vitamin M was coined for the active factor.

Somewhat similar studies were carried out by other workers using the chick, in which a highly purified diet gave rise to macrocytic anaemia (Stokstad & Manning, 1938; Hogan & Parrot, 1939, 1940). The condition was cured by a substance present in yeast, liver and cereals; the new factor was designated Vitamin B_c, and the anaemic chick provided a convenient assay organism for the subsequent identification of hematopoietic substances.

The use of micro-organisms gave rise to a third line of research at this time, demonstrating the presence of growth substances in yeast and liver essential for the lactic acid bacterium *Lactobacillus casei* (Snell & Peterson, 1940), and in spinach leaves, a similar substance utilised by *Streptococcus lactis R* (Mitchell, Snell & Williams, 1941, 1944). It was Mitchell and his colleagues who first used the term 'folic acid' (from the Latin *folium* 'a leaf') for the material which they had derived from four tons of spinach leaves.

The stage was now set for the chemists to draw together the threads of these investigations by isolating and synthesising the compound or compounds whose presence had been revealed by the nutritional studies. The isolation of a crystalline material from liver by Pfiffner *et al* (1943) was followed in 1945 by the synthesis of a structurally and functionally identical pteridine derivative by Angier and his co-workers (Angier *et al*, 1945; Subbarow *et al*, 1946) working at Lederle Laboratories.

i) General Chemistry of the Folates.

The pteridines, first examples of which were isolated from butterfly wings by Hopkins (1889), and structurally elucidated by Purrmann fifty years later (Purrmann, 1940), are widely distributed in nature being commonly found as pigments in the eyes and wings of

insects, and in the skins of amphibia and reptiles (Blakley, 1969). All pteridines are derivatives of the bicyclic nitrogenous ring system, pyrimido (4,5-b)-pyrazine, known simply as 'pteridine', shown in Figure 1.1. The general term "pterin" (from the Greek pteron "a wing") has in the past been applied indiscriminately to insect pigments in general, but in modern literature its use is restricted to compounds which are derivatives of 2-amino-4-hydroxypteridine; most of the naturally occurring pteridines fall into this category (Blakley, 1969).

The compound synthesised by Angier and his colleagues in 1945 was a 6-alkylpterin for which they proposed the name "pteroylglutamic acid"; its structure is shown in Figure 1.2. The derivation of the name pteroylmonoglutamic acid from the constituent groups of the molecule is obvious. However, as Blakley (1969) pointed out, it has the disadvantage of clumsiness when used with prefixes indicating the presence of substituent groups and it suggests an undue importance for pteroic acid, a compound not known to occur naturally. The older term, folic acid, is more convenient but the Commission on Biochemical Nomenclature (IUPAC-IUB Commission, 1966) allows its general use for any member of the family. In order to avoid ambiguity the following convention will be used throughout this text.

1. The general term 'folate' refers to derivatives of the compound shown in Figure 1.2. The structures of the folates together with their

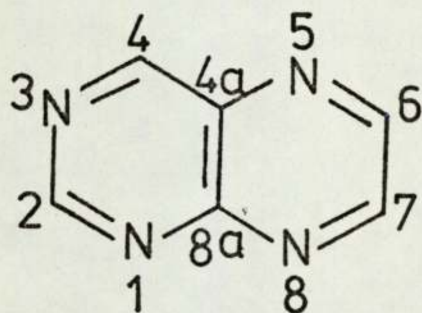


FIGURE 1.1. Pyrimido (4,5-b)-pyrazine; "Pteridine".

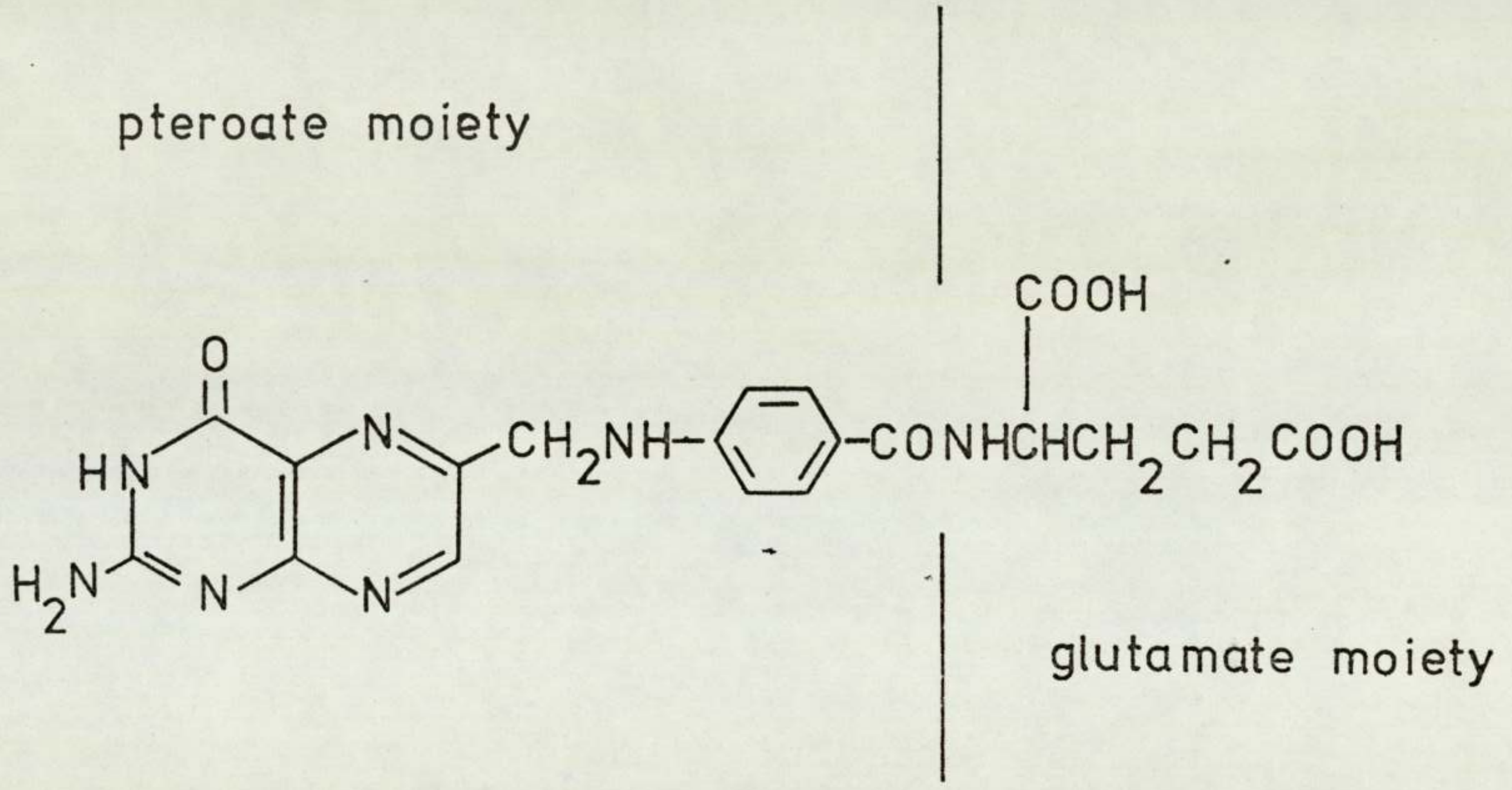


FIGURE 1.2. Pteroylmonoglutamic Acid; "Folic Acid" PteGlu.

numbering, their abbreviation in accordance with the IUPAC-IUB convention, and their common synonyms are given below.

2. The use of the term 'folic acid' is restricted to the compound shown in Figure 1.2. and is used synonomously with pteroyl-L-glutamic acid, abbreviated to PteGlu, throughout the text.

3. From time to time it will be necessary to refer to the various conjugates of folic acid (see Figure 1.9.). These will be indicated by the abbreviation PteGlu, together with the appropriate suffix. Thus $PteGlu_2$ for pteroyldiglutamic acid.

Folic acid occurs in the solid form as yellow spear-shaped crystals with a molecular weight of 441.4. It is generally described as sparingly soluble in aqueous and organic solvents; (a full discussion of the compound's solubility characteristics will be reserved for a subsequent section). Solutions of folic acid are said to be stable for long periods in the dark, but exposure to ultra-violet light gives cleavage at the 9,10 position to yield a pteridine and a free aromatic amine. The stability of folates under storage is discussed in the materials section below, and their behaviour during incubation under physiological conditions is discussed fully in Appendix II.

ii) Derivatives of Folic Acid.

Soon after the isolation and synthesis of folic

acid it was realised that many naturally occurring folates were complex derivatives of the parent material. Folate derivatives may vary as to the number of glutamate residues present in the molecule, the state of oxidation of the pteridine nucleus, or the nature and position of any one-carbon substituents. Generally speaking, all the folate derivatives are highly polar polybasic acids having rather similar physical characteristics at physiological temperatures. However, the presence of substituents and the reduction of the pteridine ring increases the lability of the molecule and this has led, in the past, to difficulties in analysis and some consequent confusion as to the identity of the compounds under study. The structures of the most important folate derivatives are given below together with some explanatory information where necessary.

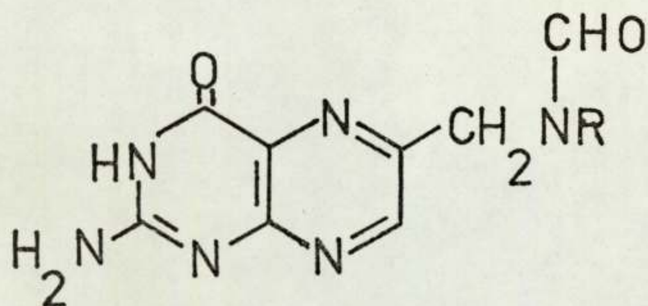


FIGURE 1.3. 10-formylfolic acid, 10CHO-PteGlu

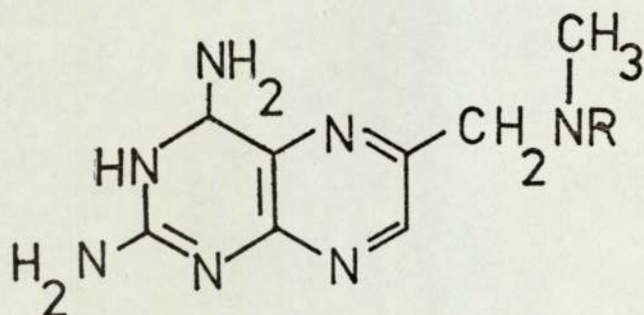


FIGURE 1.4. 4-amino, 10-methylfolic acid "Methotrexate".

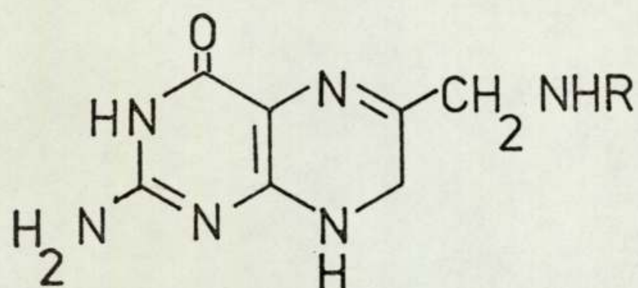


FIGURE 1.5. 7,8-dihydrofolic acid; H_2PteGlu.

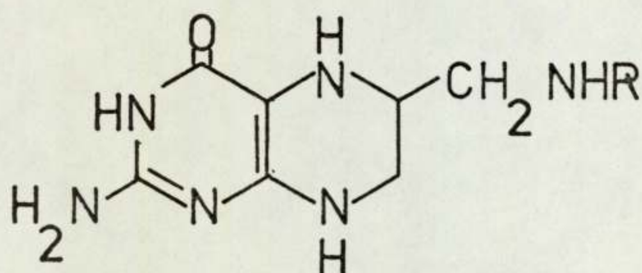


FIGURE 1.6. 5,6,7,8-tetrahydrofolic acid; H_4PteGlu.

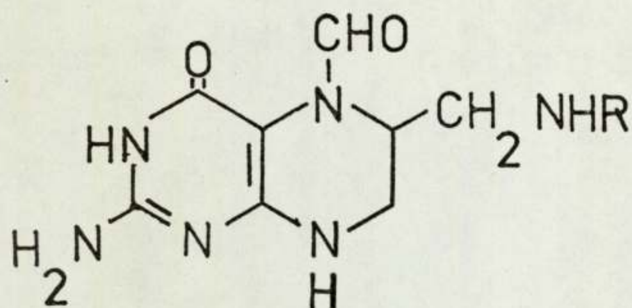


FIGURE 1.7. 5-formyltetrahydrofolic acid; 5-CHO-H_4PteGlu.
"Citrovorum factor"; "Folinic acid".

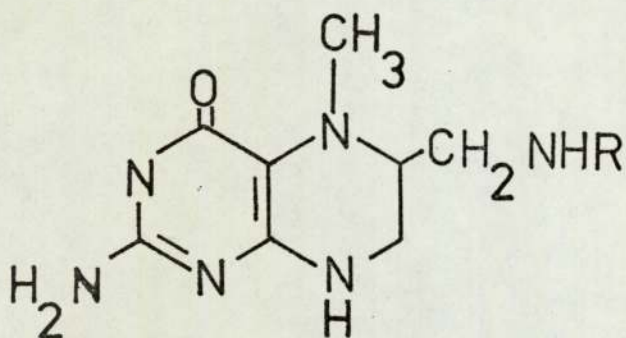


FIGURE 1.8. 5-methyltetrahydrofolic acid; 5CH_3-H_4PteGlu.

iii) Folate Polyglutamates.

During the early work on growth factors from various sources, it was shown that the chick anti-anaemia factor present in yeast extract was inactive as a growth promoting substance for L. casei (Blinkley et al, 1944). The material, which was of a high molecular weight but was apparently non-protein in nature, could be rendered microbiologically active by treatment with a crude enzyme extract prepared from hog kidney. Subsequent work (Pfiffner, Calkins, Bloom & O'Dell, 1946) showed that the inactive form of vitamin B_c (folic acid) was linked to six glutamate residues in a gamma-peptide chain: this material they termed "Vitamin B_c Conjugate". The effect of enzyme extracts, termed "conjugases", was to break the gamma peptide linkages, thus liberating free folic acid. A smaller conjugate described at the time as "fermentation L. casei factor" was isolated from Corynebacterium sp. (Hutchings et al, 1948) and shown to be $PteGlu_3$; this appears to be the major folate form in bacteria.

It should be noted that the only naturally occurring polyglutamate which has been isolated and fully identified is $PteGlu_7$, and in most cases identification of the naturally occurring forms has been based on comparison with these artifically synthesised compounds. Artificial folate polyglutamates have been synthesised with from 2 to 7 constituent glutamic acid residues. Of the two principal methods, the solid phase technique (Krumdieck & Baugh, 1969) is fast and convenient but it

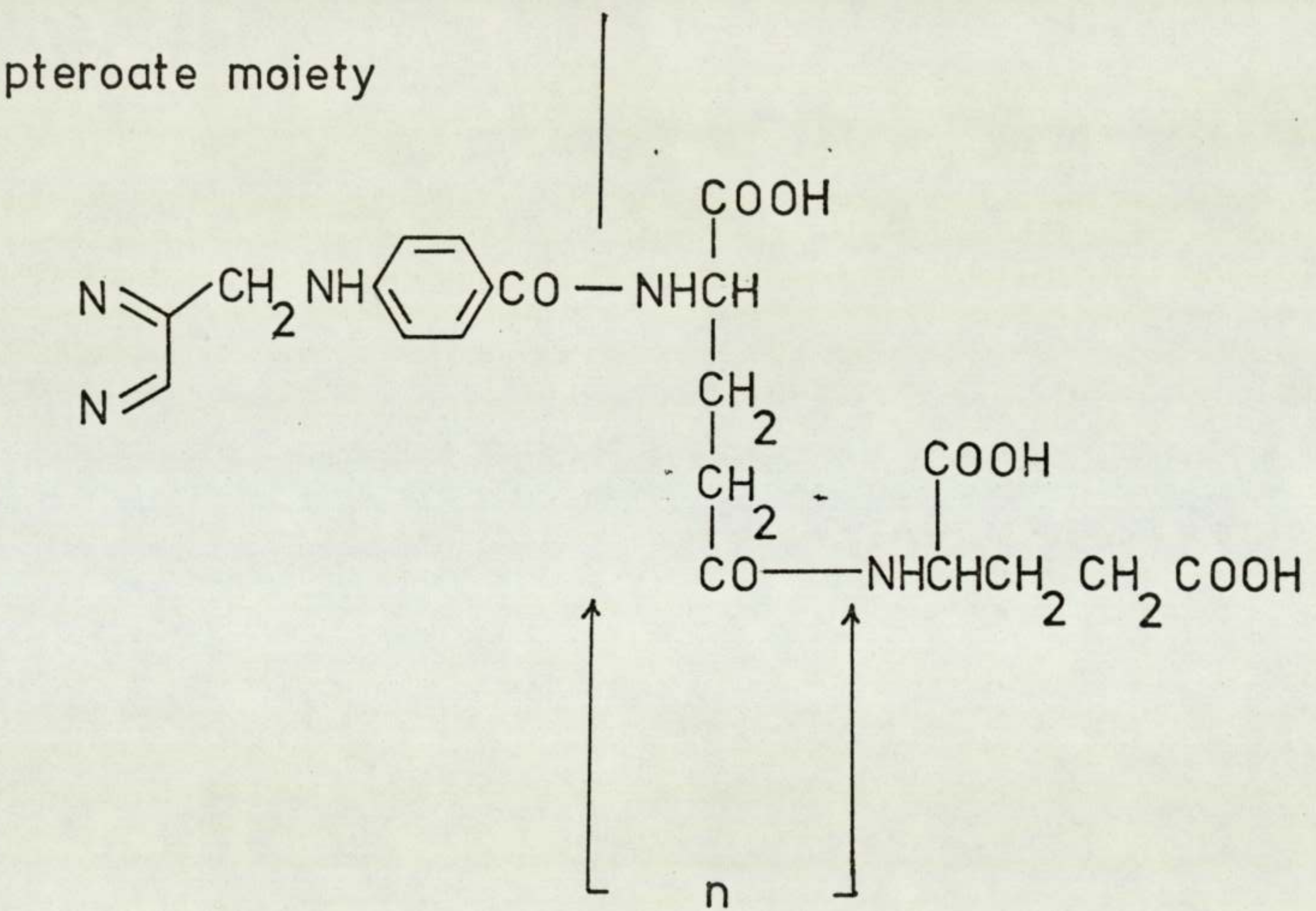


FIGURE 1.9. Folate polyglutamate, $PteGlu_n$ ($n = 1$ to 6).

has been shown to yield a final product contaminated with lower peptides and is probably less satisfactory than conventional techniques (Meienhofer *et al*, 1970; Godwin *et al*, 1972).

It is commonly claimed that as much as 75% of naturally occurring folates are in the polyglutamate form although this conclusion seems to be based more on inference than rigorous chemical identification. (Perry, 1971; Chanarin, 1969; Blair, 1969). The general structural formula for the folate polyglutamates is given in Figure 1.9.

iv) Occurrence and Biological Significance of the Folates.

The metabolic role of the folates is to act as co-enzymes for the transfer of one-carbon fragments in a number of essential synthetic pathways including those of purines and pyrimidines and of a number of amino acids. The main metabolic interconversions in which the folates are involved are summarised in Figure 1.10.

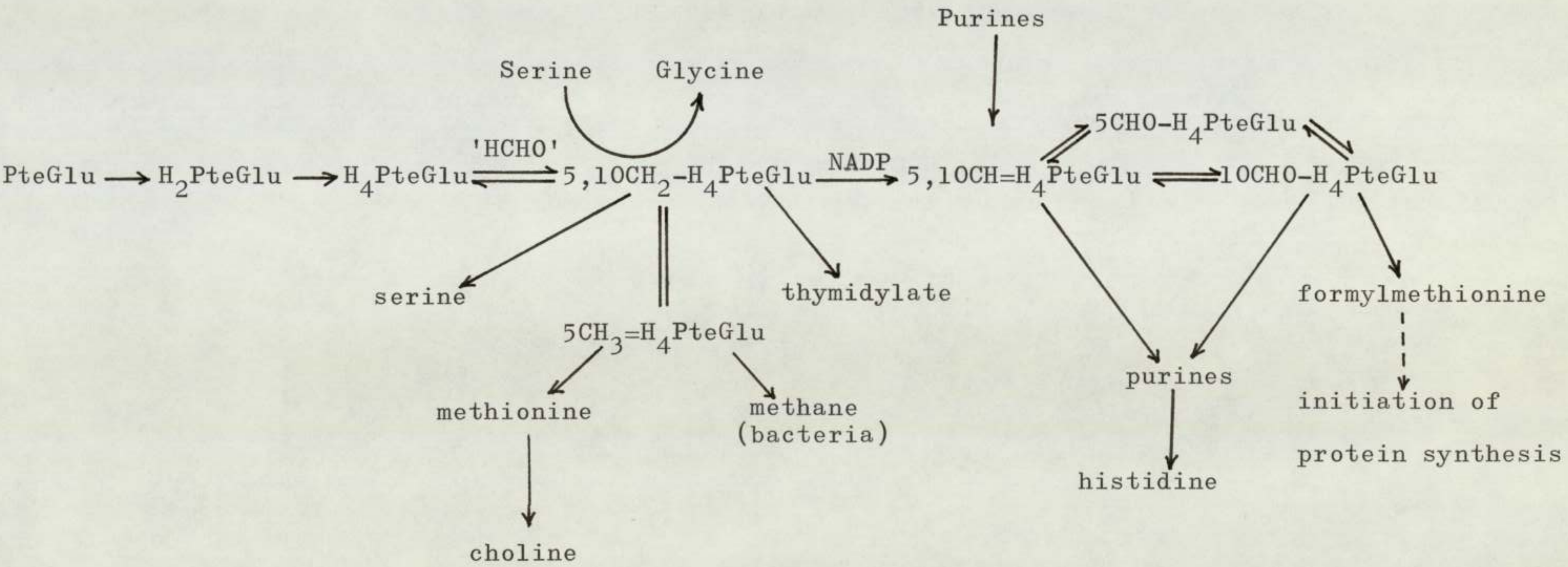
As far as is known at present folates function as co-enzymes only after reduction of the 5,6 and 7,8 double bonds to yield the tetrahydrofolate forms. In general it is the monoglutamyl folates which act as co-enzymes, but a system utilising polyglutamyl forms has been described in *Clostridium* (Wright, 1956), and Blakley (1957) reports that the activity of some folate co-enzymes increases with extension of the peptide chain length.

Reduction of folate and dihydrofolate by reduced

FIGURE 1.10.

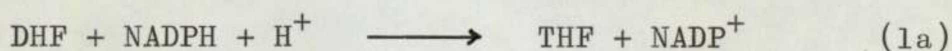
METABOLIC FUNCTIONS OF FOLATE DERIVATIVES.

(modified from Blakley, 1969)



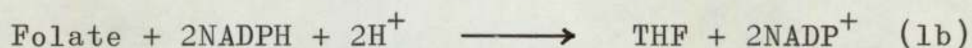
nicotinamide adenine dinucleotide phosphate (NADPH) is mediated by the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NAD(P) oxidoreductase (EC1.5.1.3)) which occurs in the liver, kidneys, spleen and intestinal mucosa of vertebrates. High concentrations of the enzyme also occur in the leucocytes of leukemia patients, in certain types of tumour, and in many microbiological sources.

The equation for the enzymic reduction of dihydrofolate is as follows:



$$DHF + NADPH + H^{+} \longrightarrow THF + NADP^{+} \qquad (1a)$$

Dihydrofolate reductase also catalyses the reduction of folate according to equation 1b.



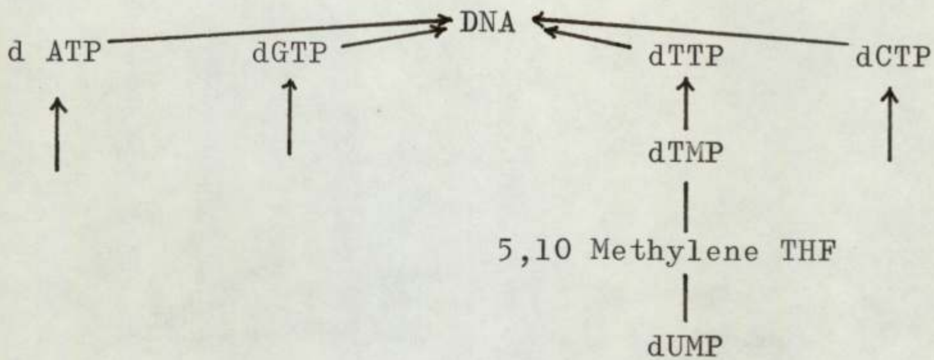
$$Folate + 2NADPH + 2H^{+} \longrightarrow THF + 2NADP^{+} \qquad (1b)$$

but this reaction proceeds at a much slower rate particularly at physiological pH. Blakley (1969) gives a comprehensive review of the occurrence and biochemistry of dihydrofolate reductase.

The folate co-enzymes occupy important positions in the metabolic pathways leading to DNA and protein synthesis, since they are not themselves synthesised by mammals their dietary importance is obvious. In man, megaloblastic anaemia is the almost invariable accompaniment of folate deficiency.

Figure 1.11.illustrates the position of 5,10 methylenetetrahydrofolate in the production of thymidylate, one of the nucleotide precursors of DNA (Wahba & Friedkin, 1962).

FIGURE 1.11.



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

(Adapted from Hoffbrand & Tripp, 1972).

It has been shown that the bone marrow cells of patients suffering from pernicious anaemia have impaired DNA synthesis and it is thought that it is the consequent disruption of cell kinetics which leads to abnormal haemopoeisis(Wickramasinghe, Chalmers & Cooper, 1967). Hoffbrand and Tripp (1972) have shown that normal lymphocytes cultured in the presence of methotrexate have reduced intracellular levels of dTTP, and increased 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.

In man, folate deficiency may occur as a result of poor diet, or because of pathological mal-absorption connected either with some generalised intestinal disease such as Tropical Sprue or Adult Coeliac disease, (Cooke, 1968) or because of a specific inability to absorb the folates themselves (Luhby *et al*, 1961; Lanskowsky *et al*, 1969; Santiago Borrero, 1973).

Opinions as to the absolute daily requirements of folate seem to be divided; the matter is reviewed by Chanarin (1969). Herbert (1962a, 1962b) has shown that a folate free diet led to early megaloblastic symptoms in man after 133 days, and that the folic acid supplement needed to prevent a fall in serum folate levels under these conditions was somewhat more than 100 μg per day. In patients suffering from megaloblastic anaemia due to folate deficiency it has been shown that the minimum dose which would initiate a haematological response varies from 5 μg in some children (Velez, Ghitis, Pradilla & Vitale, 1963) to 200 μg in other studies in adults. (Hansen & Weinfeld, 1962).

Dietary folates occur as complex mixtures of reduced and substituted forms and a substantial percentage becomes available to L. casei only after treatment with conjugase, indicating the presence of polyglutamates.

There is good evidence that a large fraction of natural folates occur in combination with binding proteins and it is possible that these macromolecular forms may, in some cases, have been misinterpreted as polyglutamates (For a full discussion of this possibility see Beavon, 1973). Folate binding proteins have been reported in milk by several workers (Metz & Herbert, 1967; Ghitis, Mandelbaum-Shavit & Grossowicz, 1969; Ford et al, 1972) and that isolated by Ford et al as a minor whey protein in goats' milk is of particular interest as it appears to play an important role in the folate nutrition of the new born kid. A proportion of serum folates have been shown by a number of workers to be protein bound (Metz & Herbert, 1967; Markannen & Peltola, 1971; Retief & Huskisson, 1969) and the folate constituents of erythrocytes seem to be combined with a protein to form "folate precursor substances" comparable in size to haemoglobin (Iwai, Luttner & Toennies, 1964). Allfrey and King (1950) have isolated a material from yeast which consists of a protein-pteroylheptaglutamate complex. It is worth mentioning here, too, that folates may be tightly adsorbed by insoluble cellulose residues, and it is possible that diets containing a high proportion of this type of material may be correspondingly low in

available folate (Luther _et al_, 1965).

The nature of the folate derivative differs between foodstuffs. Liver and yeast, which are the richest sources of dietary folate, contain respectively, 300 μg and 2 mg of folate per 100 gm moist weight, and it was these sources which led to the original isolation of folic acid (Toepfer _et al_, 1951). Green vegetables also contain substantial quantities of folate, about 90% of which is present as the 5- or 10-formyl derivatives and it is likely that less than 10% of the average dietary folate intake is in the form of free folic acid (Santini, Brewster & Butterworth, 1964). From the figures of Chanarin (1969) and Butterworth _et al_ (1963) it would seem that the average western diet provides about 700 μg of folate per day, as determined by _L. casei_ assay after treatment with conjugase. From this the intestine must absorb the minimum daily requirement of between 100 and 200 μg. As to the relative availability of the various forms of dietary folate there is some controversy and this will be dealt with under the general heading of Absorption in the second part of this section.

The last two decades have seen an immense amount of research devoted to all aspects of intestinal absorption and, as might be expected, numerous attempts have been made to establish the physiological basis of folate uptake. Despite this interest, no clear mechanism has emerged and although certain characteristics

of the absorptive process now seem firmly established, much of the published work is contradictory. This thesis describes the use of *in vitro* techniques in an attempt to clarify some of the areas of controversy and to establish a consistent model for intestinal folate absorption.

The second part of the introduction reviews in some detail the current state of knowledge as regards folate absorption both in man and in laboratory animals.

Section I (2).

Because of their clinical importance, the folates have attracted attention from a wide variety of research groups whose interests have ranged from the clinical to the purely biological and chemical. This has resulted, no doubt, in a broad inter-disciplinary approach, fruitful both in ideas and interpretation, but it has also led to a somewhat complex literature. This review will summarise the current state of knowledge as regards the intestinal absorption of folates; aspects likely to have a direct bearing on the physiological mechanisms involved will be dealt with under a series of sub-headings. By this means it is hoped to highlight the various areas where agreement has been reached as well as those aspects which remain controversial.

No attempt will be made here to describe in detail the various techniques which have been employed. Most of the *in vitro* methods will be discussed fully in Section 2 and reviews of the *in vivo* animal techniques and experimental methods used in man are provided by Parsons (1967) and Chanarin (1969).

i) The Chemical Structure of Absorbed Folates.

As we have seen, it is very likely that a large proportion of dietary folate is ingested in the form of polyglutamates and protein bound forms; it has been claimed in fact that as much as 75% of dietary folate occurs as $PteGlu_7$ (Perry, 1971). The pterolyheptaglutamate molecule has a molecular weight of 1215 and contains eight carboxyl groups, all of which are ionised at normal intestinal pH. Such a large hydrophylic molecule is a poor candidate for rapid transport across membranes and it would seem reasonable to suppose that it, like other nutritional macromolecules, might undergo degradation prior to absorption. Soon after the isolation of $PteGlu_7$ from yeast, experiments were carried out to test this possibility. It was shown that a purified yeast extract of $PteGlu_7$, which was microbiologically inactive prior to ingestion, gave rise to a urinary excretion product in man which was active for L. casei. (Bethell et al, 1947; Swedenseid et al, 1947). These workers also observed that purified yeast folate was absorbed more efficiently than the crude extract, an effect they attributed to the presence of a natural inhibitor of the enzyme responsible for deconjugation. Such an inhibitor has never been isolated although other workers have claimed that it can be separated from folate polyglutamates by column chromatography (Hoffbrand, 1969).

Rosenberg and Godwin (1969) have described

experiments in which purified yeast extract (95 - 98% $PteGlu_7$) was given orally and shown to produce a rise in L. casei-active serum folate. Treatment of the serum with folate conjugase in vitro did not increase the L. casei activity. Other workers have claimed that $PteGlu_7$ may be absorbed intact by man (Cooperman & Luhly, 1965; Benerjee & Chatterjea, 1966), but using synthetic polyglutamates labelled in various positions in the glutamate chain, Butterworth, Baugh and Krumdieck (1969) observed cleavage to PteGlu when $PteGlu_7$ and $PteGlu_3$ were given orally. Absorption of $PteGlu_7$ was significantly less efficient than the triglutamate or monoglutamate forms. Perry and Chanarin (1968; 1972) have used serum and excretion levels as well as erythrocyte incorporation and haematological responses to assess the relative absorption of equimolar oral doses of PteGlu and $PteGlu_7$ derived from yeast, and have concluded that $PteGlu_7$ was absorbed and utilised to about one third the extent of free folic acid.

Baker, Frank and Sobotka (1964) have suggested that the addition of two glutamate groups to the monoglutamate form was an essential prerequisite for its absorption and that higher conjugates must be deconjugated to the tri-glutamate form. They suggested that a defect in this mechanism is the causitive factor in the folate malabsorption of tropical sprue. No further evidence has appeared to support this hypothesis however.

In general, animal studies have confirmed the

principle of cleavage of polyglutamates as a prerequisite, or an adjunct, to absorption. Baugh et al (1971) used isolated segments of dog intestine to observe the appearance of folate in the venous blood after absorption of synthetic polyglutamates of various chain lengths from the perfused lumen. The rate of absorption was found to be roughly in inverse proportion to chain length, and PteGlu was the principal absorbed form. However, $PteGlu_2$ appeared in the mesenteric vein when $PteGlu_2$ or $PteGlu_3$ were present in the lumen. No form other than monoglutamate was absorbed when $PteGlu_7$ was the substrate in the lumen and the absence of the diglutamate under these conditions was difficult to explain. A similar observation *in vitro* was made by Rosenberg *et al* (1969) using the everted sac technique. When $PteGlu_7$ was placed in the mucosal solution, free PteGlu appeared on both sides of the sac wall but no $PteGlu_7$ was transported into the serosal solution.

It seems clear from these various types of study that in man, the rat, and the dog, folate polyglutamates are absorbed only very sparingly in the intact form although substantial quantities become available for absorption after deconjugation by intestinal enzymes. The site of this deconjugation and its functional relationship to the transport mechanism is unknown. It has been shown that in the hamster the folate conjugase of the intestinal mucosa is localised primarily in the lysosomal fraction (Hoffbrand & Peters, 1969), but

substantial quantities of the enzyme appear to be present in the brush border fraction of rat mucosal homogenate (Rosenberg & Godwin, 1969). A recent preliminary communication claims that less than 1% of conjugase activity in rat mucosal cells is located in the brush border, and that deconjugation of PteGlu occurs intracellularly (Halsted and Gotterer, 1974). A complete evaluation of this work must await the publication of full experimental details.

Most of the studies on folate absorption have used unconjugated folic acid. Apart from considerations of cost and availability, this seems to be justified by the fact that it is a very readily absorbed form. In the absence of contrary evidence it has been generally assumed that free folic acid and folate derived from deconjugation of polyglutamate both share the same transport mechanism. This view is supported by the observation that in adult coeliac disease the absorption of both PteGlu and $PteGlu_7$ are impaired to a similar extent though jejunal biopsy conjugase activity is only slightly depressed in comparison with normal levels. (Rosenberg _et al_, 1974).

Considerable controversy has arisen as to whether or not monoglutamate forms undergo metabolism during the process of absorption from the lumen. Whitehead and Cooper (1967) demonstrated by umbilical vein catheterisation that PteGlu is absorbed unaltered from the human intestinal lumen and appears as the intact

molecule in the portal vein. Butterworth, Baugh and Krumdieck (1969) working with leukemia patients, could find no evidence for reduction or methylation of plasma folate during the first two hours after the oral administration of either folic acid or synthetic polyglutamates. In a recent study by Leeming et al (1972) it was shown that oral administration of small doses of folic acid is followed by a slow rise in serum levels of 5-methyltetrahydrofolate, but that the conversion occurs at some site other than the intestine.

Chanarin and Perry (1969) observed that after an oral dose of dihydro- and tetrahydrofolic acid the only form detectable in the systemic circulation was methyltetrahydrofolate. An oral dose of folic acid was only partially methylated, substantial quantities appearing in the blood stream unaltered. Although these workers claimed that the site of methylation was the intestine itself, this conclusion is open to doubt as their observations were made on blood which had passed through the liver. More recently, Whitehead, Pratt, Viallet and Cooper (1972) have used the umbilical vein catheter technique to observe the prehepatic folate input from the intestine after the oral administration of 5-formyltetrahydrofolic acid. This study shows that conversion to the 5-methyl form occurs at some stage during intestinal absorption and that even large doses are fully methylated under these conditions.

Baugh and his colleagues (1971), in the course

of *in vivo* work with isolated intestinal segments of the dog, found no evidence for reduction or substitution of the plasma folate derived from either monoglutamate or polyglutamates in the lumen. Smith, Matty and Blair (1970) using everted sacs of rat intestine incubated in the presence of tritiated PteGlu found the predominant form transported into the serosal solution was unaltered folic acid, although a minor radioactive component was present which was not identified.

Cohen (1965), employing everted sacs of hamster intestine, recorded some methylation of PteGlu by microbiological assay of the serosal solution, but it is very likely that the methylfolate observed was derived from the tissue itself, or from lumenal micro-organisms (Blair *et al*, 1973). Perry and Chanarin (1973) have described everted sac studies in which the transport of folic acid, dihydro-, and tetrahydrofolic acid was found to result in the appearance of 10-formyl PteGlu as well as the formyl and methyl derivatives of tetrahydrofolic acid. This they ascribe to the need for a formylation step prior to methylation during transport. These results have been the subject of criticism on grounds of methodology and interpretation (Blair & Beavon, 1973; Blair *et al*, 1973). In another recent study employing the everted sac technique Selhub, Brin and Grossowicz (1973) have also reported the occurrence of tetrahydrofolic acid

and its methyl and formyl derivatives in the serosal solution after the uptake of PteGlu. This metabolism, they concluded, was not a prerequisite for transport, as even low concentrations of methotrexate were able to abolish reduction and substitution without any effect on the overall uptake of folate.

The most detailed study to date on this topic is that of Olinger, Bertino and Binder (1973). Using sheets of rat intestine mounted in lucite chambers they observed that at mucosal folate concentrations of 7, 20 and 2000 nMolar, 44%, 34% and 2% of PteGlu transported out of the mucosal compartment underwent metabolism to 5-methyltetrahydrofolic acid. The occurrence of 10-formyl folates was not recorded. Again, the addition of methotrexate abolished reduction and methylation but did not reduce the transport of PteGlu. Methylation was found to be associated with the jejunum as opposed to the ileum and coincided with the presence of high concentrations of dihydrofolate reductase.

Clearly the jejunum is an active site of metabolism of folates in mammals, though it is much less certain that this is an essential step in their absorption from the lumen. The observations by three independent groups that the *in vivo* absorption of PteGlu occurs without detectable metabolism (Whitehead & Cooper, 1967; Butterworth, Baugh & Krumdieck, 1969; Leeming *et al*, 1972) argue strongly against this, as does the work of Olinger, Bertino and Binder (1973) which demonstrates that even at

very low concentrations, and after long periods of incubation, less than half the absorbed folic acid is reduced and methylated during transport. The addition of one carbon fragment to PteGlu is accomplished only after reduction to the tetrahydrofolate form, and it seems reasonable to suppose that this might occur at some site in the intestinal wall after absorption from the lumen. The methylation of PteGlu would be rate-limited by the reduction step mediated by dihydrofolate reductase, and since this process is very slow for unreduced folate, this would account for the much more efficient metabolism of reduced forms such as 5-formyltetrahydrofolate.

It is difficult to account for all the contradictions which appear in these studies, but, as has been repeatedly pointed out (Blair, 1969; Leeming *et al*, 1972), techniques such as microbiological assay and chromatography of folates must be employed with great caution. Furthermore, the lability of the reduced folates may not always have been fully understood by some workers.

ii) Site of Absorption.

The two earliest *in vitro* studies, both of which used everted sacs of rat intestine, came to contradictory conclusions about most of the aspects of folate absorption with which they concerned themselves. Turner and Hughes (1962) have not recorded any difference in the rate of folate absorption for proximal and distal

intestine, although they do not appear to have examined the matter in detail. On the basis of their other observations, discussed below, they concluded that in common with a number of other vitamins, folic acid was absorbed by passive diffusion. Herbert and Shapiro (1962) used a combination of tracer studies and microbiological assay to study the rate of folic acid transfer by everted sacs from the proximal and distal third of the small intestine of the rat. They concluded that with a favourable concentration gradient net mucosal-serosal transfer occurs throughout the intestine, but more rapidly in the proximal third. With equal mucosal and serosal concentrations net transfer is abolished in the distal third but not in the proximal third. This they interpreted as evidence in favour of an active transport mechanism associated with the proximal jejunum.

More recently, Smith, Matty and Blair (1970) and Selhub, Brin and Grossowicz (1973) have observed that sacs from the upper intestine transported folic acid into the serosal solution more rapidly than sacs from the most distal region.

In general, *in vivo* animal techniques have supported the idea of a localised transport system. Burgen and Goldberg (1962) used *in vivo* cannulation of rat intestine and observed that transport from the lumen of the ileum was about 60% of that in the jejunum. They commented that although the difference was statistically significant it did not merit a more detailed investigation. Yoshino (1968) and Hepner (1969) both used perfused

segments of intestine *in vivo*, and both observed that absorption was more rapid in the jejunum than in the ileum. Hepner made the additional observation that transport seemed to be saturable in the jejunum and at high concentrations absorption from the ileum was therefore more rapid.

Recently a much more detailed study by Izak *et al* (1972) has confirmed that the jejunum is the principal site of absorption in the rat. Using intact animals, sections of intestine were "shunted", or removed entirely, and the rate of absorption from the functional portions was observed in normal, folate-deprived, and folate-loaded rats. It was concluded that absorption from the jejunum was 5 to 6 times that of the ileum, and that 25 to 40 cm. of intact jejunum were required for normal absorption. Furthermore, in folate depleted rats the absorptive capacity of the ileum was doubled, although it never reached that of the jejunum.

In vitro techniques have provided three more interesting observations in animals other than man. Olinger, Bertino and Binder (1973) used a combination of the everted sac technique and the lucite chamber to compare mucosal influx with transmural flux. They observed that although the rate of mucosal uptake was greater in the jejunum than in the ileum, the transmural flux of folate across the ileac mucosa was greater than across the jejunal mucosa.

An interesting inter-species difference appears to have been indicated by the work on isolated cells of Hepner and Herbert (1969) and Momtazi and Herbert (1973). Whereas the earlier work with isolated mucosal cells from the guinea pig revealed no difference in absorption rate between cells from the jejunum and cells from the ileum, jejunal cells from the rat absorbed folic acid several times more rapidly than the ileac cells. (Momtazi & Herbert, 1973).

In man it has been observed that folate malabsorption is associated with diseases affecting the jejunal mucosa but not with diseases of the ileum, and this is taken as evidence that the jejunum is the main site of folate absorption. This simple situation is complicated by the work of Franklin and Rosenberg (1973) who have observed folate malabsorption associated with ulcerative colitus, a disease primarily affecting the colon. It is not yet clear however, whether or not this implies a hitherto unobserved jejunal lesion.

Hepner *et al* (1968) employed a double lumen perfusion technique to study in some detail the rate and location of human folate absorption. Amongst their conclusions was that the principal absorption site of crystalline PteGlu was the upper jejunum, and that no absorption could be observed in the ileum. This was not, however, the observation of Baker *et al* (1969). These workers compared the absorption of PteGlu and polyglutamate in normals, with that in two elderly patients suffering

from major intestinal resections. They observed that normal subjects were able to absorb all the folate forms. In the patient with the most extensive resection all of the small bowel other than the first 10 cms of jejunum were absent and no absorption of PteGlu or polyglutamate was observed. In the second patient only the jejunum had been removed, and it was found that although PteGlu was absorbed, polyglutamates were not. It was concluded that although monoglutamate folate forms were absorbed throughout the small intestine, a normal jejunum was necessary for the absorption of conjugated forms, as only in the jejunum was sufficient conjugase available to release the monoglutamate (see previous section).

The combined evidence of these studies indicates that in rat, and probably in man, absorption of folate from the lumen occurs most rapidly in the jejunum and least rapidly in the distal ileum. In interpreting this observation it may be important to recall the findings of Olinger, Bertino and Binder (1973) that mucosal-serosal flux was more rapid in the ileum. As will be discussed later, there is strong evidence that folate is retained within the gut wall after absorption and this effect may itself be a localised phenomenon.

iii) The Kinetics of the Uptake Process.

One of the most frequently quoted diagnostic characteristics of a structurally specific transport mechanism is that saturation of rate occurs at high substrate concentrations. In systems where this is coupled with the ability to transport substrate against a concentration gradient it is usually concluded that an energy dependent transport system is at work.

Turner and Hughes (1962) used the everted sac and the isolated _in vitro_ segment to investigate the absorption of a number of vitamins, including PteGlu. They found no evidence for accumulation of folic acid against a concentration gradient and no net flux in either direction when equal concentrations of folate were present either side of the gut wall. These findings were confirmed in a similar study by Spencer and Bow (1964). Both groups worked at concentrations between 10^{-8}M. and 10^{-6} M. and produced no evidence for saturable uptake. Herbert and Shapiro (1962) claimed that net mucosal-serosal flux occurred in the absence of a favourable concentration gradient in jejunal sacs, and that movement of labelled folate occurred against a concentration of unlabelled folate. Owing to the absence of experimental details it is difficult to assess these conclusions.

Smith, Matty and Blair (1970) reported a saturable uptake process in the rat jejunum and tentatively assigned a Michaelis-Menten K_m of 0.7×10^{-6}M. Smith (1973) has distinguished between the accumulation

of folic acid at low concentrations by the gut wall, and the overall transport into the serosal solution. The former she found to be a saturable process between 10^{-8}M and 10^{-7}M, but she suggested that at higher concentrations a passive process was predominant and no evidence was found for a saturable mechanism for transmural transport into the serosal solution.

Both of these studies drew attention to the fact that folate was accumulated by the gut wall and that at low external concentrations this led to high concentration ratios which would suggest uphill transport if all the intracellular folate was in a freely diffusible form. Tissue accumulation has been confirmed in the everted sac preparation by Selhub, Brin and Grossowicz (1973) but using higher mucosal concentrations than Smith they have shown that the transport of folic acid into the serosal solution is saturable at about 3 x 10^{-6}M and above. Blair, Johnson and Matty (1974) have recently confirmed that both tissue accumulation and serosal transfer are saturable processes in the rat jejunum.

One advantage of the everted sac technique is that it provides an easy test for transport against a concentration gradient in that transported material accumulates in a relatively small volume of the serosal solution. In the case of actively transported materials such as certain sugars and amino acids, high concentration ratios are rapidly achieved. Smith, Matty and Blair (1970) and Selhub, Brin and Grossowicz (1973) have both

observed serosal:mucosal concentration ratios greater than 1, but both have suggested that they may have been due to equilibration of ionised folic acid under the influence of the transmural potential difference which existed in their preparations.

The *in vivo* studies of Burgen and Goldberg (1962), Yoshino (1968) and Hepner (1969) all produced evidence for a saturable mechanism of uptake from the jejunal lumen, but Yoshino was not convinced of the existence of an active transport process. A contradictory finding has recently been provided by Halsted and Mezey (1972) who studied the absorption of tritiated PteGlu from perfused loops of rat jejunum over a concentration range of 5.7×10^{-8}M to 5.7×10^{-4}M. Uptake was found to be linear over this concentration range.

Hepner and Herbert (1969) appear to have observed saturation kinetics for the absorption of folic acid by isolated guinea pig jejunal cells; but in a similar study, Momtazi and Herbert (1973) found a linear uptake over a somewhat larger concentration range in jejunal cells from the rat.

To summarise these findings, it appears that the majority of studies which have looked for a saturable uptake process have demonstrated one, the exceptions being Halsted and Mezey (1972) and Momtazi and Herbert (1973). It must be noted that those techniques which observe the disappearance of folate from the lumen of perfused intestine do not easily distinguish between the uptake by the gut wall and the overall transfer into the

circulation. Several of the later everted sac studies emphasize that some retention mechanism exists in the gut wall for folic acid and this was recently confirmed by another _in vitro_ technique (Olinger, Bertino & Binder, 1973). Baugh _et al_ (1971) used an _in vivo_ technique to study the appearance of mono-glutamyl folate in the blood after the perfusion of polyglutamates into the intestinal lumen of dogs. They recorded that as much as 48% of the absorbed material was bound within the gut wall. This retention phenomenon is of considerable interest as any attempt to understand the mechanism of mucosal absorption must distinguish between active absorption against a concentration gradient, and passive transport into a compartment from which substrate is removed from osmotic activity by physical binding.

The absorption of folic acid from the small intestine of man was studied by Hepner _et al_ (1968) using a double-lumen perfusion technique. They concluded that folic acid was absorbed primarily in the upper jejunum by a saturable active transport process. These conclusions have however been extensively criticised and must be regarded as unconfirmed (Sladen, 1968; Helbeck, 1968; Scott & Orsi, 1968; Matty & Blair, 1968; Hepner _et al_, 1968b).

iv) The effect of inhibitors.

Inhibitors of biological transport systems fall into two principal groups, classified functionally as the metabolic inhibitors and the competitive inhibitors. Metabolic inhibitors are thought to act by interference with some step in the physiological mechanism which supplies metabolic energy necessary for accumulation against an electro-chemical potential; significant inhibition of a transport system in the presence of such an agent is usually taken as an indication of energy dependent transport. The other class of inhibitors function in transport systems which are substrate specific, and are assumed to depend upon competition during the formation of an enzyme-substrate complex, or a mobile carrier-substrate complex. Such inhibitors are usually structural analogues of the experimental substrate.

Methotrexate has the rare distinction of at least three potential roles as an inhibitor of folic acid transport, though none have been unequivically established so far. The very high affinity of methotrexate for dihydrofolate reductase results in virtually irreversible binding and a consequent blockage of this enzyme in cellular metabolism. If reduction of folate was a critical step in the transport process, or if a carrier having a similarly high affinity was involved, then methotrexate would be a very effective inhibitor indeed. Alternatively the toxic effect of aminopterin and

methotrexate on the intestine might be expected to lead indirectly to reduced transport, particularly if an energy dependent process was involved (Zamchek, 1960; Hepner, 1969).

Burgen and Goldberg (1962), Yoshino (1968) and Halsted and Mezey (1973), have all used methotrexate as a candidate for competitive inhibition in *in vivo* perfusion studies and have all concluded that it had no effect on intestinal absorption as observed by their techniques. Hepner (1969), on the other hand, observed that folic acid absorption was inhibited by the presence of methotrexate and that the degree of inhibition increased with concentration. This he attributed to competitive inhibition either by competition for a carrier or by blockage of reduction as a step in folate absorption.

In vitro techniques have again provided a more detailed picture of the effects of methotrexate on folate absorption and the accompanying metabolism in the jejunum. Olinger, Bertino and Binder (1973) have shown that though methotrexate inhibits jejunal conversion of folic acid to 5-methyltetrahydrofolate by the blockage of DHF-reductase it has no effect on mucosal uptake, and actually increased the transmural movement of folic acid. These results are partially confirmed by Selhub, Brin and Grossowitcz (1973). Methotrexate was found to inhibit reduction of folic acid at low concentrations, and would substantially reduce tissue accumulation at high concentrations (3×10^{-6}M Methotrexate). No effect on

transport of folic acid into the serosal solution could be demonstrated.

From these studies it seems reasonable to conclude that methotrexate has no direct effect on the passage of folic acid across the mucosal-membrane in the rat and that if, say, a carrier mediated transfer system exists it has little or no affinity for methotrexate. In this regard it is interesting to note the work of Leslie and Rowe (1972), who, working with an isolated mucosal cell system which did not accumulate folates, demonstrated the presence of a protein binding site for folic acid which had no affinity for methotrexate.

Hepner's results (Hepner, 1969) are puzzling in the light of subsequent literature. An interesting additional observation of his was that methotrexate given subcutaneously 3 hours prior to the infusion of PteGlu also diminished its absorption from jejunal segments. Hepner himself attributed this to metabolic inhibition of an energy dependent "pump", as is discussed below.

Clearly, methotrexate is able to inhibit reduction of folate by DHF-reductase at some site in the gut wall (Olinger, Bertino & Binder, 1973) and this may or may not be related to its inhibition of tissue accumulation. At any rate no inhibitory effect on transmural flux has been demonstrated *in vitro* and this is consistent with the conclusion that reduction of folate is not a prerequisite for absorption. Recently, Freedman *et al* (1973) have extended this work from rat

to man using the tritiated folic acid urinary excretion test. Oral doses of methotrexate in pharmacological amounts had no effect on the absorption of small oral doses of folic acid.

Several types of metabolic inhibition study have been carried out and the usual variety of conclusions drawn. Turner and Hughes (1962) measured folic acid transfer across everted sacs and *in vitro* segments in the presence of cyanide, phlorizin, azide, iodacetate and DNP. They concluded that no adverse effect on transport occurred and in fact a small increase in the mucosal-serosal transfer of folic acid and other B-group vitamins was detected in the presence of the metabolic inhibitors. Yoshino (1968) was also unable to detect any effect of DNP on the uptake of crystalline PteGlu, and this was one of the results which led him to conclude that folic acid was absorbed passively.

A quite different set of results and conclusions was provided more recently by Kesavan and Noronha (1971). Using intact rats and everted sacs they have reported that folate absorption is sensitive both to whole-body x-irradiation and the presence of DNP in the incubation medium, and that these effects were reversed by the addition of ATP. These results they attribute to the presence of an energy dependent folate uptake mechanism, as the deconjucation of polyglutamate forms appears to be unaffected; they provide an interesting parallel to those of Hepner who attributed the effect of subcutaneous

methotrexate to its known interference with the aerobic metabolism of the intestine (Hepner, 1969; Zamcheck, 1960).

A puzzling observation was made by Smith (1973), who showed that folate accumulation in the gut wall *in vitro* was inhibited both by fluoride ions, and by methotrexate in phosphate buffered medium but not in bicarbonate buffer. No explanation was suggested and it is not known whether this was a metabolic effect or not.

Enhancement of folic acid absorption in the presence of glucose seems well established. Such an effect has been reported by Smith, Matty and Blair (1970) in everted sacs, Momtazi and Herbert (1973) in isolated rat jejunal cells and by Gerson *et al* (1971) using the triple lumen perfusion technique in man.

The inhibitors discussed so far have been used as a result of established physiological practice; another group of substances, which have been shown to give rise to the symptoms of folate deficiency when used clinically have attracted interest in recent years. The subject of drug-induced megaloblastic anaemias is very complex and improperly understood and no detailed discussion will be attempted here. However it has been suggested that two classes of drugs which induce folate deficiency, the anti-convulsants and some oral contraceptives, may interfere with the intestinal absorption of dietary folate, and it will be appropriate

to include a brief discussion in this section.

It has been known for a number of years that long term treatment of epilepsy with anti-convulsants such as diphenylhydantoin leads to low serum folate levels and, on occasion, megaloblastic anaemia; this has led some workers to suggest as a mechanism a direct interference with folate uptake (Hawkins & Meynell, 1958; Klipstein, 1964; Meynell, 1966). In humans, it has been reported that the absorption of oral doses of PteGlu is inhibited by diphenylhydantoin, evidence being obtained by serum folate measurements and triple lumen intubation experiments (Meynell, 1966; Dahlke & Mertens-Roesler, 1967; Gerson *et al*, 1972). Furthermore, it has been reported that the absorption of natural yeast folate conjugates was impaired by a simultaneous oral dose of diphenylhydantoin (Rosenberg *et al*, 1968; Hoffbrand & Nechelles, 1968), and since the activity of folate conjugase enzymes *in vitro* was shown to be suppressed by the same drug it was suggested that the impairment of intestinal deconjugation of polyglutamates was responsible for malabsorption. However, all these findings have been contradicted by other groups and the matter remains open (Baugh & Krumdieck, 1969; Houlihan *et al*, 1972; Perry & Chanarin, 1972).

Benn *et al* (1971) have used a different approach to the problem and suggested an alternative mechanism for the effect of diphenylhydantoin. Having

ascertained that both phenytoin sodium and sodium bicarbonate impair the absorption of a simultaneous oral dose of PteGlu in man, they then demonstrated by direct intraluminal monitoring that administration of sodium bicarbonate and chronic use of phenytoin both induced an increase in the jejunal pH. The mechanism by which phenytoin sodium alters the jejunal pH is unknown, but a theoretical argument for its effect on folate absorption was suggested and this will be discussed in the context of a general theory of folate absorption in another section. It should be noted here though that the impairment of folate absorption by sodium bicarbonate has been disputed by another group (Perry & Chanarin, 1972).

Decreased absorption of folic acid in the presence of diphenylhydantoin has been demonstrated *in vitro* by Hepner (1969) and Momtazi and Herbert (1973).

There have been a number of reports of low serum folate levels and acute megaloblastic anaemia in patients using oral contraceptives, and the situation is rather similar to that of anti-convulsive therapy, as are the explanations which have been suggested. This, as well as many other aspects of drug induced folate deficiency and megaloblastic anaemia is discussed in detail in a recent review article (Stebbins, Scott & Herbert, 1973).

Megaloblastic anaemia is a common complication of chronic alcoholism (Herbert, Zalusky & Davidson, 1963) and this is probably the result of various effects of

alcohol on cellular metabolism as well as of the poor diet which often accompanies the condition (Stebbins, Scott & Herbert, 1973). Experimental evidence for intestinal malabsorption associated with alcoholism has been provided, though it appears that as the malabsorption can be reversed by an improved diet it is more likely to be an intestinal defect associated with poor nutrition than a specific interference by alcohol with a folate absorption site (Halsted, Griggs & Harris, 1967; Halsted, Robles & Mezey, 1971).

v) Folate Malabsorption in Intestinal Disease.

The most common folate malabsorption syndrome is probably that associated with Adult Coeliac Disease, also known as non-tropical sprue or idiopathic steatorrhea (Cooke, 1968). The disease is characterised by lesions of the small intestine which are at their most severe in the duodenum and proximal jejunum, but becoming milder down the length of the intestine. The lesions take the form of villous atrophy leading to a 'flattened' mucosa often showing a characteristic mosaic pattern when viewed under low power microscopy. A reversal of these symptoms is brought about by a gluten-free diet, recovery being more satisfactory in the less severely affected lower jejunum and ileum. These gross histological changes are accompanied by disorders in the mucosal cells themselves including structural irregularities of the nucleus and brush border.

The symptomology of the condition consists of general ill-health and disturbances of physique associated with the malabsorption and defective metabolism of a variety of dietary components. Anaemia is said to be present in a majority of untreated cases and this is associated with the low serum folate levels found in most cases (Klipstein, 1966; Chanarin, 1969). Chanarin (1969) has provided an analysis of the literature on the occurrence of folate malabsorption in coeliac disease and has shown that of 281 patients, 252 were shown to be suffering from defective folate absorption and that all the techniques commonly used in its measurement gave similar results. The mechanism of folate malabsorption in coeliac disease is not known, but the matter is discussed in detail in Section 4.

Tropical sprue is a somewhat similar condition of unknown aetiology, but which is endemic in many tropical areas. Once again the disease leads to macrocytic anaemia due to folate malabsorption, but it is of particular interest that many of the pathological changes associated with the condition may be reversed, and in mild cases cured, by prolonged administration of folic acid. It has been reported that a factor present in calf jejunum and normal human jejunal fluid is capable of reversing the folate malabsorption of tropical sprue when given orally in combination with folic acid (Baker _et al_, 1968).

As one might expect, poor folate absorption is associated with several other intestinal defects which affect the upper small bowel (Hoffbrand, 1971). Franklin and Rosenberg (1973) have shown that ulcerative colitis and granulomatous colitis, diseases not otherwise known to affect the upper intestine, are accompanied by low serum folate levels resulting from folate malabsorption. They also report that malabsorption is aggravated by the use of the drug salicylazosulfapyridine during therapy, and they have shown that this material impairs folate uptake by rat intestine *in vitro*.

Abnormalities in the bacterial flora of the jejunum have been shown to disturb the serum folate levels in some patients, either by reducing the availability of normal dietary folate, or on the contrary, by contributing to the intestinal folate content by bacterial synthesis (Hoffbrand *et al*, 1971). That no such effects have been demonstrated in relation to ileac bacteria may be taken as further evidence that it is the jejunum which is of primary importance in human folate absorption.

Of much greater interest are the specific defects of folate absorption which are occasionally reported. Luhby *et al* (1961), Lanskowsky *et al* (1969) and Santiago-Borrero (1973) have all described subjects in whom normal development was impaired from birth by an inability to absorb dietary folate or small doses of PteGlu. This has led to recurrent macrocytic anaemia, and varying degrees of mental retardation, but the acute

symptoms are controlled by large oral doses of PteGlu or by regular intramuscular injections. These conditions are, happily, rare, but apart from the suffering they cause their great interest lies in the fact that they seem to be quite specific congenital defects in the intestinal uptake of folate, and are not associated with visible abnormalities in the jejunal mucosa. Furthermore, Lanskowsky et al (1969) have shown that whereas the normal plasma:cerebro-spinal fluid folate ratio is 1:3, the CSF folate level in their patient remained at 0 even in the presence of artificially high blood folate levels.

vi) Intestinal Absorption of 5-Methyltetrahydrofolic Acid.

Most of the work cited so far has been concerned with the transport of folic acid. Before passing on to a brief discussion of folate transport at sites other than the intestine, it is important to mention the few reports which have appeared on the subject of the intestinal absorption of 5-methyltetrahydrofolate.

Strum et al (1971), using in vitro techniques, have investigated most of the transport criteria discussed above. They report that absorption of labelled 5-methyl THF 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 anaerobiosis, 10^{-4}M DNP, glucose and sodium concentration. Competition

studies were carried out with 50 nMolar, 5-methyl THF in the presence of 500 nMolar folic acid, methotrexate and 5-formyl tetrahydrofolic acid. No inhibitory effect was observed, though in the presence of 5-formyl THF a significant stimulation of 5-methyl THF transfer was recorded. On the basis of these observations it was concluded that the absorption of 5-methyl THF in the intestine did not involve a specific transport system and could probably be accounted for by passive diffusion.

An important and so far unique observation in the folate field is described by Weir *et al*, (1973). Having prepared pure samples of the two diastereoisomers of 5-methyl tetrahydrofolate, they showed that the naturally occurring isomer, which is biologically active for micro-organisms, was preferentially absorbed from the intestine, and preferentially reabsorbed by the kidney in man. They suggest that in the human intestine there exists a transfer system which is structurally specific for the naturally occurring diastereoisomer of 5-methyl-tetrahydrofolic acid. This interpretation has, however, been recently criticised (Blair & Matty, 1974).

vii) Other Folate Transport Systems.

Apart from a few very specialised systems which will be discussed below, little is known about the other sites of folate transport in the mammal.

Considerable data has been accumulated on the rate of clearance from the plasma of an injected dose of

folate in healthy and abnormal human subjects and this is well summarised by Chanarin (1969). Briefly, PteGlu and its derivatives are taken up from the plasma at varying rates, the disappearance of PteGlu being more rapid than that of its reduced 5-formyl and 5-methyl derivatives. Labelled folate may be subsequently displaced from the tissues by a cold folate flushing dose but the amount that can be displaced decreases with time. The mechanism of tissue accumulation is poorly understood but it appears to involve binding to dihydrofolate reductase and, no doubt, to other binding proteins. On the basis of the rapidity and selectivity with which folic acid is removed from the plasma, it has been concluded by some workers that specific "pump" mechanisms must exist within the tissues (Johns, Plenderleith & Hutchinson, 1963; Johns & Plenderleith, 1963; Johns et al, 1961).

Goresky et al (1963) have studied the renal reabsorption of folic acid in detail, using the single renal intra-arterial injection technique in the anaesthetised dog. They conclude that though much plasma folate is protein-bound and therefore non-filterable, glomerular filtration of the unbound fraction occurs and is followed by reabsorption in the renal tubule. Uptake of folate by the tubular cells appears to be a saturable process inhibited by methotrexate. Accumulation of folate occurs intracellularly and the stored material can be displaced into the tubule lumen by a process described by the authors as counter-transport.

The processes of folate exchange in a number of tumour cells have been examined in some detail. Hakala (1965 a,b) has reported that the uptake of methotrexate by sarcoma 180 cells is a process of passive influx counterbalanced by an energy dependent efflux. The net transport into the cell was increased by the presence of DNP, which was thought to inhibit active efflux, or by the presence of calcium ions or low pH in the extracellular medium which, it was claimed, reduced the net charge on the methotrexate molecule, thereby increasing its lipid solubility and enhancing passive influx. In the Yoshida sarcoma cell methotrexate uptake appears to be an active process, exhibiting saturation and competition with folic acid (Divekar *et al*, 1967).

The kinetics of folate exchange in the L1210 Leukemia cell and the Ehrlich ascites cell have been described in a series of detailed and careful papers (Goldman, Lichtenstein & Oliverio, 1968; Lichtenstein, Oliverio & Goldman, 1969; Goldman, 1971). Folate absorption in these cells appears to be a complex system, involving both passive and energy dependent processes as well as intracellular binding and metabolism. However, Goldman (1971) appears to have established the existence of carrier-mediated hetero-exchange diffusion in these cells and has thus provided one of the most well defined descriptions of folate transport yet achieved.

Section I (3).

The aims of the present study.

In considering the movement of a solute across the mucosal epithelium of the intestine it is necessary to distinguish between non-mediated transfer, in which the solute passes across the mucosal barrier by simple diffusion in the non-aqueous phase, and "facilitated" transport in which the solute passes via specific channels, assumed to be discrete components of the epithelial cell membrane. This latter concept is usually invoked to explain the localisation and saturability of an intestinal transport system. The existence or otherwise of a specific folate transport mechanism is the fundamental problem which emerges unanswered from the literature. The overall strategy of this study has been to investigate more fully those areas of the literature which seemed contentious, and to seek new information in directions which might be expected to lead to crucial mechanistic tests.

The localisation of folate transport in the proximal half of the small intestine seems well established and this point has not been investigated any further during this study. As has been shown, there is much evidence to suggest that the jejunal absorption of folic acid is a saturable process but many workers have merely recorded that the percentage absorption of an intraluminal dose is reduced at high concentrations; consequently the first priority of this project was to confirm the saturability

of the uptake process and to try to define the kinetics with some accuracy.

The occurrence of substrate competition and exchange diffusion at membrane surfaces is well established for many biological transport systems (Wilbrandt & Rosenberg, 1961) and the occurrence of such phenomena is strongly indicative of a specific transport mechanism, usually described in terms of "carriers". Methotrexate has been the choice of most previous workers as a candidate for competitive inhibition despite the fact that, as is discussed above, it is likely to have other effects on the intestine besides competition for hypothetical carriers. Competition studies in this work have been undertaken using 10-formylfolic acid, a compound not previously employed for this purpose.

Besides the unexplained acceleration of folic acid flux in the presence of methotrexate (Olinger _et al_, 1973), and of 5-methyl THF transfer in the presence of 5-formyl THF (Strum _et al_, 1971) there has been no previous suggestion of counter-transport effects connected with folate transfer in the intestine, and no attempt appears to have been made to detect them. Since this seemed a possibly fruitful line of investigation, a series of experiments was put in hand to test for the possibility of counter-transport at the mucosal surface.

There has been no agreement in the literature as to the effect of metabolic inhibitors on folate transport in the intestine. Experiments were therefore

put in hand to investigate the matter.

Leslie and Rowe (1972) raise two problems with their work on isolated mucosal cells. First there is the fact that the existence of a membrane protein with a strong binding affinity for folate could be interpreted as circumstantial evidence for a carrier transport system; at the same time the inability of viable mucosal cells to accumulate folate is puzzling if the transepithelial absorption route lies intracellularly. In view of the reports of other workers that isolated intestinal cells capable of folate absorption have been prepared by other methods (Hepner & Herbert, 1969; Momtazi & Herbert, 1973), it seemed that the malfunction of folate transport in the cell preparation of Leslie and Rowe might provide a significant clue to the transport mechanism in functionally normal cells. With this in mind, a series of experiments was undertaken using isolated cells prepared by dis-aggregation with hyaluronidase, and attempts were made to correlate their folate transport characteristics with their histological appearance.

Section 2 of this thesis is a general description of the physiological and chemical techniques used through-out this study, together with some discussion of the factors which led to their use. The methodological details peculiar to specific experiments are included, where necessary, in Section 3, together with the results obtained in each experiment. A detailed discussion of the results forms Section 4, which places the conclusions drawn from this work in the context of specific models for membrane transport.

SECTION 2.

GENERAL METHODS AND MATERIALS.

Part 1. Materials.

Part 2. Physiological techniques.

Part 3. Chemical techniques and methods of assay.

Section 2. (1)

i) Animals.

All the animals used in this study were male Wistar rats, between 180 and 210 gms in weight, bred and reared in the animal house at Aston University. Animals were transferred from the animal house to the laboratory the day before use, and housed in standard animal cages overnight. This seemed to be important; it was the author's impression that animals transferred and used immediately on the same day gave results for folic acid transport significantly different to those of animals kept overnight. No attempt was made to confirm this impression but an effort was made to ensure uniformity of treatment as far as possible. All the animals used in the kinetic studies were deprived of food for about 18 hr. overnight prior to sacrifice, and all 5 litter-mates in each experimental group were used on the same day. In later experiments, notably those with isolated cells, animals were deprived of food for about 4 hrs. on the morning of sacrifice. All animals were allowed water *ad libitum* prior to use.

The constituents of Heygates 41 b diet, on which the rats were fed throughout this study, is shown in Table 2.1.

TABLE 2.1.

Composition of Heygates Diet 41B.

	%	Vitamins:	
Crude protein	17.069	B_{12}	14.09 μg/kg
Crude oil	2.732	E	19.25 mg/kg
Crude fibre	4.352	Thiamine	6.201 "
Digestible oil	2.114	Riboflavin	3.499 "
Digestible fibre	1.723	Niacin	55.1 "
Arginine	0.801	Pantothenic acid	14.99 "
Lysine	0.877	Choline	1311 "
Methionine	0.309	Biotin	0.904 "
Cystine	0.261	Folic acid	0.838 "
Tryptophan	0.192	Pyridoxine	5.95 "
Histidine	0.326	Inositol not less than	220 "
Leucine	1.089	A	10,220 I.U /kg
Tyrosine	0.326	D_3	2,556 "
Isoleucine	0.710	Iron	65 mg/kg
Phenylalanine	0.674	Manganese	32 "
Threonine	0.555	Copper	7 "
Valine	0.837	Iodine	4.18 "
Glycine	0.981	Cobalt	0.89 "
Calcium	1.3	Zinc	8.29 "
Phosphorus	0.72		
Ca:P	1:0.6		
Sodium	0.575		
Chloride	0.154		

ii) Folates.

Pteroyl-L-glutamic acid was obtained from Koch Light Ltd. and used throughout the study. Pteroyl-D-glutamic acid, methotrexate and pteroic acid were gifts from Lederle Laboratories Ltd. 10-Formylfolic acid was synthesised in this laboratory by Dr. J.R.G. Beavon (Beavon, 1971).

Radioactive pteroyl-L-glutamic acid and 5-methyltetrahydrofolic acid was obtained from The Radiochemical Centre, Amersham. Folic acid-2-C14 is supplied by the manufacturer as the potassium salt, having a specific activity of about 55 mCi/mmol and an isotopic abundance of about 87% at carbon atom 2. The specified radiochemical purity is 98% and this was confirmed by thin layer chromatography as described in Part 3 of this section.

Folic acid has been shown to be stable for at least a year in aqueous solutions with a pH about 7.4, stored in the dark at room temperature (Biamonte & Schneller, 1951). All the folate compounds used in this work were stored for long periods as the dry solid. Solutions prepared for a particular experiment were stored frozen at $-20^{o}C$, except for 5-methyltetrahydrofolic acid which, being less stable than the other compounds, was used as the undiluted labelled material immediately after opening the manufacturer's vial. There was no detectable decomposition of folic acid during 60 minutes incubation in the absence of tissue.

iii) Physiological Salines.

Standard Krebs-Ringer solution was used for transport studies throughout this work (Umbreit, Burris & Stauffer, 1951). The solution contained NaCl (127 mM), KCl (5.1 mM), KH_2PO_4 (1.3 mM) $MgSO_4.7H_2O$ (1.3 mM), $CaCl_2$ (1.0 mM). The solution was buffered to 7.4 with 11 parts of 0.1 molar phosphate buffer per 100 parts of the above solution. This solution, with the addition of glucose (28 mM), is referred to as "the standard incubation medium" throughout the text.

A second incubation medium (Leslie & Rowe, 1972) containing NaCl (120 mM), K_2HPO_3 (3.0 mM), $MgCl_2$(1.0 mM), $CaCl_2$ (1.0 mM) and Tris-Cl buffer (20 mM, pH 7.4) was used, with the addition of 1 mg/ml hyaluronidase and 1 mg/ml bovine serum albumin, as an isolation medium for mucosal cells.

Inorganic saline constituents (analar grade) and glucose were obtained from Fysons Ltd. Hyaluronidase, and bovine serum albumin (fraction V) were supplied by Sigma Chemical Co.

iv) Scintillators and Associated Chemicals.

Scintillation cocktails were obtained from Nuclear Enterprises Ltd. (NE 220) and Fisons Ltd. (Dioxan Cocktail 'D'; C^{14} Absorber 'P'). Other materials were obtained from Koch Light Laboratories Ltd. (ethanolamine, 2,5-diphenyloxazole, 1,4-di-(2-(5-pheny-loxazoyl))-benzene, and scintillation grade toluene).

Section 2. (2).

In vitro intestinal preparations are based on the assumption that useful observation can be made upon tissues which, deprived of their normal vascular circulation, derive their metabolic requirements from an artificial bathing medium. This usually requires that while the mucosal surface is exposed to a well stirred nutritive medium, the serosal surface is available for the collection of the transported material. In working with this kind of preparation the intestinal physiologist must ensure that his preparations maintain sufficient functional integrity to remain of relevance to the *in vivo* situation which is his ultimate interest.

Despite the ease and simplicity of preparations such as the everted sac, *in vitro* techniques are open to considerable methodological variation and this may have a substantial influence on their physiological behaviour. Levine *et al* (1970) pointed out that almost nothing was known about the structural integrity of the everted sac during incubation, despite its prominent role in the physiological literature in the years since its introduction. Levine and her co-workers examined the histological appearance of everted sacs of rat and hamster intestine after various periods of incubation at different temperatures, and compared material from animals sacrificed by decapitation with that of animals killed under anaesthesia. Their conclusion was that in animals

killed by decapitation the structural dissolution of the mucosal epithelium began after a few minutes of incubation at 37° and led to complete destruction after 1 hour. A disturbing implication of their work was that this destruction was a result of water absorption by the tissue and that although it could be minimised by killing the animal under anaesthesia and incubating the tissue at 27° this merely reflected the fact that the normal transport activity of the tissue was suppressed under these conditions. More recent work by Gibaldi and Grundhofer (1972) has attempted to correlate these observations with the transport of various drugs. It was reported that whereas the rate of transport of lipid soluble neutral substances remained constant during 2 hours of incubation, the permeability of the sac towards ionised compounds increased continuously, and this may parallel the destruction of the mucosal epithelium which is the rate limiting barrier for the transport of polar molecules. These studies raise serious questions and suggest that not only is the usefulness of *in vitro* intestinal tissues restricted to short periods of incubation, but that their behaviour is very likely to vary with the details of their preparation.

In general the approach in this study has been to develop preparations which seem both viable and functionally intact by the standards of what seem to be the most competent reports in the literature, and then

to aim for consistency in their use throughout the project. At the same time, the possible pitfalls of *in vitro* preparations have been borne in mind. The work of Levine *et al* sounds a note of warning, and in particular it stresses the fact that the method by which the animal is killed may be of considerable importance. Appendix I. carries this discussion further and describes an experiment designed to decide upon the best method of sacrifice. The gross destruction of the mucosal epithelium described by Levine *et al* is clearly visible to the naked eye when it occurs. In the author's experience, the pale "mushy" appearance of the sac associated with mucus and cellular material in the incubation medium, has rarely been observed within the first two hours of incubation at 37°C, although the effect may be very quickly induced by incubating the tissue at higher temperatures. Nevertheless, with few exceptions the experiments in this study were restricted to incubation times of 30 minutes, and in many experiments, much less. No gross damage was observed during this period and the preparations have retained a pink appearance with the villi visible to the naked eye on close examination. Appendix III describes viability studies carried out on the tissue.

i) Preparation of the Everted Intestine.

Animals were killed by a single blow on the head followed immediately by decapitation. The abdomen was opened with a mid-line incision and the entire small intestine was removed by severing it at the duodeno-jejunal flexure, and at the ileo-caecal junction. Most of the mesentery came away as the intestine was removed; no attempt was made to strip away the remaining fat. The intestine was then immediately transferred to a beaker containing 50 ml. of oxygenated saline, (0.9% NaCl) at 0-4°C., and the lumen was flushed through with 50 ml. of the same solution delivered from a wash bottle via the jejunal opening.

At this stage it will be as well to mention the considerations which led to the use of chilled tissue as opposed to tissue maintained at 37°C. Fisher and Parsons (1949, 1950) have stressed the vulnerability of the rat intestine to damage by anoxia. During the development of the continuously perfused isolated segment they observed that even very brief interruptions in the blood supply to the intestine, prior to the establishment of perfusion, led to vigorous motor activity and a marked acid pH shift in the mucosal perfusate. These symptoms they attributed to predominantly glycolytic respiration extended long after the period of anoxia. Other observations have shown that in cat intestine mucosal lesions are induced by mimicked shock leading to low blood flow in the villous tips, and that these are

prevented by intraluminal perfusion with oxygenated saline (Ahren & Haglund, 1973). On the other hand, Evans _et al_ (1971) have reported that the aerobic metabolism of isolated mucosal cells from the rat is quickly re-established after long periods of anoxia.

In any case, it is obviously impossible to maintain an intact blood supply during the process of eversion and preparation of sacs and rings, and some degree of restricted oxygenation is unavoidable. One way of circumventing the problem is to lower the temperature of the tissue so that oxidative metabolism is minimised, and the physiological processes leading to tissue damage are reduced (Turner & Hughes, 1962). The use of chilled tissue may be objected to, however, on the grounds that it may itself damage the cells, or that the muscular rigour it causes may stiffen the tissue and make it susceptible to damage during eversion. In practice it was found that although the tissue is certainly stiffened by chilling, this was not instantaneous and no difficulty was encountered if eversion was carried out immediately after the lumen was flushed through. Levine _et al_ (1970), who maintained their material in ice-cold buffer during the entire period of preparation, reported that the tissue was apparently quite undamaged by the eversion process, prior to incubation. Reference to the literature shows that a number of workers have carried out successful physiological observations using tissues maintained in ice-cold medium, and this practice has been

used consistently throughout the present study. (Bronk & Parsons, 1965 a,b; Bamford, 1966; Turner & Hughes, 1962).

At this stage the distal half of the intestine was usually discarded, and the jejunum was slipped onto a surgical quality stainless steel rod (1.5 mm. diameter, 40 cm. length) tied off at the distal end, and slipped back over the ligature. The first 3-4 cm. of everted jejunum were discarded to avoid the use of damaged tissue, and the required length of intestine was then placed in a glass trough of oxygenated saline at 0-4°C. During the early stages of this work some attempt was made to use the vertical hook apparatus described by Turner (1959) but this was abandoned as it seemed to offer no particular advantage.

ii) Everted Sacs.

Sacs were prepared by cutting the first 40 cm. of jejunum into sections of the appropriate length, and selecting the required number at random. The sacs were ligatured at one end and a blunted needle, fitted to a 5 ml. disposable syringe, was introduced at the other and securely ligatured in place. The appropriate volume of serosal solution was then introduced (for example, 0.5 ml. in the case of a 7 cm. sac), the needle was withdrawn and the ligature was tightened and secured. A bubble of oxygen was not introduced with the serosal solution. Sacs were manipulated and filled on Whatman No. 1 filter

paper, saturated with cold saline on a disposable polythene sheet. This had the advantage of localising any radioactive contamination. Care was taken never to allow everted intestine to come into contact with dry filter paper as this can be seen to damage the mucosal surface.

In most experiments the sacs were incubated in 25 ml. Erlenmeyer flasks, containing 5 ml. or 10 ml. of incubation medium, open to the atmosphere and gassed continuously with 5% CO_2 in O_2. Unless otherwise stated the flasks were maintained at 37°C in a Griffin 100 series shaking incubator running at 100 oscillations per minute.

In many experiments it was necessary to measure the fluid movements during incubation and this was accomplished by means of appropriate weighings, before and after filling the sac and before and after collecting the final serosal fluid. The weighing scheme was as follows: -

W_i = weight of empty sac + 1 suture

W_{ii} = weight of filled sac + 2 sutures

W_{iii} = weight of filled sac + 2 sutures after incubation

W_{iv} = weight of empty sac + 2 sutures after incubation

then

$W_{ii} - W_i$ = initial serosal fluid + 1 suture

$W_{iii} - W_{ii}$ = total water uptake

$W_{iv} - W_i$ = increase in wet weight of tissue + 1 suture.

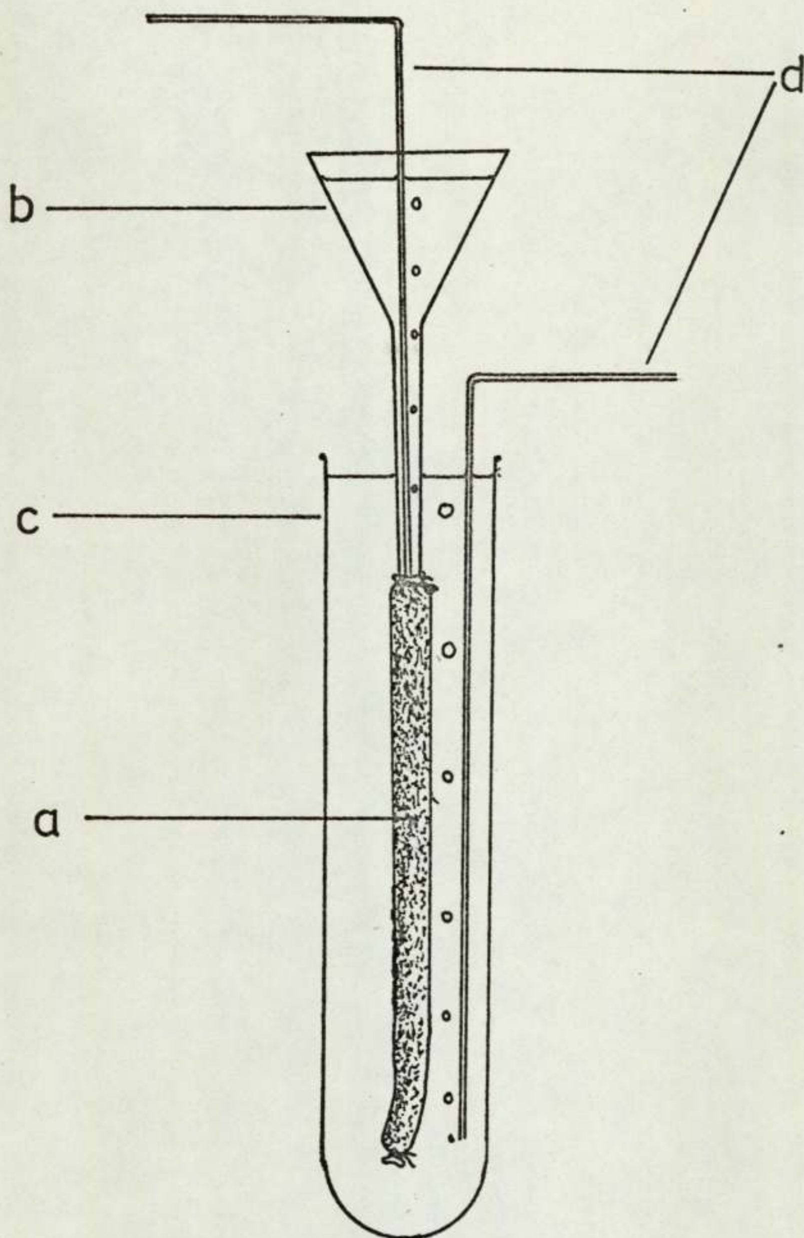
The weight of the sutures was estimated by determining the average weight of wet and dry sutures of standard length. Weighing was carried out on 0 - 1 gm and 0 - 5 gm torsion balances (White Elec. Inst. Co.). Extraneous fluid was removed by gently blotting the lower end of the suspended sac once, with moist filter paper. Treatment and assay of the tissue and serosal fluid are described in Part 3 of this section.

iii) Cannulated Sacs.

A version of the cannulated sac was employed in some of the initial transport experiments, and for the measurement of transmural potential differences.

A section of everted intestine (7 - 10 cms. initial wet length) was ligatured to a glass funnel as shown in Figure 2.1. The lower end was tied off and the serosal fluid was introduced via the cannula, care being taken to exclude bubbles and ensure a continuous column of fluid. After filling, the sac was immersed in 20 ml. of mucosal fluid, the serosal volume was adjusted to give a hydrostatic pressure of about 3 cm, and both sides of the sac were gassed continuously with 5% CO_2 in O_2 delivered via polythene tubing.

For transport studies serial sampling of the serosal solution was carried out by means of a tuberculin microsyringe; for transmural potential difference measurements electrical contact was maintained with the



<u>FIGURE 2.1.</u> The cannulated sac apparatus. The everted sac (a) is ligatured to the cannula (b) and immersed in the vessel (c). The serosal and mucosal fluids are gassed via the tubes (d).

serosal and mucosal solutions, via saturated KCl-Agar bridges led away to calomel half cells.

iv) Rings of Intestinal Wall.

The method used to study the uptake kinetics of folic acid was a version of the isolated ring technique of Agar Hird and Sidhu (1954) and Crane and Mandelstam (1960), though the manner of preparation differs in some details. The principle of the isolated ring is that during the early stages of intestinal uptake the transported substrate accumulates in the tissue underlying the mucosal epithelium. The technique has the advantages that the tissue can be used very quickly after its removal from the animal, the rate of transport is measured over very short time periods when the tissue is at its most viable, and local variations in the permeability of the intestine are averaged out by randomising the tissue.

The jejunum was exsected and everted as previously described and the first 40 cm. of jejunum were cut, with scissors, on the surface of a cool glass plate, into rings about 2 mm. in length. Regions of lymphoid tissue - "Peyer's Patches" - were discarded at this stage. The material from a single animal was then randomised by gently swirling and distributed amongst 5 (usually) 25 ml. Erlenmeyer flasks containing 5 ml. of incubation medium previously warmed to 37°C (unless

otherwise stated). Flasks were maintained at 37° in a Griffin 100 series shaking incubator running at 100 oscillations per minute. At appropriate intervals, flasks were removed from the incubator and the contents were emptied onto the scintered disk of a small Hirsch funnel. The material was rapidly flushed with 100 ml. of chilled saline (0.9% NaCl.) delivered in three portions with gentle swirling agitation. Each sample was then gently blotted and packed into filter paper buckets of known dry weight, freeze dried overnight, and assayed by the tissue oxidation technique, modified after Kalberer and Rutschmann (1961), described in Part 3.

During the initial experiments it was found that if the rings were allowed to exceed 3 mm. in length they tended to collapse during the rinsing stage to form an "envelope" of tissue. This led to rather high and variable uptake values due to inadequate washing. If the tissue was allowed to stand between rinses some reduction in activity was observed, due, presumably, to back diffusion of absorbed material. However, once the technique was standardised and used routinely very consistent results were obtained. For the kinetic determinations it was usual to use a minimum of 5 animals for each rate measurement. 5 identical experiments were carried out on the same day, and the results averaged as described in Section 3, Part 1.

v) Mucosal Scrapes.

The mucosal scrape preparation, first described by Dickens and Weil-Malherbe (1941) and in more detail by Crane and Mandelstam (1960), was used for some purposes. In the present study, 10 cm. lengths of everted intestine were laid on the surface of a cool plate, and the mucosa was removed by a single stroke of a microscope slide, pushed away from the body at a slight angle while the intestine was held with forceps. The mucosal scrape was transferred to a beaker of ice-cold saline as before, and the remaining intestine was discarded.

The material produced by this technique appeared, under low power microscopy, to consist of intact villi and a little attached muscular material. No further disaggregation was carried out and incubation was similar to that of the other preparations.

vi) Isolated Mucosal Cells.

The use of isolated mucosal cells is becoming increasingly common though it is doubtful whether a wholly satisfactory preparation has yet been achieved. Techniques differ mainly as to the method by which the cells are detached from the basal membrane and disaggregated from one another. The principal methods are enzymic dissolution (Perris, 1966; Kimmich, 1970), mechanical disruption (Momtazi & Herbert, 1973) and chelation with agents such as EDTA or citrate (Evans *et al*,

1971; Stern & Jensen, 1966). In the present study isolated cells were produced by a modification of the technique of Leslie and Rowe (1972), itself adapted from the techniques of Kimmich (1970) and Perris (1966), in which the basement membrane of the mucosal epithelium is dissolved by incubation with hyaluronidase.

The jejunum was removed from two (or three) rats, flushed, and everted in the usual way. The everted material was then cut into lengths of about 4 cm. and placed in the isolation medium, consisting of the Tris buffered saline described in Part 1 of this section, to which had been added 1.0 mg/ml bovine serum albumin and 1.0 mg/ml hyaluronidase. Incubation was carried out in the Griffin 100 series shaking incubator running at 100 cycles per minute, for 30 minutes at 37^oC.

After 30 minutes the pieces of jejunum were placed in 25 ml. of enzyme-free medium and gently agitated in a shallow polythene dish. It was usually necessary to brush away the surface of the mucosa with a polythene utensil having a rounded spade-like tip about 5 mm. across, constructed for the purpose by pulling out and flattening a piece of polythene tubing over a bunsen flame. This process produced a cell suspension consisting of individual cells and small clumps. After harvesting by this method the cells were poured off into 15 ml. polythene centrifuge tubes maintained at 4^oC until all the cells were collected. The tubes were then centrifuged at setting 4 - 5 for 2 - 3 minutes in a

bench centrifuge (M.S.E. Ltd.) The supernatant was poured off, replaced with fresh enzyme-free medium at 4°C and the cells were gently re-suspended and centrifuged as before. This was repeated twice to remove residual hyaluronidase, and the cells were finally transferred to a single tube and spun down to form a loose pellet.

For transport experiments the cells were added to 10 mls. of the standard incubation medium, as used for the whole tissue preparations, containing appropriate concentrations of folic acid. Incubations were carried out in 100 ml. polythene beakers under continuous oxygenation as usual. At appropriate time intervals, duplicate 0.5 ml. aliquots were pipetted off and rapidly transferred to 15 ml. polythene centrifuge tubes containing 10 ml. of ice-cold saline (0.9% NaCl). The tubes were then centrifuged at setting 6 - 7 for 1 minute, the supernatant was poured off and the process was repeated. Unless otherwise stated in Section 3, 3 such washes were employed in the standard procedure.

All isolated cell samples were assayed for radioactivity using the automatic tissue oxidation technique described in Part 3 of this section. After washing, the cells were divided amongst an appropriate number (4 or 6) of tissue oxidiser "boats", and freeze-dried overnight as usual.

Section 2. (3).

At an early stage, several methods of assaying the radioactivity of tissue samples were compared. Sacs and everted rings have been homogenised in 5% acetic acid using a motor driven ground glass homogeniser, but this technique was time consuming and inconvenient. Moreover, only the clear supernatant can be counted and, as is discussed in Section 4, there is a strong possibility that much of the absorbed activity remains bound to the residual tissue. The method was found to give inconsistent results and it was abandoned. A proprietary tissue solubiliser (Koch Light TS.1) has been tried but intestinal tissue appears to be rather resistant to such treatment even after several hours of incubation at 50°C. For these reasons a modified version of the tissue oxidation technique of Kalberer and Rutschmann (1961) was adopted and is described in detail below. The effective, but somewhat inconvenient method was finally replaced with a Beckman Tissue Oxidiser, and this was used for the experiments with isolated cells.

i) Tissue Combustion.

The modified tissue combustion apparatus of Kalberer and Rutschmann (1961) is shown in Figure 2.2.

together with the method of making the paper sample baskets.

The everted rings of intestinal tissue and their use in folate accumulation experiments are described elsewhere in the text. In most experiments the jejunum of a single animal provided 5 separate samples of tissue, each of which (50 - 80 mg.) was packed, after washing, into 1 basket, frozen, and freeze-dried overnight to constant weight. In order to ensure complete absorption of CO_2, the whole sample was not allowed to exceed 250 mg. and the dry weight of the tissue was determined by subtracting the known dry weight of the paper basket.

The sidearm of the combustion apparatus contained 25 ml. of a CO_2 absorbing solution consisting of 140 ml. of monoethanolamine made up to 1 litre with methanol. The tubes were cooled in solid CO_2/acetone before use, attached to the flask by means of springs, and the whole apparatus was thoroughly purged with oxygen. The sample was placed in the nichrome spiral attached to the glass stopper, the fuse was lit, and the assembly was plunged into the flask and held firmly in place until combustion was complete. The stopper was then clipped in place and the apparatus was inclined to allow the absorber to flow into the flask. 30 minutes equilibration time was allowed for complete absorption to occur, after which the absorber was tipped back into the side arm and the apparatus dismantled and washed with a copious jet of water.

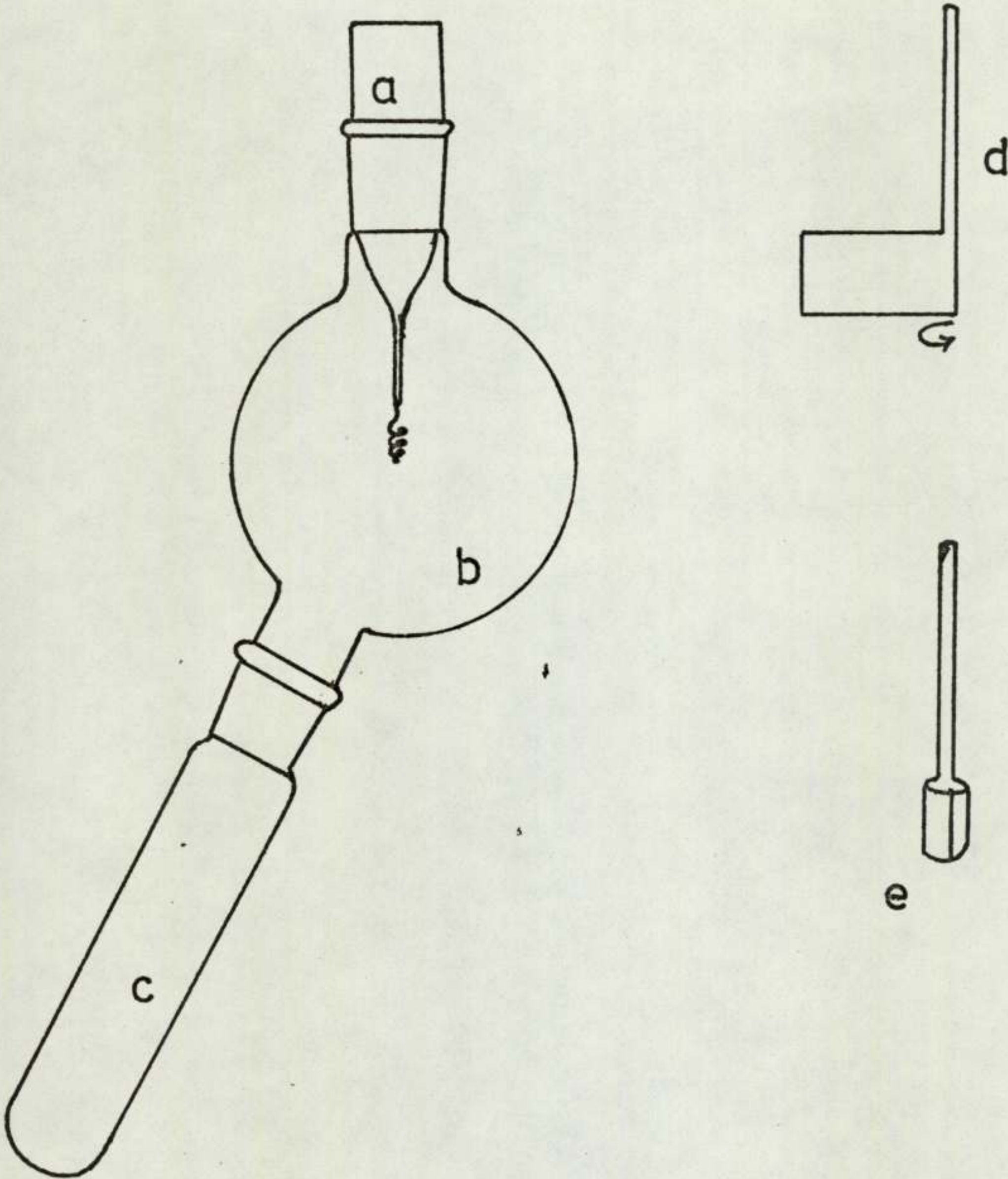


FIGURE 2.2. Tissue combustion apparatus. The complete vessel comprises the stopper and nichrome spiral (a), the combustion flask (b) and the side-arm (c). The method of making the paper basket is shown in (d) and (e).

The activity of the absorbing solution was determined by mixing 3.0 mls. of absorber with 7.0 mls. of a scintillator consisting of 2,5-diphenyloxazole (4 gm.) and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (100 mg.) dissolved in 1 litre of toluene (Kalberer & Rutschman, 1961). In some cases these samples separated into two phases in which case methanol was added dropwise with agitation until a homogenous solution was obtained. Samples prepared by this method were found to be prone to chemi-luminescence and it was usually found to be necessary to allow 12 hours before stable count rates were obtained. The use of methanol causes heavy quenching, and the necessity of adding variable quantities of methanol to some samples necessitated internal standardisation of each counting vial. To this end, an accurately weighed quantity of n-(1-^{14}C) hexadecane was added to each vial after the initial counting. The samples were then recounted to accumulate a minimum total of 10,000 counts.

Radioactivity was determined using the expression: -

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

where A = activity of hexadecane, (Ci/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)

Calculations were done on an Olivetti Programma 101 desk top computer.

ii) Use of the Tissue Oxidiser.

The Beckman Biological Material Oxidiser operates on a principle essentially similar to that outlined above, 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 completed 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 complete combustion and preparation of a tissue sample within about 5 minutes.

In the present study the Tissue Oxidiser was used routinely for the determination of carbon-14. Before use the equipment was allowed to reach its thermostatically controlled operating temperature, and was primed in accordance with the manufacturer's instructions using 500 mg. aliquots of mannitol. The oxygen flow rate was kept steady throughout by means of an adjustable flow meter, and the apparatus was checked and standardised periodically using accurately weighed aliquots of ^{14}C-hexadecane.

The amount of material which can be burnt in the apparatus is restricted by the capacity of the catalyst bed to fully oxidise the sample within the pre-set cycle time. Consequently care was taken to keep the sample weight well within the manufacturer's recommended range. The CO_2 effluent was collected in 15 ml. aliquots of absorber-scintillator, transferred to standard vials and counted as usual.

Two scintillator-absorbers were used routinely throughout this work. A solution prepared by dissolving 2,5-diphenyloxazole (4 gm) in a mixture containing monoethanolamine (500 ml), methanol (500 ml) and toluene (1000 ml), was used with success. However, since this solution tends to separate into two phases and requires the addition of extra methanol it was later replaced with Fison's ^{14}C absorber, "P". This was found to have better solubilising ability and a higher counting efficiency.

For the assay of whole tissue, aliquots (about 100 mg. dry wt.) of tissue were weighed and burnt in the re-usable "boats" supplied by Beckman Ltd. It was found that isolated cells could be conveniently pippeted into the "boats" and dried _in situ_. The dry weight of each cell sample was determined by weighing the "boats" before and after combustion. To ensure accuracy, the samples were kept in a silica-gel desiccator during storage before the weighings.

iii) Fluid Samples.

Fluid samples were counted in NE 220 (Nuclear Enterprises) or Dioxan Cocktail "D" (Fisons). In most cases 0.1 ml. aliquots of neutral saline were counted in 10 ml. of scintillator. Such samples, when agitated, gave clear stable solutions with a little solid precipitate in most cases. Counting efficiency was about 65% with amplifier gain at 30, lower discriminator setting of 0.8v and upper discriminator setting of 5.0v.

iv) Thin Layer Chromatography.

Thin layer chromatography (T.L.C.) was used for the separation and identification of folates in several experiments. In all cases, pre-spread commercial plates ("Polygram Foils", Machery-Nagel) were used having a 0.1 mm, MN 300 or MN 300 UV cellulose layer. Single spots were applied, dried in air, and the chromatograms were run in air-tight glass solvent tanks. At the end of the run the foils were dried in air and spots were located visually as fluorescent or absorbent areas under 254 or 366 nm u.v. light. Details of the solvent systems used are given in Table 2.2. Positive identification of a species was made only after co-chromatography with a standard marker compound in the three solvent systems. The standard procedure adopted was to apply single spots of test solution and marker solution, and run all the chromatograms simultaneously under identical conditions.

TABLE 2.2.

Chromatography Solvent Systems

Solvent Number	System
1.	n-Butanol/acetic acid/water, 4:1:5 v/v. Equilibrated at room temperature for 12 hours prior to use.
2.	n-Propanol/ammonium hydroxide (s.g. 0.880)/water, 200:1:99 v/v. Made immediately before use.
3.	0.1M phosphate buffer, pH 7.0.

v) Autoradiography.

Autoradiography was used for the identification of radioactive folic acid and its metabolites in some experiments. Thin layer chromatograms were prepared as described above, dried, and fastened to a glass plate by means of adhesive tape. X-ray sensitive photographic film (Ilford Industrial 'G', or Kodak 'Kodirex') was then laid directly on the cellulose surface, aligned square, and pressed firmly into place with a second glass plate. The whole assembly was then fastened with elastic bands, wrapped in light excluding black paper and placed in a light tight box. Exposure times varied with the circumstances of the experiment but in cases where there was uncertainty as to the activity of the applied solutions two autoradiographs were set up simultaneously so that a "pilot" experiment was available as a guide to the required exposure period.

After exposure the autoradiographs were dissembled and the films developed in Kodak DX80 (5 to 10 minutes), stopped by immersion in 3% v/v aqueous acetic acid (2 minutes) and fixed in Kodak FX 40 (10 minutes). The films were then washed thoroughly and dried in air. Spots were identified by comparison with standard marker compounds and by reference to Rf values reported in the previous literature.

In some cases permanent visual records of the autoradiograms were produced with a recording densitometer (Joyce-Loebl) which plots, in effect, spot-density against elution distance on graduated chart paper.

vi) Microbiological Assay.

The most sensitive assay technique for low concentrations of folates is microbiological assay, a method which is based upon the accurate measurement of the growth response by micro organisms having specific folate requirements. This technique was used for a few experiments in this study, the assays being performed by Mr. K. Ratanasthien.

The method used was the standard procedure as described by Toepfer et al (1951) and by Chanarin (1969). Briefly, the culture tubes containing the solution to be assayed are innoculated with the micro-organism and incubated for 18 hours at 37°C to obtain a measurable growth response. At the termination of the incubation the turbidity of the 'test' tubes is compared with standard tubes containing known concentrations of folate. The organisms used were Lactobacillus casei, NCIB 8010 grown in Q-Ess medium. Streptococcus faecalis NCIB 8043, grown in Difco 0319-15 medium, and Pediococcus cerevisiae NCIB 7838 grown in Difco 0456-15 medium. The response of these micro-organisms to the principal folates discussed in this work is shown in Table 2.3.

TABLE 2.3.

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).

SECTION 3.

EXPERIMENTAL RESULTS.

Section 3.

i) Preliminary Results.

A series of preliminary experiments were undertaken in order to observe the general behaviour of the preparation and ensure that the techniques employed were optimal for the purposes of the present study.

Figure 3.1. indicates the time course of the serosal transport of folic acid by everted sacs during a relatively long period of incubation. The concentration of folic acid in the mucosal solution was 10^{-5}M, 1 sac per animal was removed from incubation at each time interval, and the serosal contents were assayed for radioactivity. Each point represents the mean and standard error of the number of animals shown in brackets; 1 sac was prepared from each animal.

These results indicate that everted sacs prepared as described in Section 2 transported folic acid into the serosal solution approximately linearly for a period of about 2 hours. For the reasons discussed in Section 2, routine incubations were restricted to periods of one hour or less.

In order to observe the time course of serosal transfer over a shorter time period, a cannulated sac technique was employed to allow continuous sampling of the serosal solution. Figure 3.2. illustrates qualitatively the appearance of folic acid in the serosal solution of cannulated everted sacs (10 cm. initial wet length). Each point indicates the mean of the number of sacs shown in brackets. One sac was prepared from each

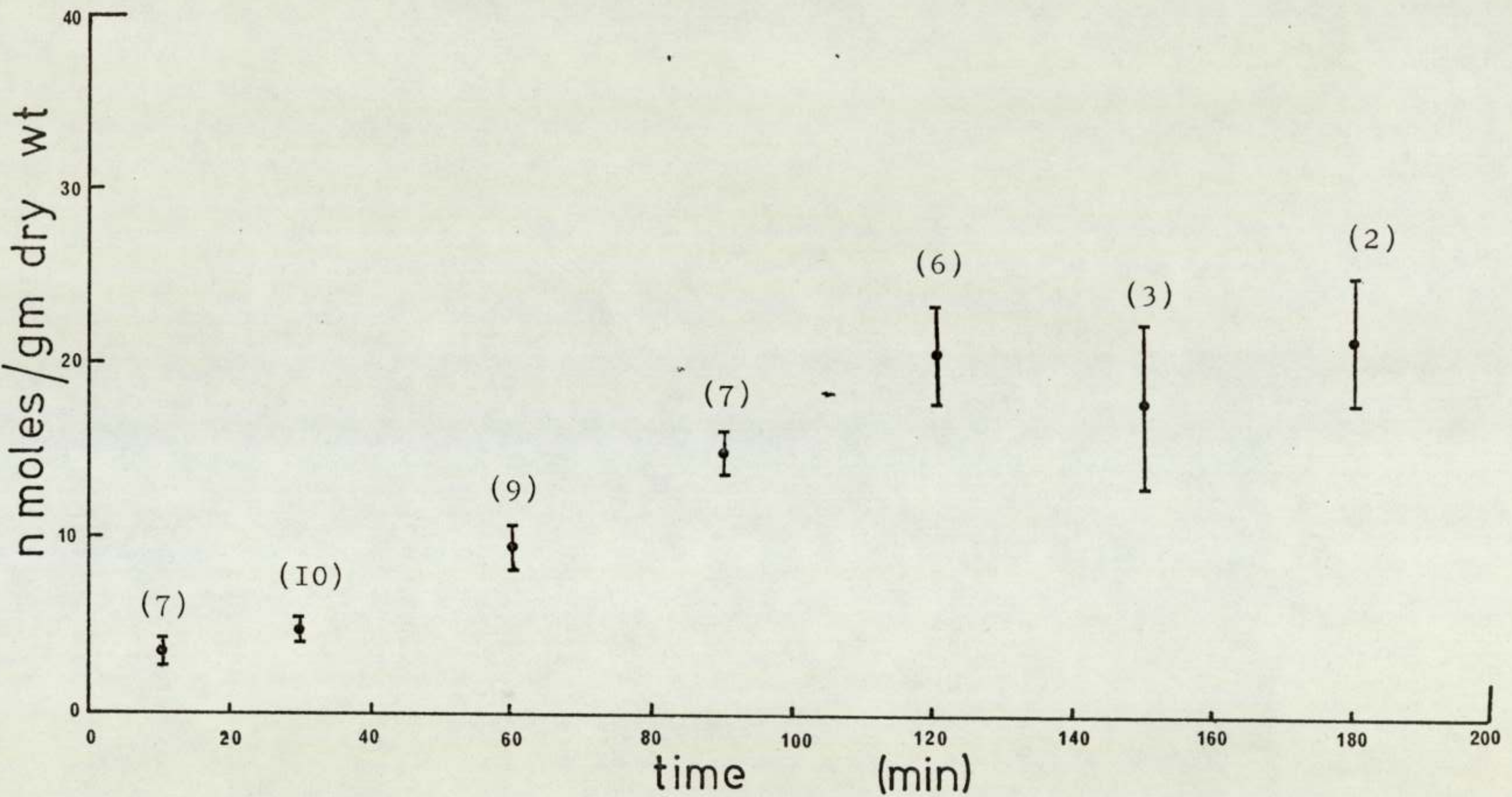


FIGURE 3.1.

Time dependent serosal transfer of folic acid by everted jejunal sacs. Each point is the mean and s.e.m. of the number of animals shown in brackets.

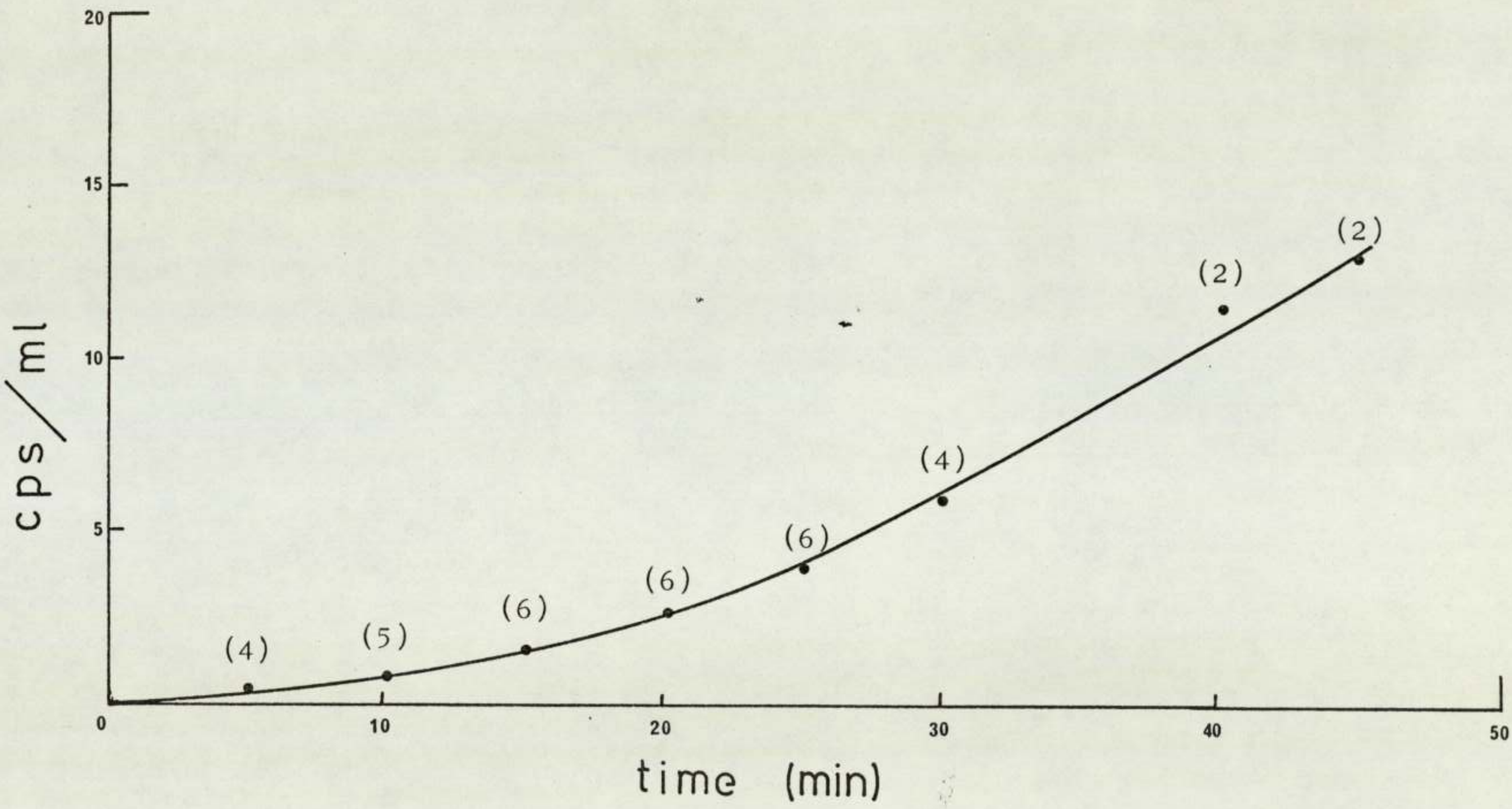


FIGURE 3.2.

Time dependent serosal transfer of folic acid by cannulated sacs of rat jejunum.

animal. Inspection of Figure 3.2. reveals that the appearance of folic acid in the serosal solution becomes linear at about twenty minutes. This type of time lag has been reported for other compounds (Wilson & Wiseman, 1954a) and presumably indicates the time required for the substance to diffuse across the successive permeability barriers of the gut wall and reach a significant concentration in the interstitial fluid. Kaplan and Cotler (1972) have used a rather similar technique for the evaluation of drug permeability, and have reported that the permeability of a substance as judged in the <u>in vitro</u> cannulated sac is in accord with its <u>in vivo</u> absorption as measured in the intact animal. It is also of interest to note that the lag period indicated in this experiment appears to be in the range reported by these workers for apparently passively absorbed drugs having permeability characteristics classed by them as good.

Figure 3.2. illustrates that the loss of absorbed material from the gut wall into the serosal solution is small during the first twenty minutes of incubation. In order to estimate accurately the initial rate of uptake by the tissue accumulation method it is important to ensure that what is measured is a truly linear uptake. Figure 3.3. illustrates the folate content of everted rings of intestine, incubated at 10^{-5}M and sampled at 5 minute intervals for 25 minutes. Each point is the mean and standard error of five experiments. Folate accumulation is clearly linear over this period

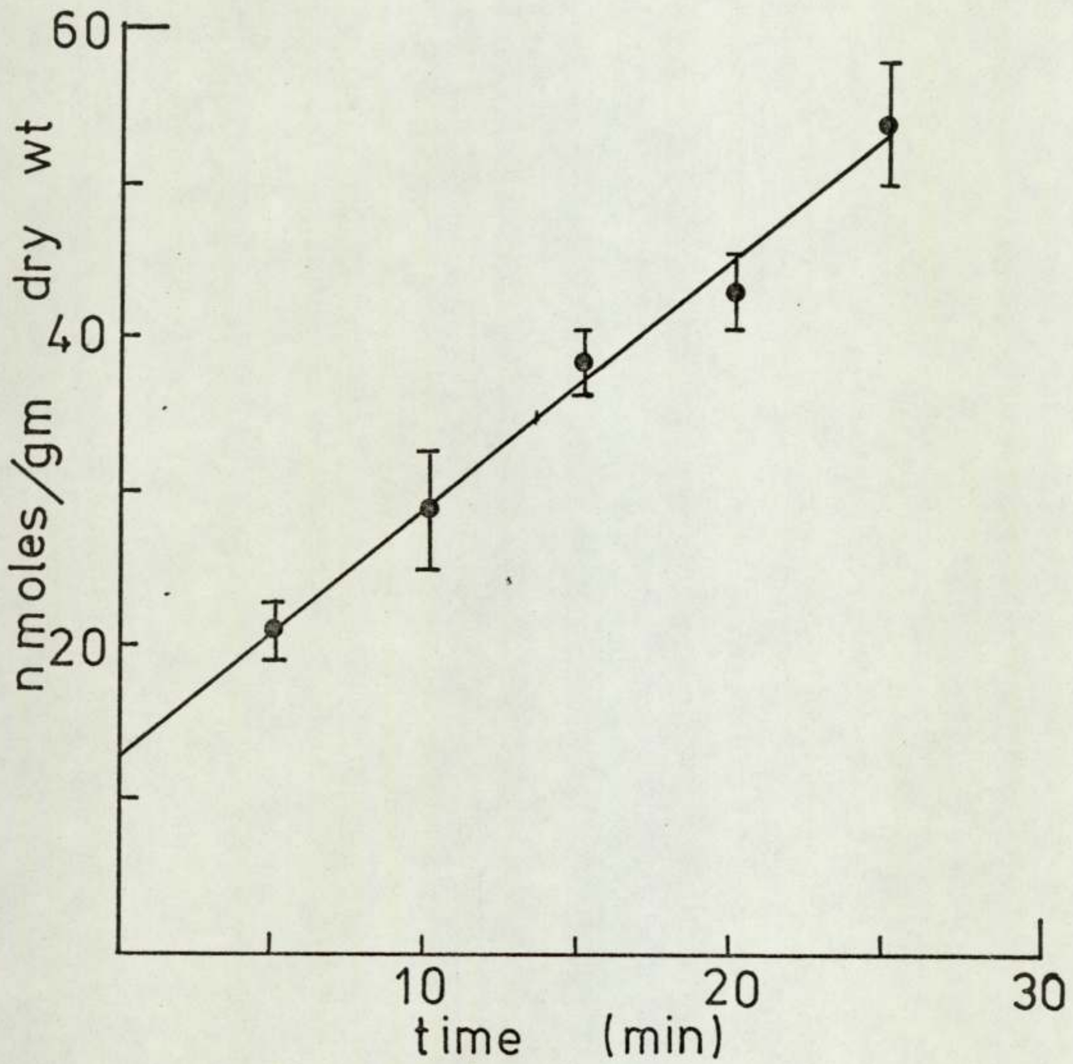


FIGURE 3.3.

Time dependent tissue accumulation of folate by everted rings of rat jejunum. Each point is the mean and s.e.m. of 5 observations.

indicating a minimal loss of absorbed material by back diffusion. A period of 10 minutes was chosen for the kinetic studies described in the next section as this permits a more accurate determination of the accumulation gradient, and ensures maximum tissue viability. The intercept on the 'y' axis of Figure 3.3. was found to be a constant feature of the routine determinations. The phenomenon is discussed quantitatively in (ii), Kinetic Studies, below.

ii) Kinetic Studies.

The rate of accumulation of folic acid in the intestinal wall was measured over an approximately 100 fold concentration range, using the everted ring preparation as previously described. The incubations were carried out for periods of ten minutes and samples were removed at two minute intervals. The results of five such experiments were averaged, and the rate of uptake was determined from the line of best fit, calculated by the method of least squares. No result was accepted which failed to give a correlation coefficient greater than 0.98.

Table 3.1. contains the results of these experiments carried out at the concentrations shown, and these data are illustrated in Figure 3.4.

As was previously mentioned, one of the characteristics of the linear uptake graphs is a positive intercept on the 'y' axis, indicating some form of rapid uptake process. Such a process might merely reflect incomplete removal of adherent folic acid during the post-incubation flushing, or it might result from rapid physical binding to some readily accessible site in the tissue. As an aid in distinguishing these two possibilities the magnitude of the 'rapid uptake' was plotted against concentration and the results are shown in Table 3.2. and Figure 3.5.

TABLE 3.1.

Rate of uptake of folic acid by everted rings of rat jejunum.

Folic Acid Concentration (Molar)	Uptake of Folic Acid (n-mole/g. dry wt. min.)	n.	Standard Error.
0.8×10^{-7}	0.04	5	0.01
2.9×10^{-7}	0.15	5	0.01
5.5×10^{-7}	0.44	5	0.01
12.0×10^{-7}	0.70	5	0.01
19.0×10^{-7}	0.97	5	0.02
51.0×10^{-7}	1.69	5	0.02
110.0×10^{-7}	1.60	5	0.04

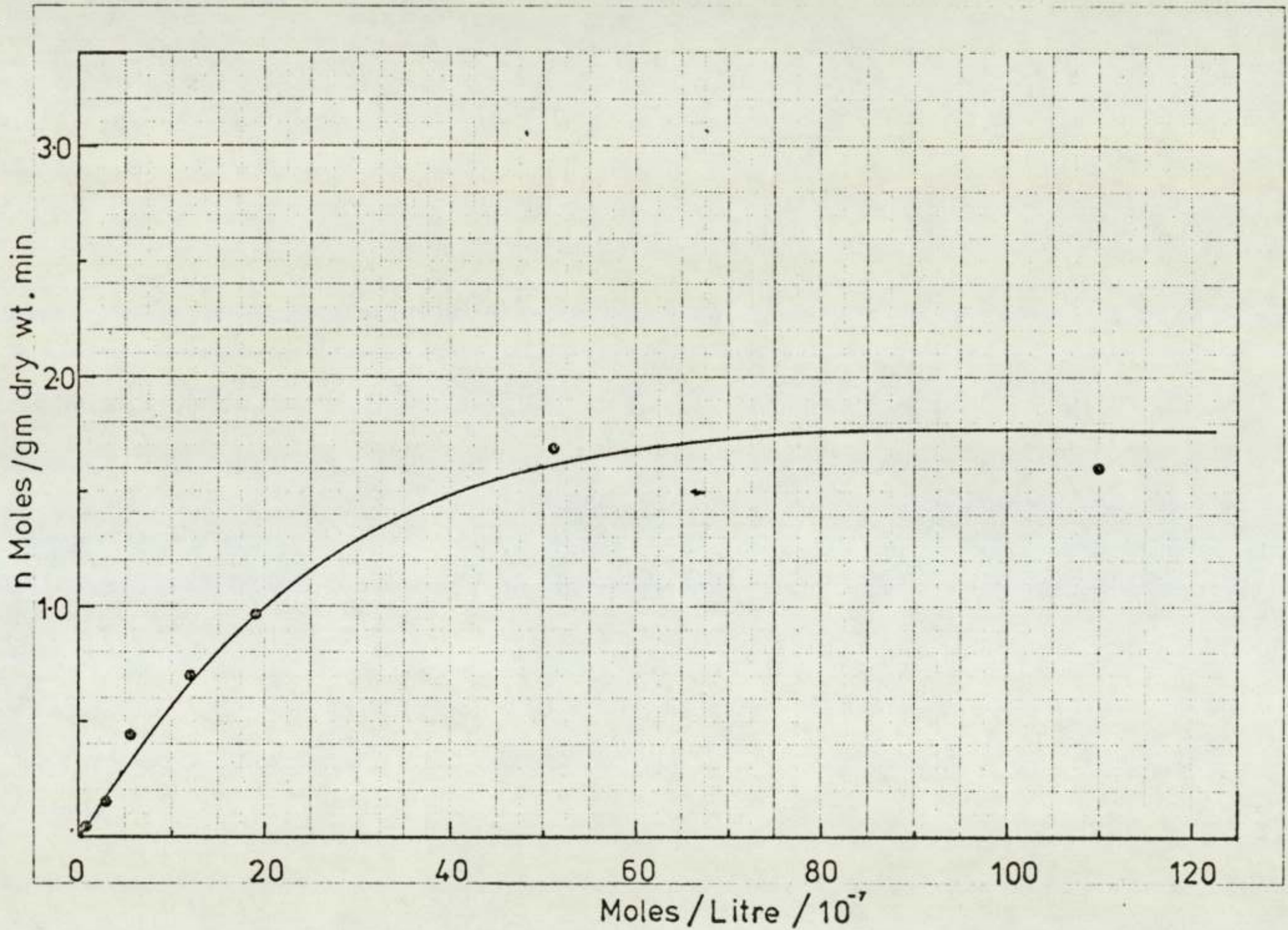


FIGURE 3.4.

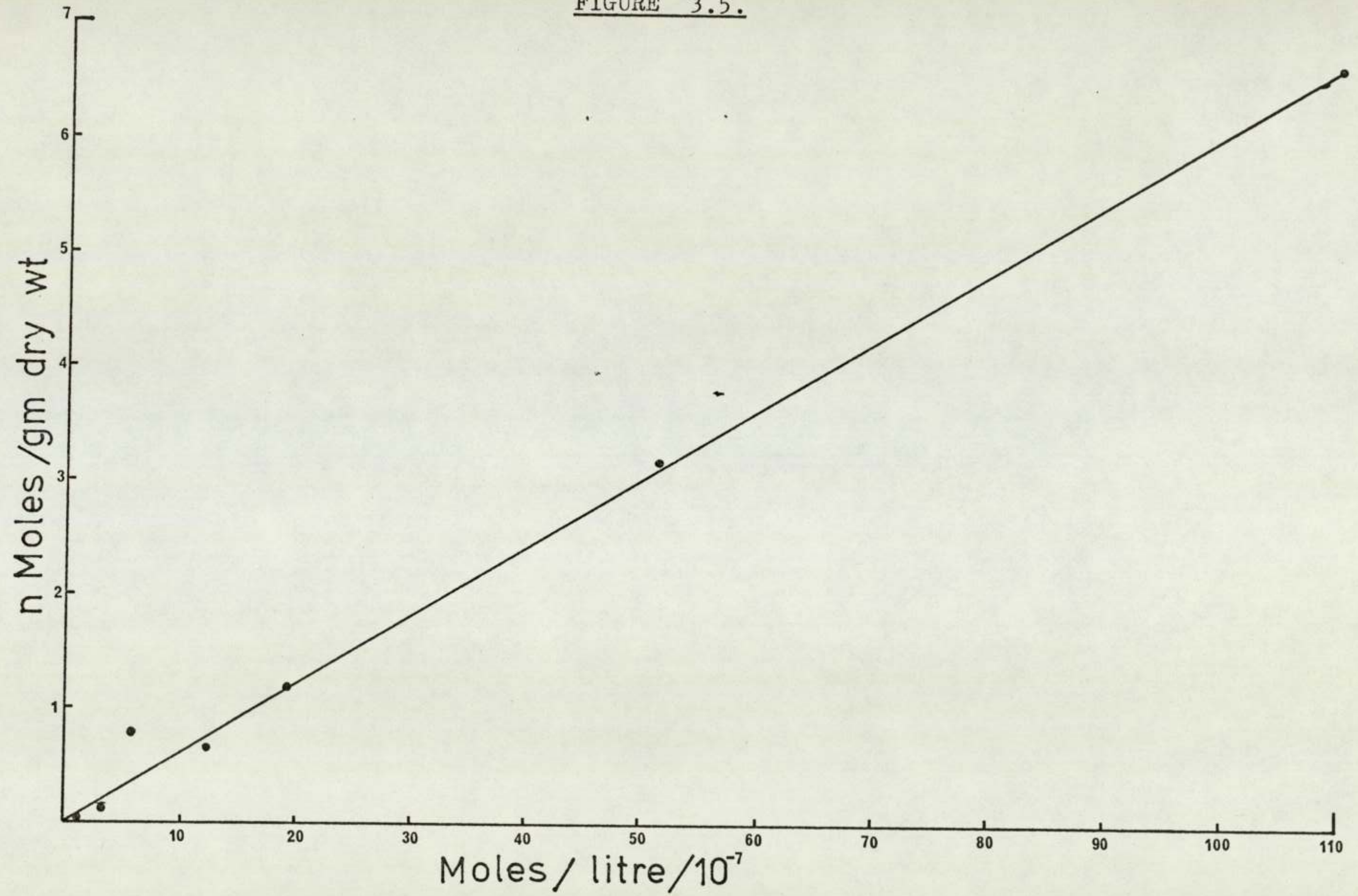
Rate of uptake of folic acid by everted rings of rat jejunum.

TABLE 3.2.

Rapid component of folic acid uptake by everted rings of rat jejunum.

Folic Acid Concentration (Molar)	Adherent Folate n/mole/gm. dry wt.
0.8×10^{-7}	0.01
2.9×10^{-7}	0.12
5.5×10^{-7}	0.76
12.0×10^{-7}	0.65
19.0×10^{-7}	1.18
51.0×10^{-7}	3.17
110.0×10^{-7}	6.67

FIGURE 3.5.



Rapid component of folic acid uptake by everted rings, plotted as a function of concentration.

As a second criterion of transport the serosal transfer of folic acid after a fixed incubation period was determined using the everted sac preparation. The data of Table 3.3. and Figure 3.6. illustrate the total serosal transfer of sacs incubated over the range of concentrations shown. In each case the value represents the mean and standard error of 'n' experiments, each of which was carried out on one animal.

TABLE 3.3

The serosal transfer of folic acid by everted sacs. Each result is the mean serosal transfer, after 30 minutes of incubation, for the number of animals shown as 'n'.

Folic Acid Concentration (Molar)	Serosal Transfer n-mole/g. dry wt.	n
7.1×10^{-7}	1.36 ± 0.18	4
13.9×10^{-7}	2.98 ± 0.54	4
27.5×10^{-7}	4.24 ± 0.61	4
56.3×10^{-7}	5.96 ± 1.10	4
106.3×10^{-7}	6.87 ± 1.15	7

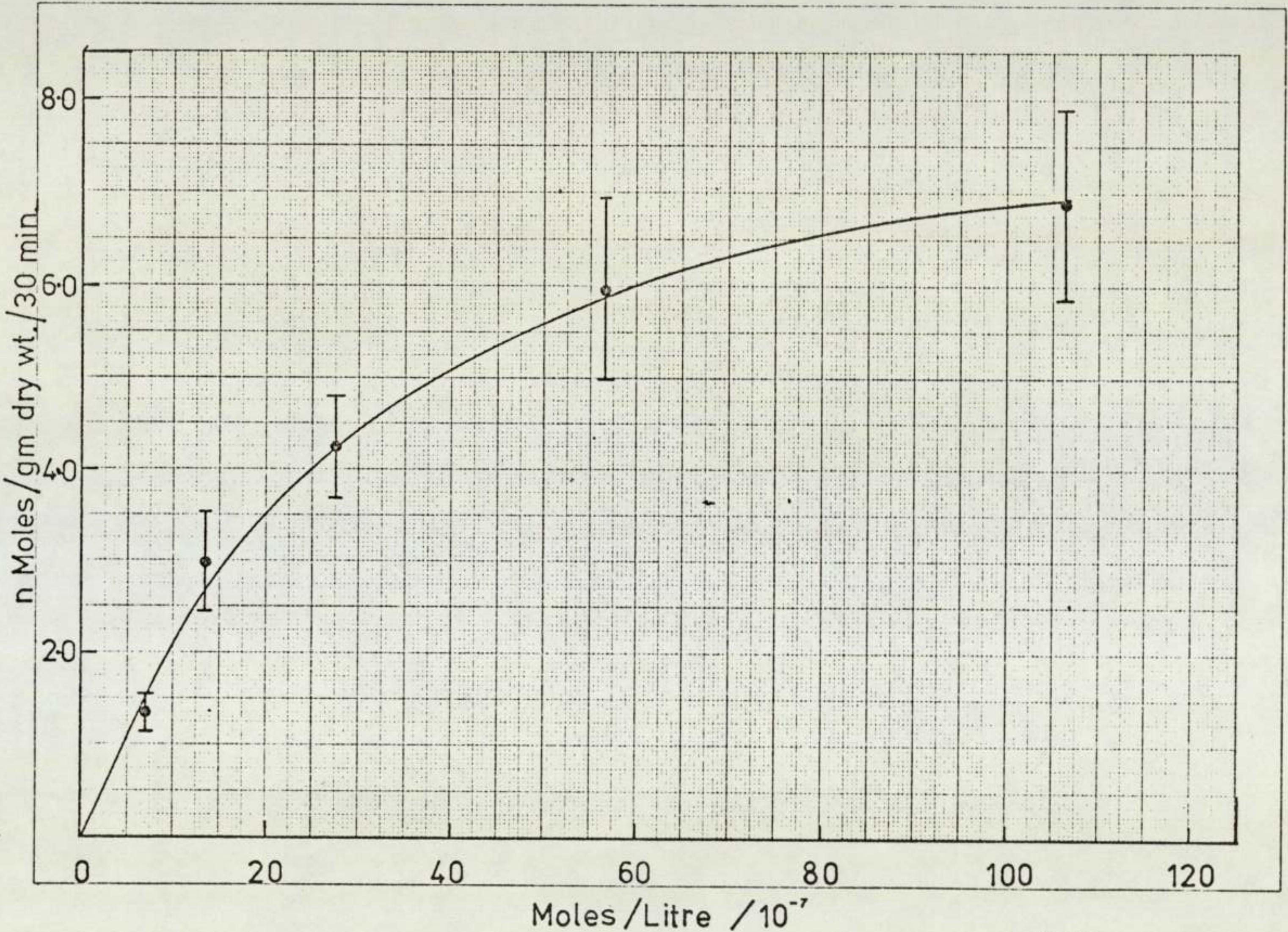


FIGURE 3.6.

Serosal transfer of folic acid by everted sacs, plotted as a function of mucosal concentration.

It should be noted that only by the technique adopted in the present study is it possible to accurately distinguish the various components of the overall uptake process. For example, if the rate of uptake had been estimated from a single measurement of the tissue content at one incubation time, the rapid component would have been superimposed on the time-dependent component and the rate of uptake would have been over-estimated, the extent of the error depending upon the incubation time chosen. Furthermore, since the rapid uptake process is unsaturable at the concentrations investigated, rates of uptake calculated from single short incubation times would be unduly "weighted" by the unsaturable transport component and would therefore display more or less linear kinetics. These points are illustrated in Figure 3.7. Both curves represent estimates of the rate of uptake plotted against concentration of folic acid. The rates for curve A were estimated from the total uptake at 10 minutes, while those in curve B were estimated from the total uptake after 1 minute. It is clear that neither estimate corresponds accurately to the actual rate of time dependent uptake, and both are close to linearity. Previous workers have not distinguished between these two aspects of the transport process; for example, Olinger, Bertino and Binder (1973) have estimated the rate of uptake of folic acid from the total tissue content of everted sacs after an incubation period of 1 minute.

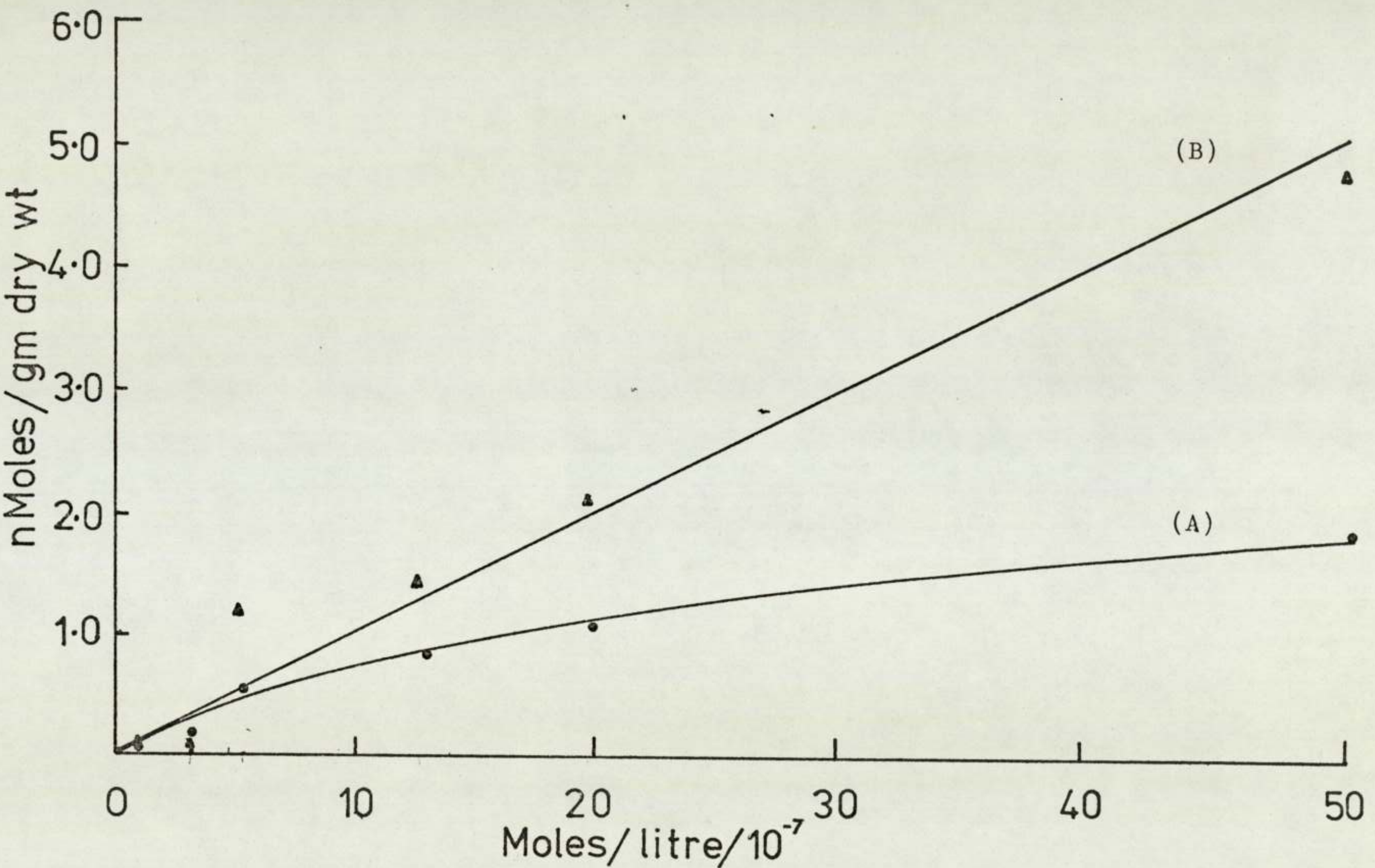
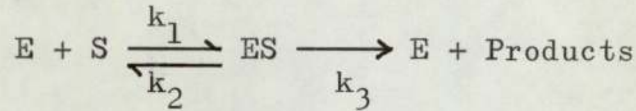


FIGURE 3.7. Rates of transport estimated from the total uptake of folic acid after 10 minutes incubation (curve A) and one minutes incubation (curve B).

Inspection of figures 3.4. and 3.6. reveals that both the tissue accumulation and the serosal transfer of folic acid under the present experimental conditions are saturable processes bearing, superficially at any rate, a kinetic resemblance to a simple enzyme-mediated reaction of the general form:



$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_3]{} E + \text{Products}$$

Such "Michaelis-Menten" reactions are described by the fundamental equation:

$$v = \frac{V.S}{K_s + S} \quad \text{. . . . . (3a)}$$

where v is the velocity of the reaction, S is the substrate concentration, V is the maximum reaction velocity and K_s is the dissociation constant of the enzyme-substrate complex. It is customary to derive the parameters V and K_s from a linear transformation of equation (i); several such transformations are available and the choice depends to a large extent on the accuracy of the experimental measurements of v.

It is generally agreed that the conventional "Lineweaver-Burk" double reciprocal plot is the least satisfactory method of determining K_s and V because of the undue weight attached to potentially inaccurate small values of v (Dowd & Riggs, 1965). For example, if v is underestimated, the effect of plotting its reciprocal is

to magnify this error and rotate the uncorrected line anti-clockwise. Table 3.4. contains the data of Table 3.1 and the equivalent reciprocals, together with the values of K_s and V derived from an unweighted regression line of y on x. Since the y intercept of this line is negative the values of K_s and V so derived are clearly meaningless. Tables 3.5.and 3.6. contain the tissue accumulation and serosal transfer data of Tables 3.1. and 3.3. together with the appropriate values of $\frac{s}{v}$ (substrate concentration/rate of uptake); figures 3.8. and 3.9. illustrate linear plots of $\frac{s}{v}$ against s, the form recommended on statistical grounds by Wilkinson (1961). The regression lines, and the values of K_s and V obtained from them, were calculated using an Olivetti Programma 101, desk top computer, which weights individual points on the assumption that s is the independent variable, and that the variance of v is independent of the magnitude of v.

Table 3.7. gives values of K_s and V for the processes of tissue uptake and serosal transfer of folic acid. The significance of these parameters will be discussed in Section 4.

TABLE 3.7.

	K_s (Molar)	V n. mole. gm. dry wt. min.
Tissue Uptake (Fig.	22.4×10^{-7}M	2.20
Serosal Transfer	29.0×10^{-7}M	0.30

TABLE 3.4.

Folic Acid Concentration (S) (Molar)	Uptake of Folic Acid (v) (n-mole/g. dry wt. min)	$\frac{I}{S}$	$\frac{I}{v}$
0.8×10^{-7}	0.04	1.25	25.00
2.9×10^{-7}	0.15	0.345	6.67
5.5×10^{-7}	0.44	0.182	2.27
12.0×10^{-7}	0.70	0.083	1.43
19.0×10^{-7}	0.97	0.053	1.03
51.0×10^{-7}	1.69	0.020	0.59
110.0×10^{-7}	1.60	0.009	0.63

slope = 20.0 k_s = - 115.9

intercept = - 0.173 V = - 5.79

TABLE 3.5.

Folic Acid Concentration (S) (Molar)	Uptake of Folic Acid (V) (n-mole/gm. dry weight. min)	$\frac{S}{V}$
0.8×10^{-7}	0.04	20.00
2.9×10^{-7}	0.15	8.19
5.5×10^{-7}	0.44	12.50
12.0×10^{-7}	0.70	17.14
19.0×10^{-7}	0.97	19.59
51.0×10^{-7}	1.69	30.18
110.0×10^{-7}	1.60	68.75

Slope = 0.458

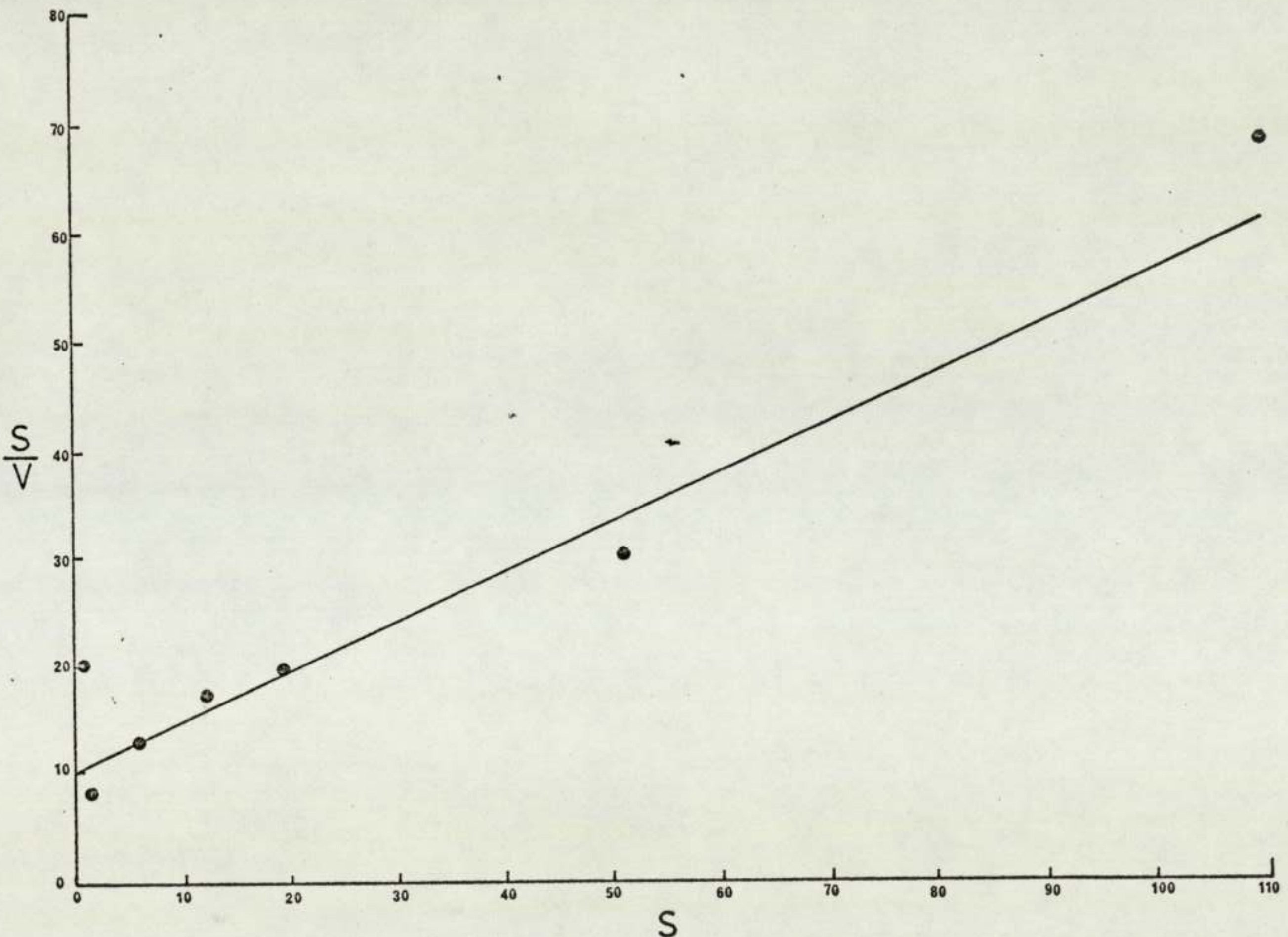
Intercept = 10.25

TABLE 3.6.

Folic Acid Concentration (S) (Molar)	Serosal Uptake (v) (n-mole/g. dry wt.)	$\frac{s}{v}$
7.1	1.36	5.22
13.9	2.98	4.66
27.5	4.24	6.49
56.3	5.96	9.45
106.3	6.87	15.47

Slope = 0.11

Intercept = 3.28



<u>FIGURE 3.8.</u>

Linear transformation of tissue uptake data.

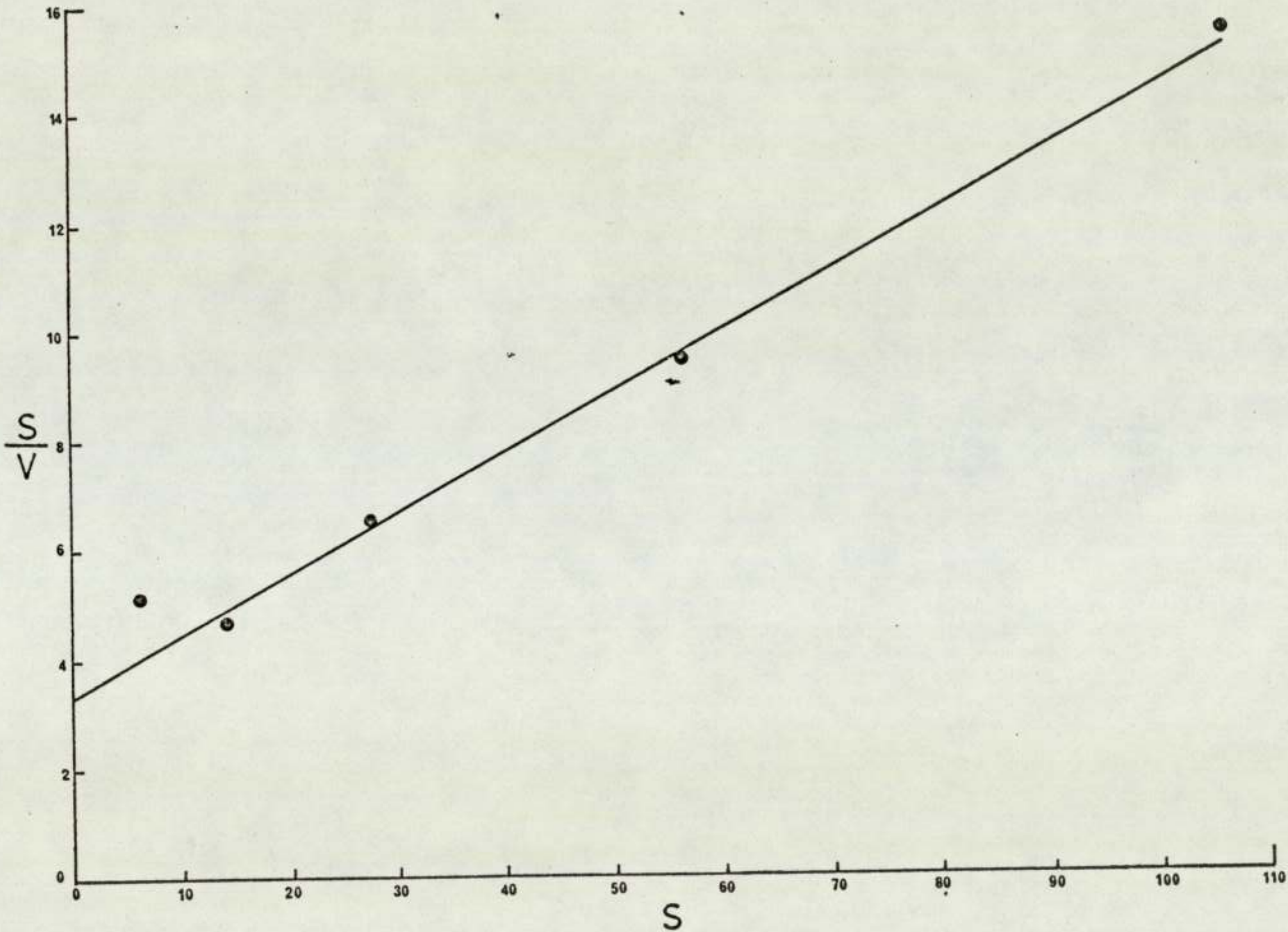


FIGURE 3.9.

Linear transformation of serosal transfer data.

To observe the effect of temperature on the tissue uptake of folic acid, incubations were carried out at 27°C, at concentrations of 10^{-5}M and 10^{-7}M, and at 0°C at 10^{-5}M. These data are tabulated in Table 3.9. Figure 3.10. illustrates the accumulation of folic acid by everted rings at 0°C. It is clear from the large standard errors and poor fit of this data ($r = 0.70$, $p > 10\%$) that reduction of the incubation temperature to this extent, brings about an impairment of the uptake process.

By comparison with the previously quoted data at 37°C, the Q_{10} values for the uptake process have been calculated at either end of the concentration range. Table 3.8. contains these values, together with the activation energies which have been calculated from them.

TABLE 3.8.

Folic Acid Concentration	Q_{10}	Activation Energy (Kcal./mole).
1.0×10^{-5}	1.88	12.5
0.8×10^{-7}	2.00	13.7

TABLE 3.9.

Effect of temperature on tissue uptake
of folic acid by everted jejunal rings.

Folic Acid Concentration (Molar)	Temperature ^{o}C	Uptake of Folic Acid n-mole/gm dry wt. min.	n	Standard Error
100×10^{-7}	27	0.85	5	0.2
0.8×10^{-7}	27	0.02	5	0.004
100×10^{-7}	0	0.38	7	-

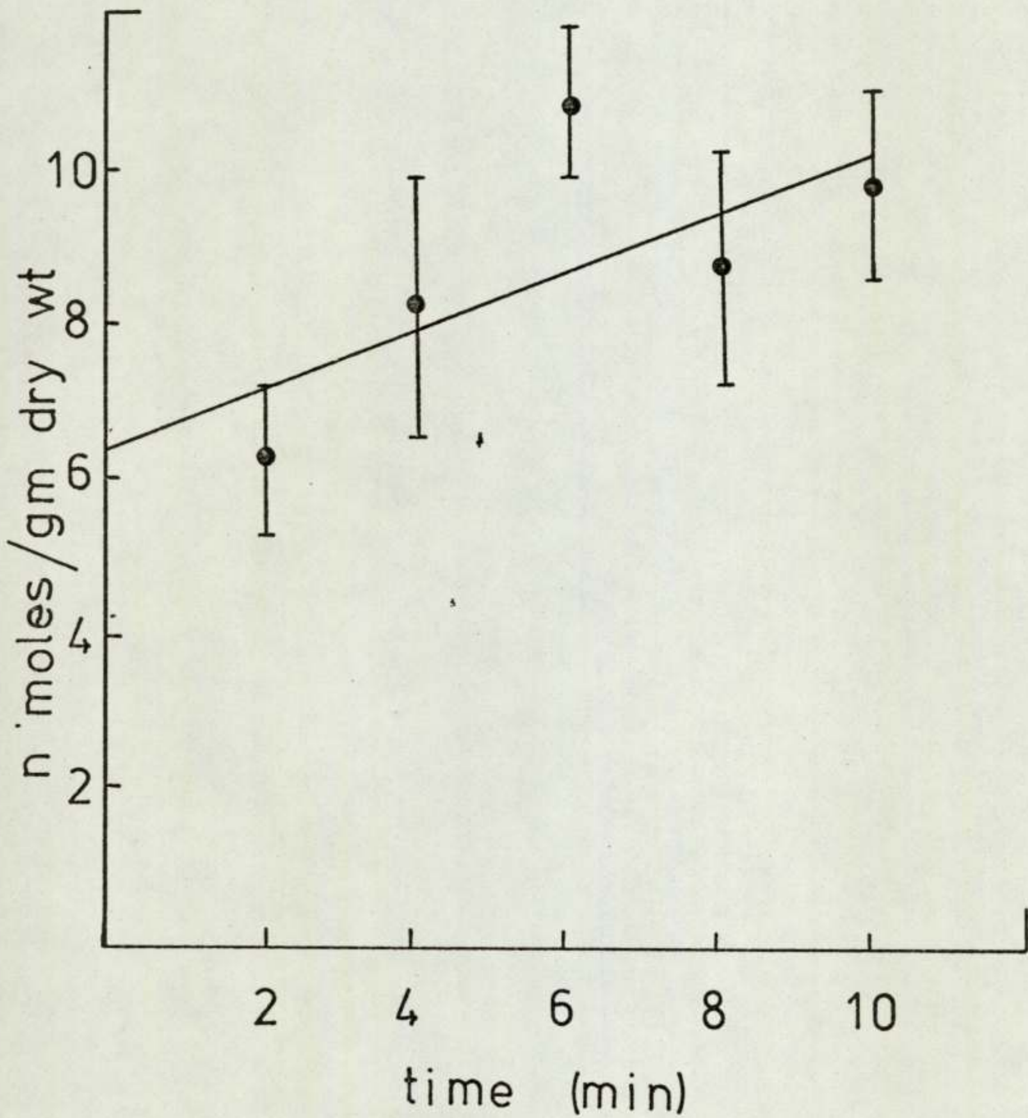


FIGURE 3.10.

Uptake of folic acid by everted jejunal rings at 0°C. Each point is the mean and s.e.m. of 7 determinations. Concentration of folic acid in the incubation medium was 10^{-5}M.

iii) Effect of Metabolic Inhibition.

From the foregoing data it is established that the *in vitro* intestinal transport of folic acid is a saturable process showing some dependence upon the temperature of incubation. The everted sac technique was used to examine the effect of inhibitors of aerobic respiration on serosal transfer, and on the accumulation of folate in the sac wall.

In these experiments, 6 sacs (4-5 cm. initial wet length) were prepared from the jejunum of each rat. Of the six, 2 were maintained as controls under normal incubation conditions, 2 were incubated in the presence of 10^{-4}M, 2:4-Dinitrophenol (DNP) and two were incubated under a continuous stream of nitrogen. Care was taken to allocate control and treatment sacs at random, and to ensure that all the other aspects of the incubation procedure were identical. The sacs were incubated under the usual conditions for 30 minutes, after which all 6 were removed and assayed for serosal transfer and tissue accumulation as described in Section 2, Part 3. The serosal fluid transfer of the control and treatment sacs was measured by the method described in Section 2, Part 2 and this provides a second index of metabolic inhibition; a discussion of the inter-relationship of water absorption and membrane transport in the intestine will be found in Appendix I.

Figures 3.11, 3.12, 3.13, and 3.14 illustrate the tissue folate concentration, the total serosal folate

transfer, the final serosal folate concentration, and the serosal fluid transfer respectively. The numerical data for folate absorption is contained in Table 3.10.

These data indicate that the suppression of aerobic metabolism by DNP or by anoxia brings about reduction in the *in vitro* intestinal transport of folic acid. This reduction is about 40-50% for most of the quoted parameters and is of only weak statistical significance when expressed in terms of total serosal transfer. During these incubations it was observed that the concurrent serosal fluid transfer of these sacs also underwent a significant reduction; the net effect being a slight loss of fluid from the serosal compartment.

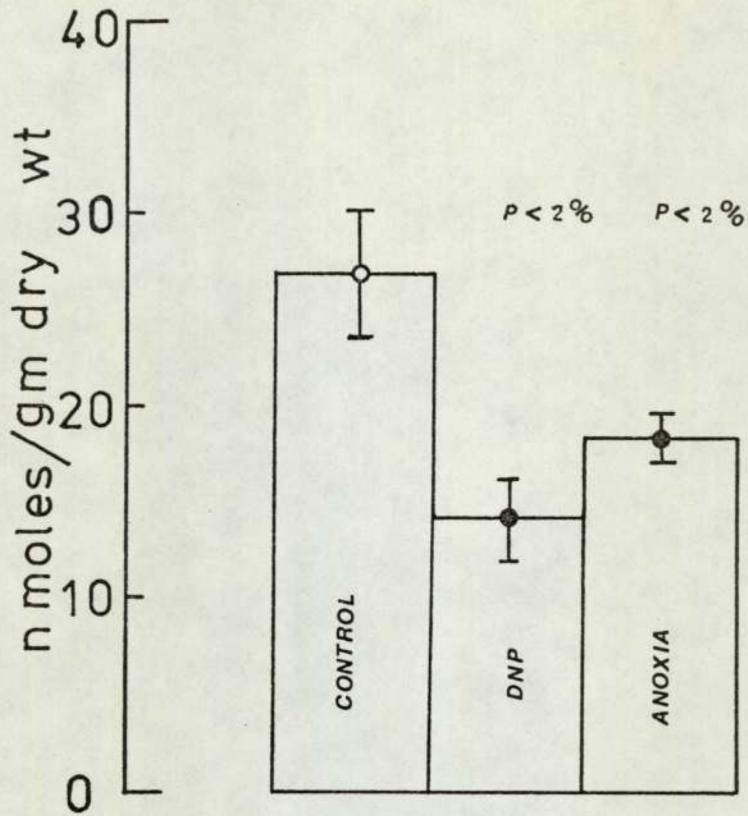


FIGURE 3.11. Metabolic Inhibition:
tissue uptake after 30 mins. incubation.

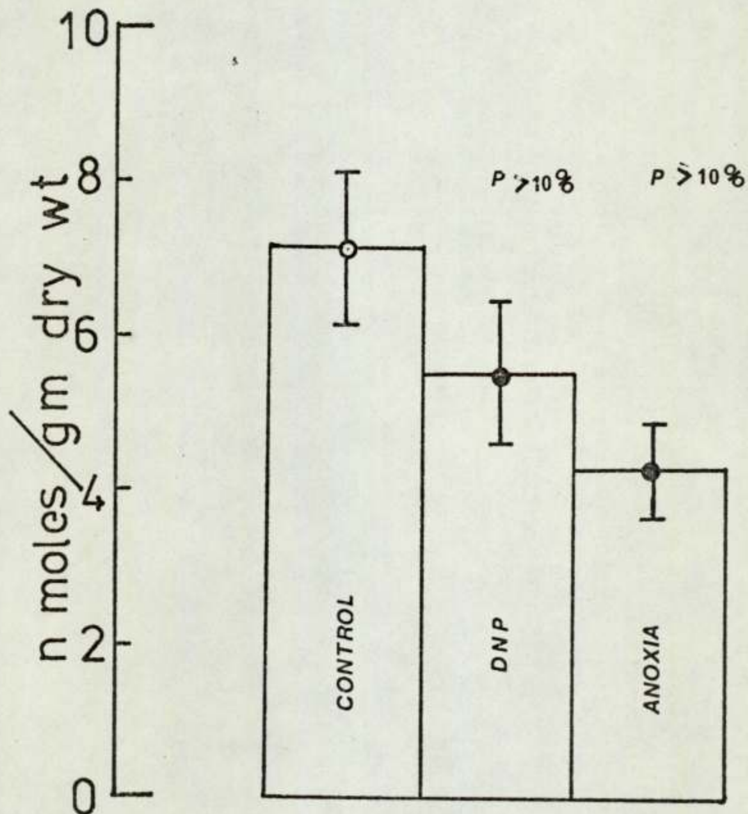


FIGURE 3.12. Metabolic Inhibition:
serosal transfer after 30 mins. incubation.

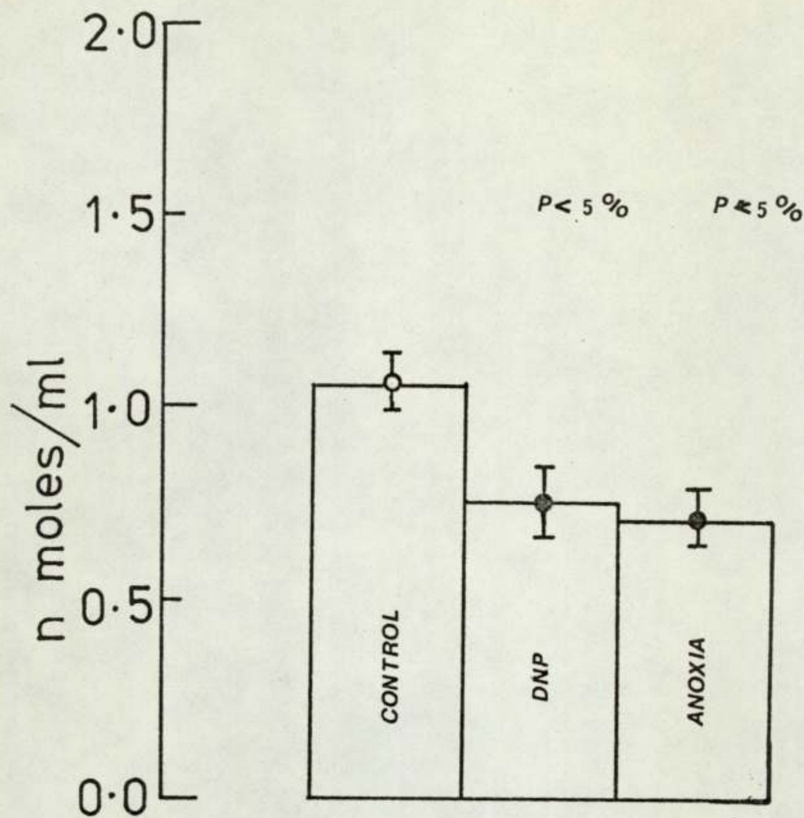


FIGURE 3.13. Metabolic Inhibition:
serosal concentration after 30 mins. incubation.

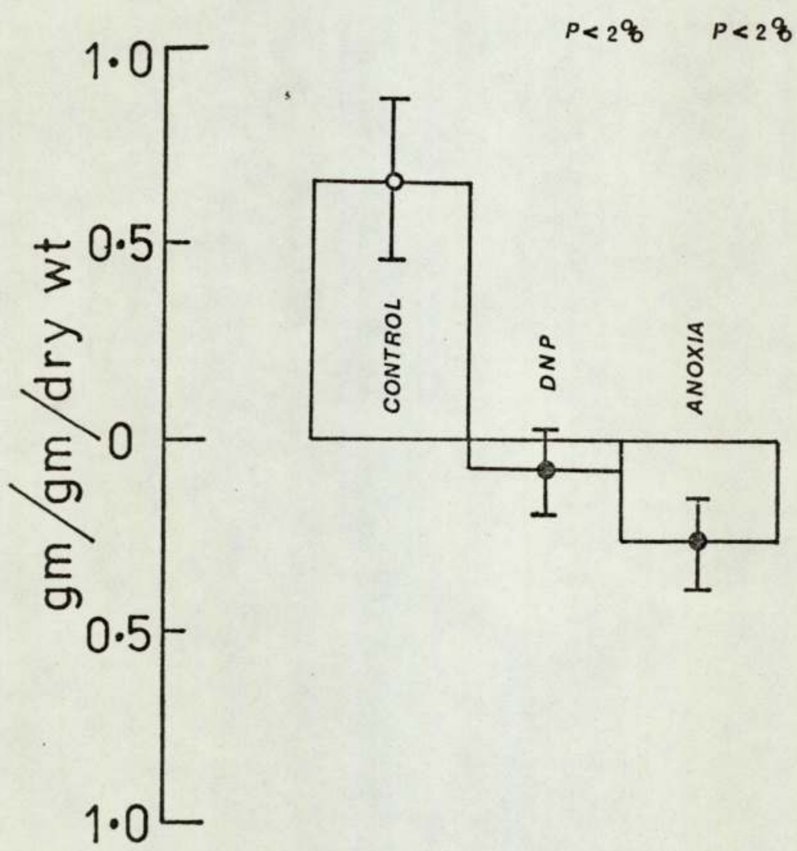


FIGURE 3.14. Metabolic Inhibition:
serosal fluid transfer after 30 mins. incubation.

TABLE 3.10

Effect of metabolic inhibition on transport of folic acid by everted sacs of rat jejunum.

	Control	DNP	P*	Anoxic	P*
i) Absolute serosal transfer (n mole/gm dry wt.)					
	7.17 ± 1.0 (5)	5.49 ± 0.93 (5)	> 10%	4.30 ± 0.55 (5)	> 10%
ii) Serosal concentration (n mole/ml)					
	1.07 ± 0.06 (5)	0.77 ± 0.08 (5)	< 5%	0.73 ± 0.08 (5)	< 5%
iii) Tissue Content (n mole/gm dry wt.)					
	27.0 ± 3.3 (5)	14.2 ± 2.1 (5)	< 2%	18.5 ± 1.2 (5)	< 2%

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

Number of animals in brackets. Each animal value is derived as the mean of 2 sacs.

iv) Competitive Inhibition.

10-formylfolic acid was chosen as a suitable subject for competitive inhibition studies with folic acid. Besides its close structural similarity to folic acid it has the advantage of relative stability during the incubation procedure and, unlike methotrexate, there is no evidence that it is toxic to tissues at the concentrations employed.

Figure 3.15 illustrates a preliminary experiment in which the serosal folate transfer of everted sacs incubated in the presence of 10^{-5}M folic acid (approximately 5.4 mCi/mMol) is compared with that of similar sacs, incubated in identical solutions, to which had been added 10-formylfolic acid, also at a concentration of 10^{-5}M. The mucosal volume was 5 ml. and the incubations were carried out for 30 minutes under the usual conditions.

TABLE 3.11.

Serosal transfer of folic acid in the presence of 10-formylfolic acid (1:1 molar ratio)

Total serosal transfer (n mole/gm dry wt.)

Control	Test
18.4 ± 5.8 (3)	15.2 ± 5.0 (3)

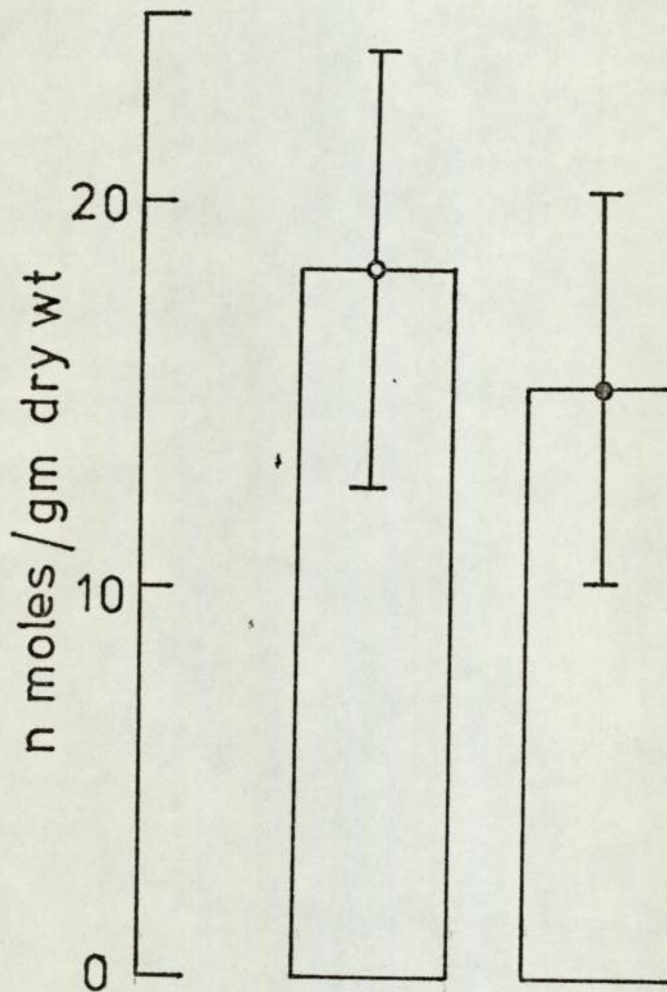
paired "t" = 1.6

$p > 10\%$

Number of animals in brackets.

Values are given as mean $\pm$ s.e.m.

FIGURE 3.15



Serosal transfer of folic acid in the presence of 10-formylfolic acid; 1:1 molar ratio; 30 mins. incubation.

The small reduction in the serosal transfer of folic acid in the presence of its 10-formyl derivative observed in this experiment is not statistically significant. Figure 3.16 and Table 3.12 illustrate a more detailed experiment in which the transport of folic acid was measured in the presence of a ten-fold higher concentration of 10-formylfolic acid. Six everted sacs were prepared from each animal and distributed at random amongst three "control" flasks containing 10^{-6}M folic acid (approximately 55 mCi/mMol) and three "test" flasks containing the same solution with the addition of 10^{-5}M 10-formylfolic acid. The six flasks were incubated under identical conditions as before.

In Figure 3.16 each pair of experimental points is derived from the means of three control and three test sacs from the same animal. The mean values for the serosal transfer and tissue accumulation of the six experimental animals are presented, and the significance of the difference of these means is assessed by the paired "t" test. As in the previous experiment there is a small reduction, of low statistical significance, in the serosal transfer of folic acid in the test sacs. However, there is a highly significant 36% reduction in the amount of folic acid retained by the gut wall.

The 10-formylfolic acid used in this study was material previously prepared by workers in this laboratory. The purity of the compound was checked by thin layer chromatography and by microbiological assay, and the level of impurity was estimated by these methods as less than 5% and less than 10% respectively.

TABLE 3.12.

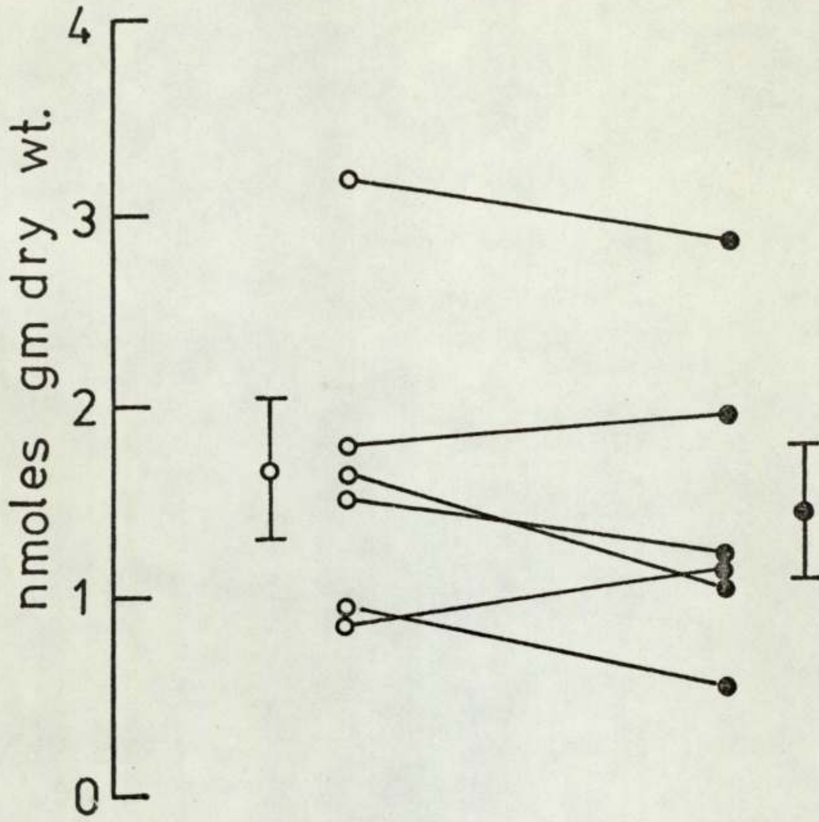
Transport of folic acid in the presence of 10-formylfolic acid (10:1 molar ratio).

Control		Test
	i) Total serosal transfer (n mole/gm dry wt.)	
1.67 ± 0.34 (6)		1.48 ± 0.34 (6)
	paired "t" = 1.35	
	$p > 10\%$	
	ii) Tissue uptake (n mole/gm dry wt.)	
7.57 ± 0.43 (6)		4.80 ± 0.14 (6)
	paired "t" = 6.04	
	$p < 1\%$	

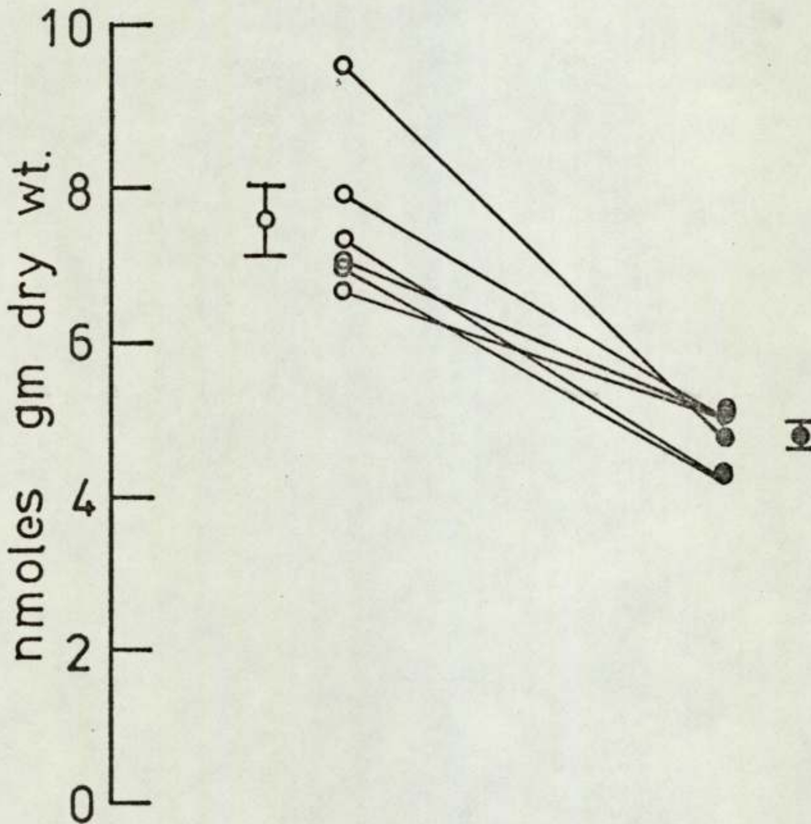
(Number of animals in brackets)

Values are given as mean ± s.e.m.

FIGURE 3.16.



Total serosal transfer in the presence of 10-formylfolic acid; 30 mins. incubation.



Tissue uptake in the presence of 10-formylfolic acid; 30 mins. incubation.

v) Efflux of folic acid from pre-loaded jejunal sacs.

A series of experiments were put in hand to observe the efflux of folic acid from the mucosal surface in the presence and absence of folic acid and related compounds.

Six jejunal sacs (approximately 4 cms. initial wet length) were prepared from each animal in the usual way. The sacs were pre-loaded by incubating for 20 minutes in the standard incubation medium, containing 1.0 or 0.5 x 10^{-6}M ^{14}C labelled folic acid. After pre-loading the sacs were washed briefly (45 secs.) with a gentle swirling agitation in warm oxygenated saline to remove extraneous activity, and transferred to glass test tubes containing 1.0 ml. of the incubation medium. Control tubes contained folate free medium, treatment tubes contained folic acid or 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 ensured by continuous gassing as before. At intervals of 5, 15 and 30 minutes 0.1 ml. 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 folate and paired with similar control groups incubated in folate free medium. The results for sacs in each group were averaged to give single treatment and control values for each animal. Differences were tested for significance by Student's paired "t" test.

Figure 3.17 and Table 3.13 illustrate the time course of folate efflux from sacs pre-loaded in 1.0 x 10^{-6}M folic acid and subsequently incubated in an unlabelled solution of 1.0 x 10^{-5}M folic acid, compared with the efflux from similar pre-loaded sacs incubated in folate free medium. At each time interval there is a consistent and significantly higher concentration of labelled folate in the treatment tubes; this indicates that the efflux of folate from the mucosal epithelium is stimulated in the presence of external folic acid.

Figures 3.18, 3.19, 3.20 and 3.21 illustrate similar experiments carried out with "treatment" solutions containing 10^{-5}M concentrations of 10-formyl-folic acid, methotrexate, pteroic acid and pteroyl-D-glutamic acid. The data for these experiments are tabulated in Tables 3.14, 3.15, 3.16 and 3.17, which also contain statistical details for each set of observations.

In a similar experiment, the pre-loading incubation was carried out with 5-^{14}C-methyltetrahydro-folic acid in the incubation medium in place of labelled folic acid. The subsequent incubation was carried out with folate free medium and 10^{-5}M folic acid as before. The results of this experiment are illustrated in Figure 3.22.

TABLE 3.13.

STIMULATED EFFLUX.

Folic Acid: Efflux in the presence of mucosal folic acid.

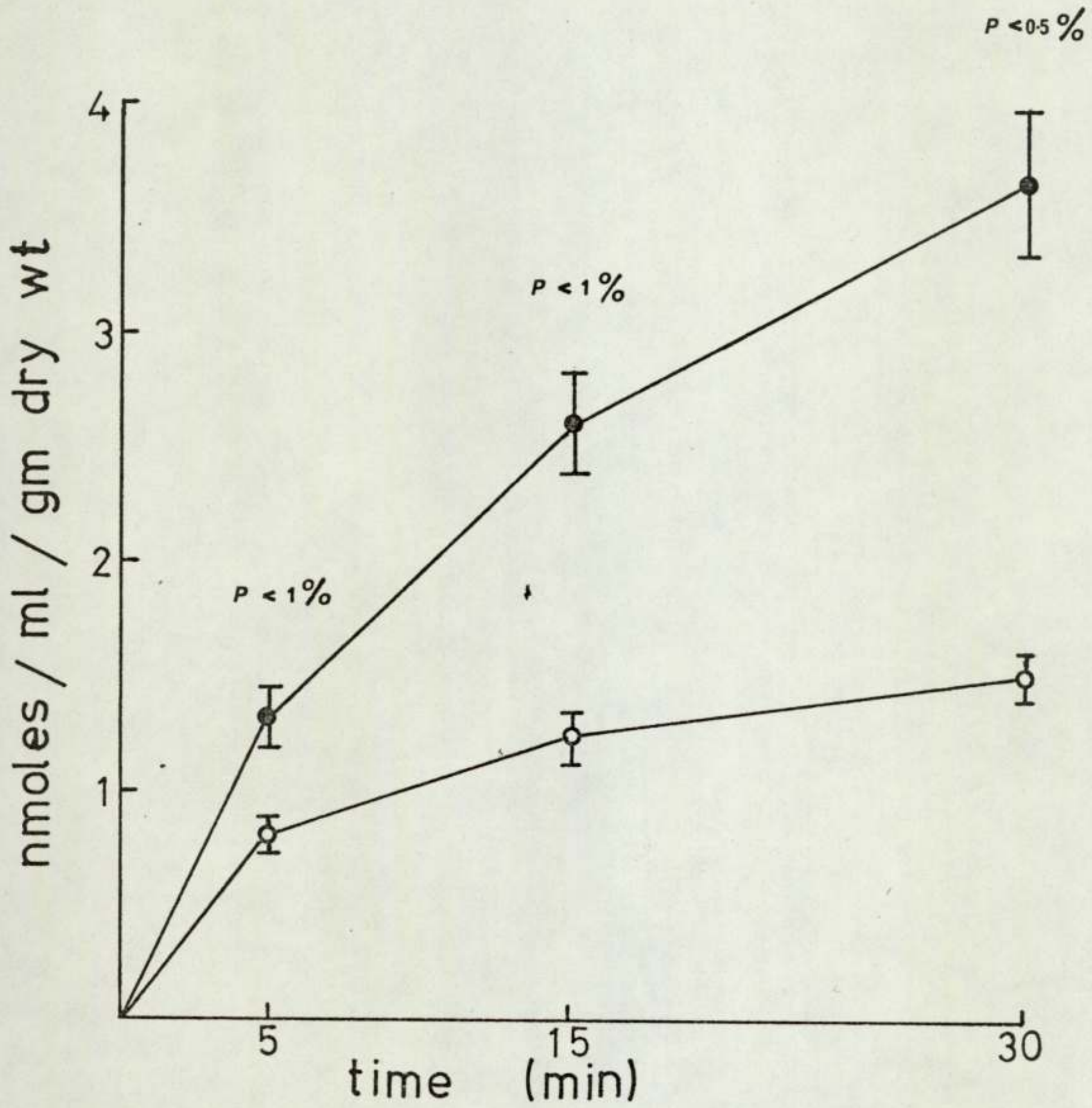
Time (minutes)	Mucosal Concentration (n. moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M Folic Acid	P*
5	0.79 ± 0.07 (5)	1.32 ± 0.12 (5)	$< 1\%$
15	1.24 ± 0.10 (5)	2.62 ± 0.24 (5)	$< 1\%$
30	1.51 ± 0.10 (5)	3.66 ± 0.35 (5)	$< 0.5\%$

Number of animals in brackets. Each individual animal value is derived from the mean of 3 sacs.

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

Values are given as mean ± s.e.m.

FIGURE 3.17.



Efflux of pre-loaded folate from everted sacs as a function of time, in the presence (•——•) and absence (o——o) of mucosal folic acid.

TABLE 3.14.

STIMULATED EFFLUX.

Folic Acid: Efflux in the presence of mucosal 10-formylfolic acid.

Time (minutes)	Mucosal Concentration (n. moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M 10-Formylfolic acid	P *
5	0.97 ± 0.09 (3)	1.37 ± 0.19 (3)	< 10%
15	1.57 ± 0.12 (3)	2.73 ± 0.38 (3)	< 10%
30	1.70 ± 0.21 (3)	3.50 ± 0.40 (3)	< 5%

Number of animals in brackets. Each animal value is derived from the mean of 2 sacs.

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

Values are given as mean ± s.e.m.

TABLE 3.15.

STIMULATED EFFLUX.

Folic Acid: Efflux in the presence of mucosal methotrexate.

Time (minutes)	Mucosal Concentration (n. moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M Methotrexate	P*
5	0.90 ± 0.10 (3)	1.47 ± 0.13 (3)	< 5%
15	1.47 ± 0.18 (3)	2.40 ± 0.30 (3)	< 2%
30	1.63 ± 0.23 (3)	3.13 ± 0.52 (3)	< 5%

Number of animals in brackets. Each individual animal value is derived from the mean of 2 sacs.

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

Values are given as mean ± s.e.m.

TABLE 3.16.

STIMULATED EFFLUX.

Folic Acid: Efflux in the presence of mucosal pteroic acid.

Time (minutes)	Mucosal Concentration (n. moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M Pteroic Acid	P
5	0.67 ± 0.09 (3)	0.63 ± 0.07 (3)	-
15	1.00 ± 0.20 (3)	0.93 ± 0.12 (3)	-
30	1.17 ± 0.24 (3)	1.07 ± 0.12 (3)	-

Number of animals in brackets. Each individual animal value is derived from the mean of 2 sacs.

Values are given as mean ± s.e.m.

TABLE 3.17.

STIMULATED EFFLUX.

Folic Acid: Efflux in the presence of mucosal pteroyl-D-glutamic acid.

Time (minutes)	Mucosal Concentration (n.moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M Pte-D-Glu	P*
5	0.67 ± 0.09 (3)	0.73 ± 0.14 (3)	>10%
15	1.0 ± 0.20 (3)	1.17 ± 0.23 (3)	>10%
30	1.17 ± 0.24 (3)	1.54 ± 0.30 (3)	>10%

Number of animals in brackets. Each individual animal value is derived from the mean of 2 sacs.

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

Values are given as mean ± s.e.m.

TABLE 3.18.

STIMULATED EFFLUX.

5-Methyltetrahydrofolic acid: Efflux in the presence of mucosal folic acid.

Time (minutes)	Mucosal Concentration (n.moles/ml./gm dry wt.)		
	Folate Free	10^{-5}M Folic Acid	P
5	0.60 (2)*	0.40 (2)**	-
15	0.70 (2)*	0.60 (2)**	-
30	0.80 (2)*	0.70 (2)**	-

Number of animals in brackets.

* Each animal value is derived as the mean of 2 sacs.

** Each animal value is derived as the mean of 3 sacs.

Values are given as $\pm$ s.e.m.

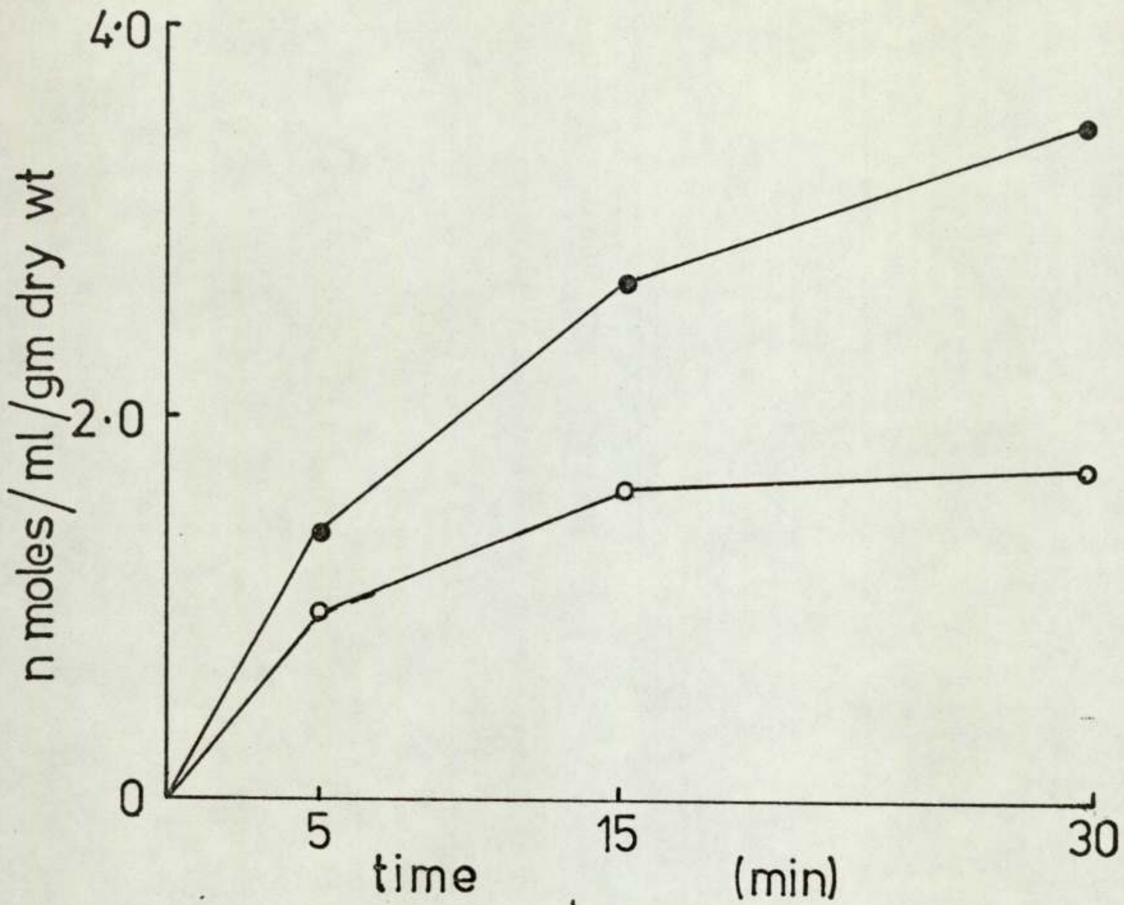


FIGURE 3.18 Efflux of pre-loaded folic acid in the presence (•) and absence (o) of mucosal 10-formylfolic acid.

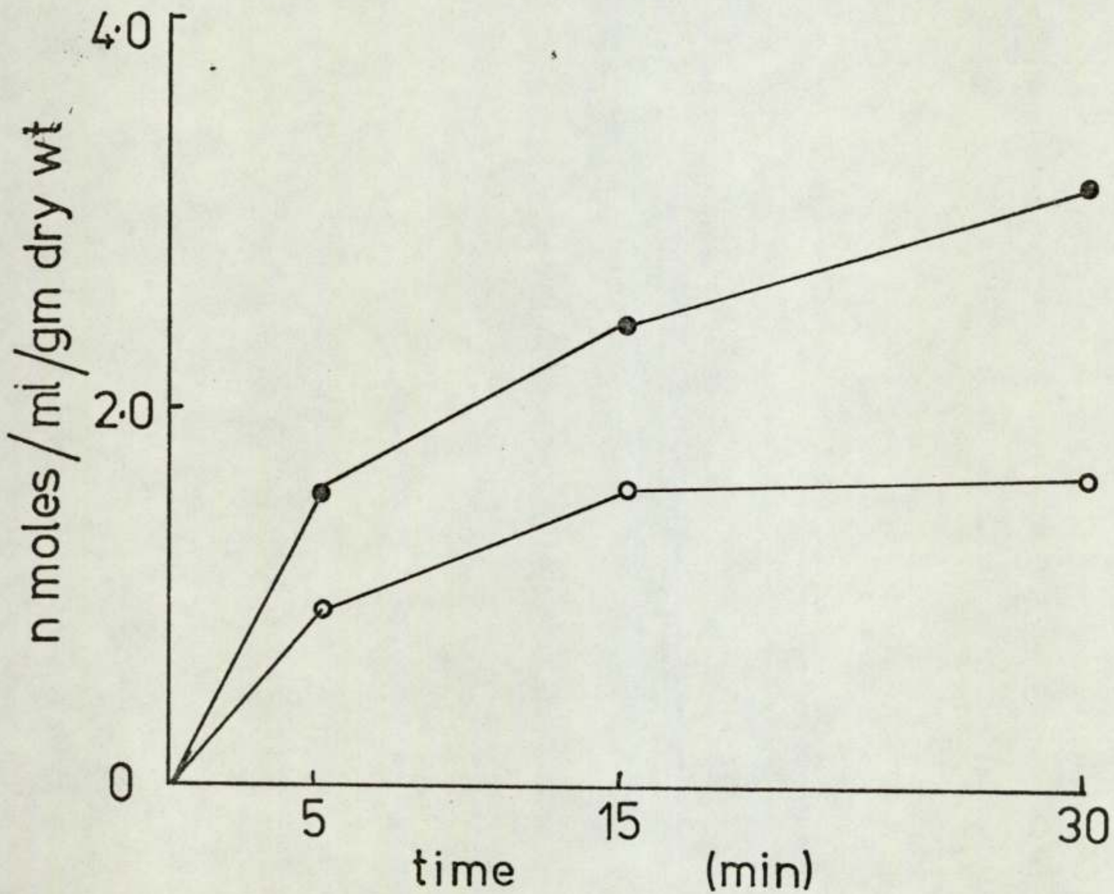


FIGURE 3.19 Efflux of pre-loaded folic acid in the presence (•) and absence (o) of mucosal methotrexate.

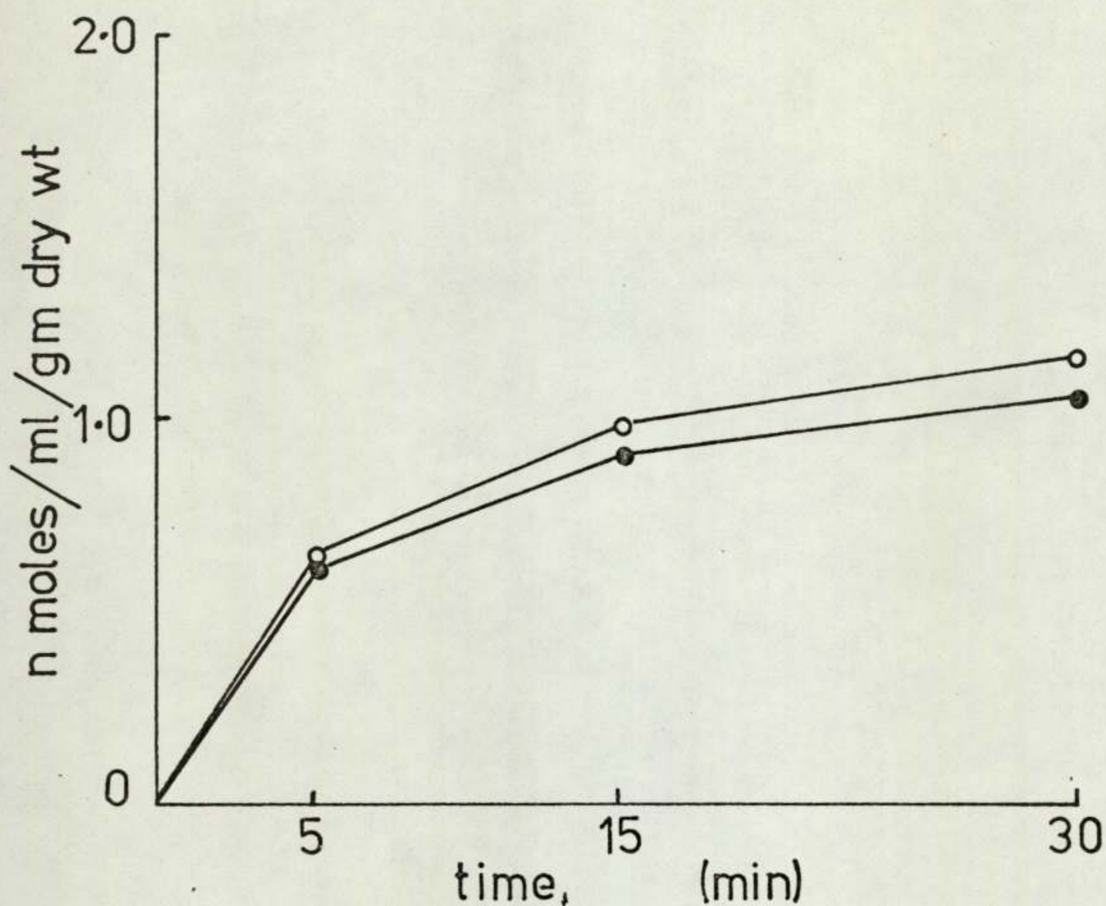


FIGURE 3.20. Efflux of pre-loaded folic acid in the presence (•) and absence (o) of mucosal pteroic acid.

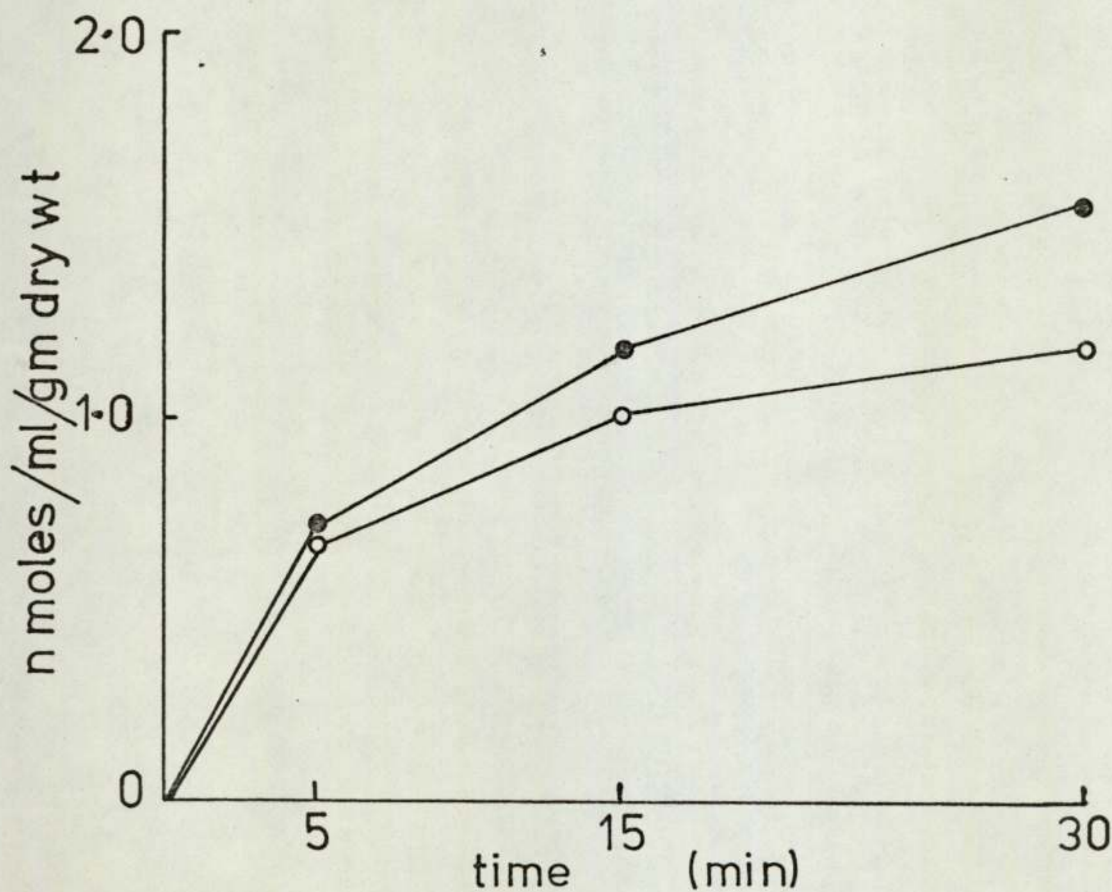
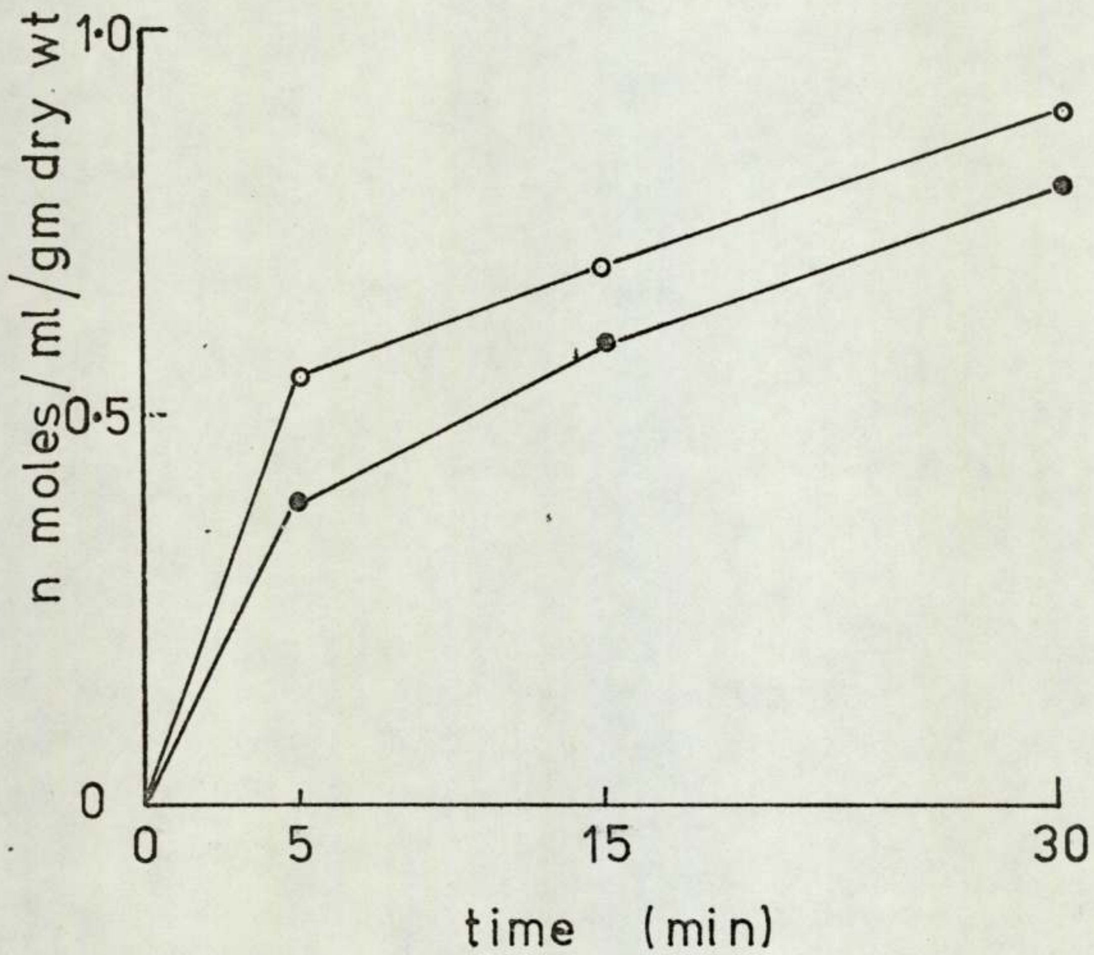


FIGURE 3.21. Efflux of pre-loaded folic acid in the presence (•) and absence (o) of mucosal pteroyl-D-glutamic acid.

FIGURE 3.22.



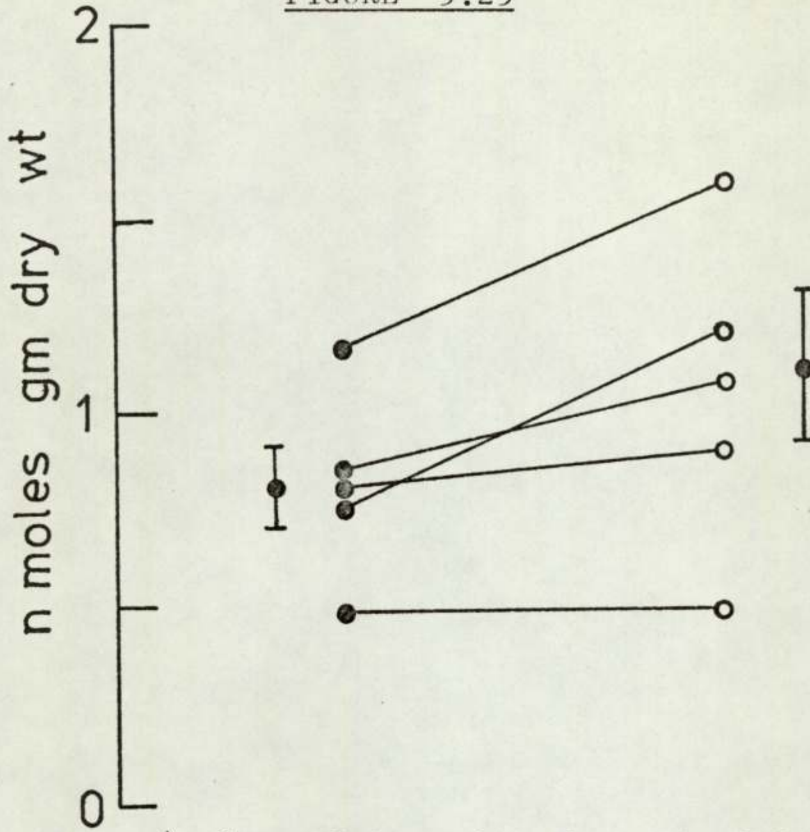
Efflux of pre-loaded 5-methyltetrahydrofolate from everted sacs as a function of time, in the presence (•——•) and absence (o——o) of mucosal folic acid.

vi) Serosal transfer.

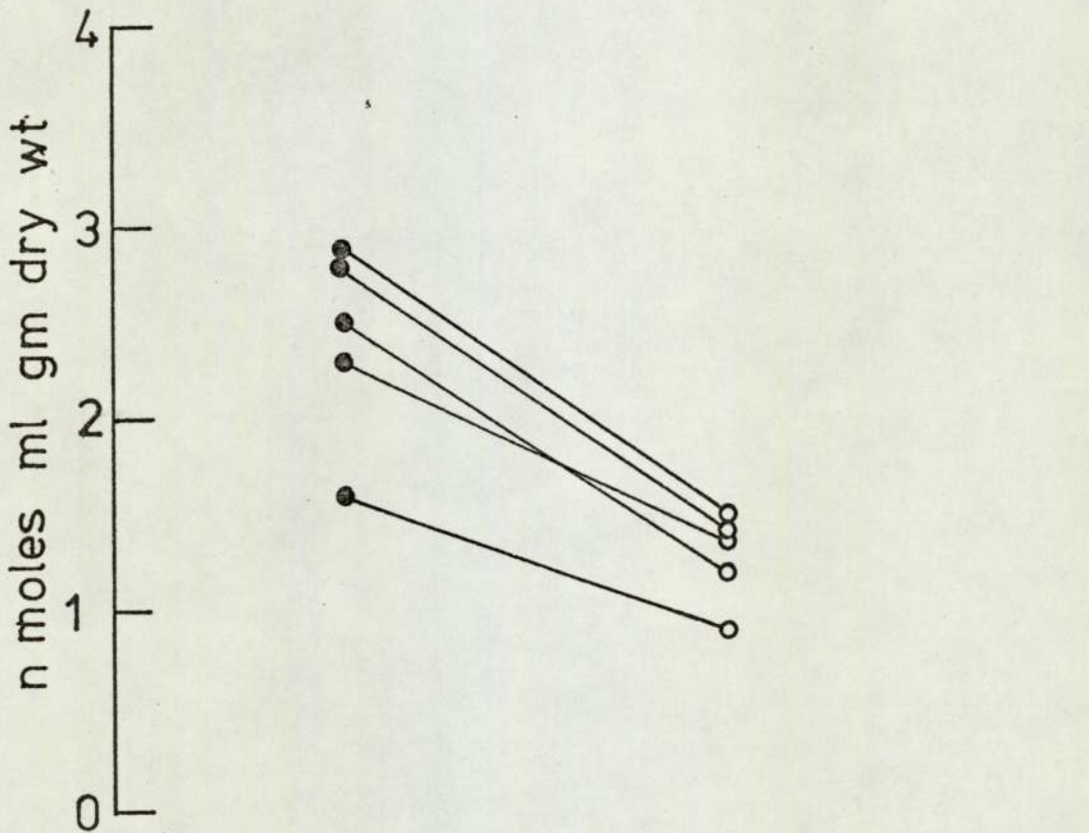
Having established that the efflux of folate from the mucosal surface of the intestine was stimulated *in vitro* by the presence of folic acid in the mucosal bathing medium, it was of interest to examine the "symmetry" of this effect. The object of this experiment was to determine whether or not the displacement of pre-loaded folate into the mucosal bathing medium was accompanied by a simultaneous displacement into the serosal compartment.

Sacs were pre-loaded as before using 0.5×10^{-6}M folic acid in the incubation medium and an incubation period of 10 minutes. The "efflux" incubation was conducted as usual except that the mucosal media for the control and test sacs was sampled at 30 minutes only. The three control sacs and three test sacs were then opened and drained and the absolute serosal transfer during the duration of the experiment was determined as usual. The three test values and the three control values were then averaged to give single results for each animal. In Figure 3.23 the data have been plotted as paired values for each animal so that the results can be followed in detail. Figure 3.23 (a) contains the serosal transfer results, and the 30 minute mucosal efflux results are given in Figure 3.23 (b) for comparison. The man and standard errors for both sets of data are given in Table 3.19 together with the statistical significance as calculated by Student's paired "t" test.

FIGURE 3.23



a) Serosal transfer of pre-loaded folate in the presence (•) and absence (o) of mucosal folic acid.



b) Efflux of pre-loaded folate from everted sacs in the presence (•) and absence (o) of mucosal folic acid.

TABLE 3.19

Serosal transfer after pre-loading and subsequent incubation in the presence of mucosal folic acid.

	Serosal transfer at 30 minutes n.moles/gm dry wt.	
Folate Free	10^{-5}M Mucosal Folic Acid	P*
1.06 (5)	0.81 (5)	< 5%

	Mucosal Concentration at 30 minutes n.moles/ml/gm dry wt.	
Folate Free	10^{-5}M Mucosal Folic Acid	P*
1.28 (5)	2.42 (5)	< 2%

Number of animals in brackets. Each animal value is derived as the mean of 3 sacs.

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

Figure 3.24 illustrates a single experiment in which the mucosal efflux of pre-loaded folate was compared in the presence and absence of a high serosal concentration of unlabelled folic acid.

The pre-loading incubation of a large (10 cms) jejunal sac was carried out as before using a $0.5 \times 10^{-5}M$ mucosal concentration of ^{14}C folic acid and an incubation period of 20 minutes. The sac was then removed from incubation, drained, washed in warm saline and cut into two identical 5 cm. sacs. The control sac was filled with standard folate free incubation medium, the test sac was filled with a similar solution also containing $10^{-5}M$ folic acid. The sacs were then set up as in the previous efflux experiments, and the mucosal bathing medium was sampled at the time intervals shown in Figure 3.24. The object of this experiment was not quantitative, but merely to determine whether the mucosal efflux was stimulated or depressed by the high concentration of cold folic acid in the serosal solution. Consequently the data has been expressed only in terms as counts per second per gram dry weight of sac.

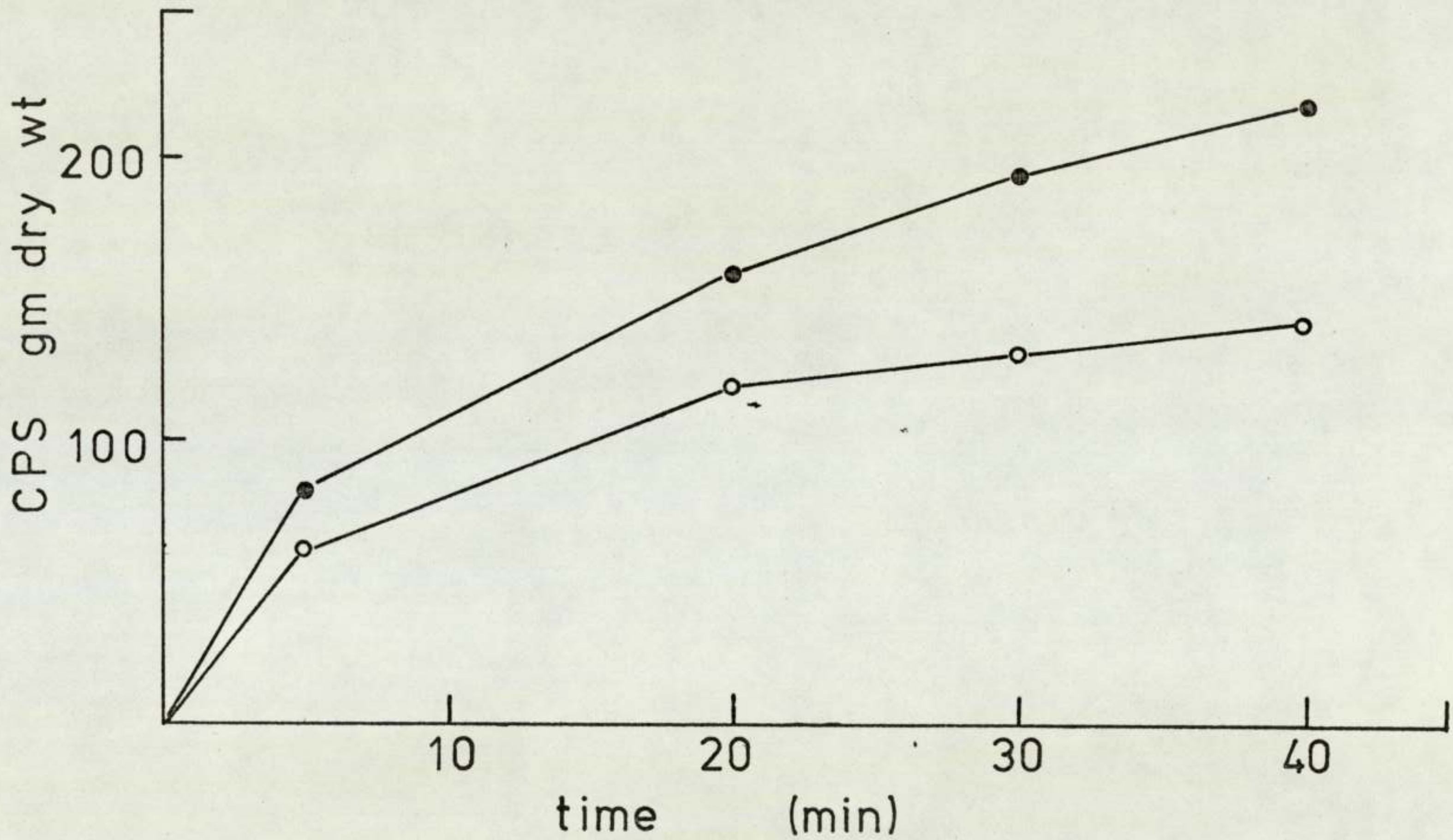


FIGURE 3.24. Mucosal efflux of pre-loaded folate in the presence (•——•) and absence (o——o) of a high serosal concentration of unlabelled folic acid.

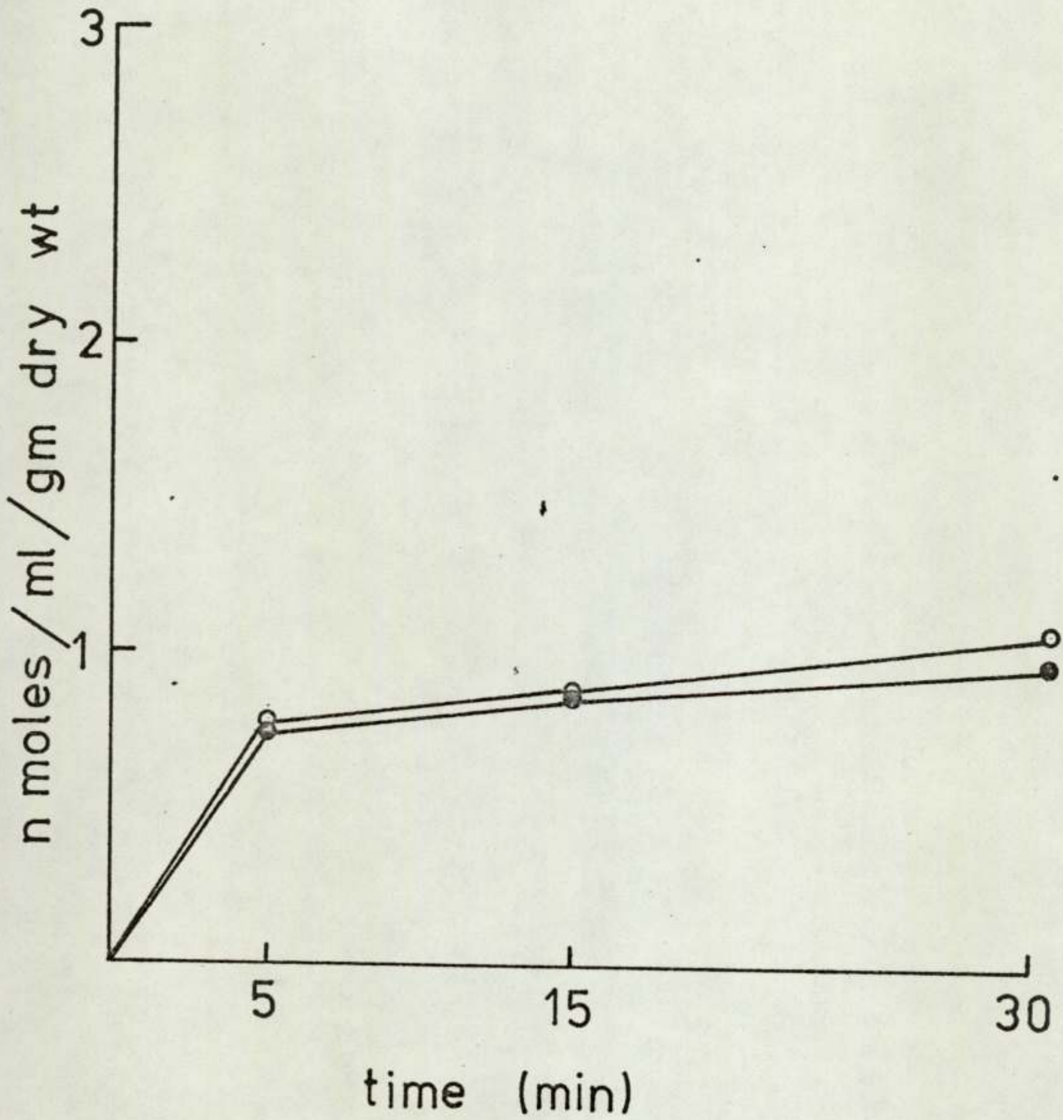
vii) Localisation of stimulated efflux.

In order to determine whether the stimulated efflux effect was localised to the jejunum, the jejunal efflux was compared with the efflux from sacs taken from the distal ileum.

In Figure 3.25 the efflux from pre-loaded sacs prepared from the most distal 10 cms. of the ileum is compared in the presence and absence of a 10^{-5}M concentration of mucosal folic acid. Control sacs are indicated by open circles, solid circles indicate test sacs.

In Figure 3.26 the experiment is carried a stage further. The jejunal efflux in the presence and absence of 10^{-5}M folic acid is indicated by the solid triangles and open circles respectively. For comparison, the efflux in the presence of 10^{-5}M folic acid from ileac sacs prepared from the same animal is indicated by solid circles. Error bars represent the range of two determinations performed on 2 animals.

FIGURE 3.25



Efflux of pre-loaded folate from everted ileac sacs in the presence (•——•) and absence (o——o) of mucosal folic acid.

TABLE 3.20

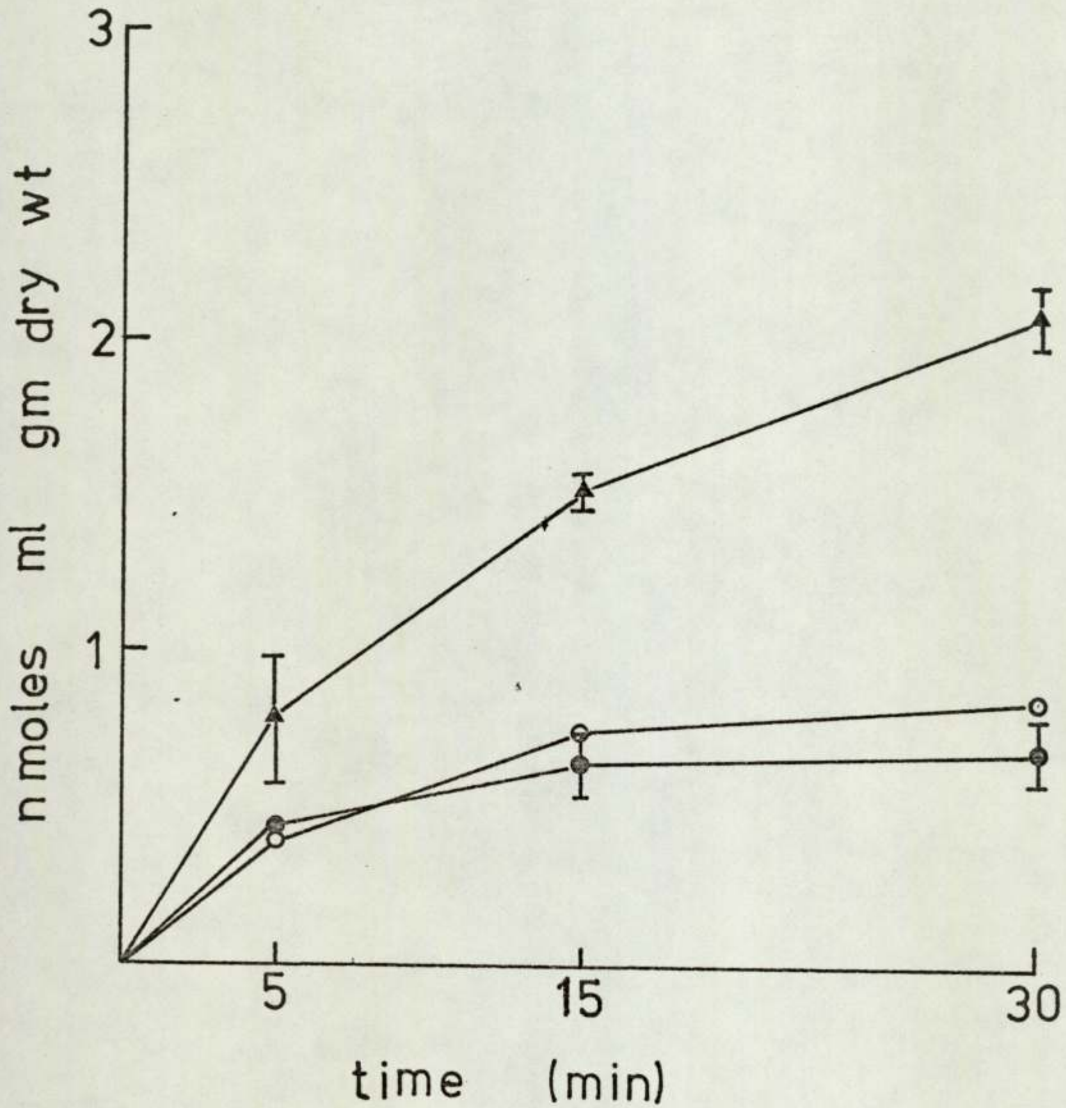
STIMULATED EFFLUX.

Folic Acid: Efflux from ileac sacs in the presence of mucosal folic acid.

Time (in minutes)	Mucosal concentration (n.moles/ml/gm dry wt.)	
	Folate Free	10^{-5}M Folic Acid.
5	0.8 (2)	0.75 (2)
15	0.9 (2)	0.85 (2)
30	1.1 (2)	1.0 (2)

Number of animals in brackets. Each animal value is derived from 1 sac.

FIGURE 3.26.



Efflux of pre-loaded folate from jejunal sacs in the presence (▲—▲) and absence (o—o) of mucosal folic acid, compared with efflux from pre-loaded ileac sacs in the presence of mucosal folic acid (●—●).

TABLE 3.21

STIMULATED EFFLUX

Folic Acid: Comparison of jejunal and ileac folate efflux.

Time (minutes)	Mucosal folate concentration (n.mole/ml/gm dry wt.)		
	Jejunum (mucosal folic acid)	Ileum (mucosal folic acid)	Jejunum (folate free)
5	0.8 (2)	0.45 (2)	0.4 (2)
15	1.55 (2)	0.65 (2)	0.75 (2)
30	2.10 (2)	0.70 (2)	0.85 (2)

Serosal folate concentration (n.mole/ml/gm dry wt.) after 60 min. efflux incubation	
Jejunum (mucosal folic acid)	Ileum mucosal folic acid)
1.7 (2)	0.17 (2)

Number of animals in brackets. Each animal value is derived from 1 sac.

viii) Transport of pteroic acid.

In view of the failure of pteroic acid to displace pre-loaded folic acid from the gut wall, a brief study was made of the transport of this compound. Single jejunal sacs were prepared as usual from three animals and incubated for 30 minutes under the standard conditions in 10^{-5}M. pteroic acid. At the end of the incubation the mucosal solutions and the serosal solutions were analysed by microbiological assay, and corrected for endogenous *S. faecalis*-active material, and the serosal:mucosal concentration ratios were calculated. In Table 3.22 the value for pteroic acid is compared with the equivalent value for folic acid obtained in a similar experiment. Values are expressed as the mean and standard error.

TABLE 3.22.

Comparative transport of pteroic acid and folic acid.

Serosal:Mucosal concentration ratio at 30 minutes.

Folic acid	Pteroic acid
0.13 ± 0.02 (6)	0.08 ± 0.01 (3)

Number of animals is shown in brackets.

In one human subject an oral dose of pteroic acid was followed by no detectable rise in serum levels.

ix) Identification of the displaced species.

The principal radioactive species displaced from the tissue in these experiments were identified by means of autoradiography.

A single jejunal sac (6 cm. initial wet length) was prepared and filled as usual. Pre-loading was carried out in ^{14}C-folic acid ($10^{-6}M$) for 20 minutes after which the sac was washed and transferred to 1.0 ml. of incubation medium containing unlabelled folic acid ($10^{-5}M$). The second incubation period was 30 minutes.

At the end of the incubation the sac was discarded and single spots of the mucosal solution were applied to thin-layer chromatography plates as described in Section 2. A marker solution containing ^{14}C-folic acid was also applied to each plate and the chromatograms were run in three solvent systems (see Page 83, Section 2). Autoradiographs were set up as described in Section 2, and exposed for 20 days.

The principal radioactive component in the mucosal solution co-chromatogrammed with folic acid in all three solvent systems. At least one other minor radioactive component was present in the mucosal solution and this was tentatively identified as 5-methyltetrahydro-folic acid by reference to previously recorded R_f values (Beavon, 1973).

x) <u>Identification of the species present in the gut wall.</u>

The nature of the folates present in the gut wall after incubation with ^{14}C folic acid was investigated by means of autoradiography.

The upper jejunum of a single rat was used to prepare everted rings as previously described. All the material was transferred to a 25 ml. Erlenmeyer flask containing 10 ml. of standard incubation medium to which had been added 10^{-5}M. ^{14}C folic acid. Incubation was carried out for 60 minutes under the standard conditions. At the end of the incubation the everted rings were drained and washed as usual and homogenised in a motor driven ground glass homogeniser with 2 ml. distilled water. The material was centrifuged and the supernatant and the final incubation medium were run on thin-layer chromatograms in 3 solvent systems as previously described. Standard solutions of ^{14}C-folic acid, and unlabelled 5-methyltetrahydrofolic acid and 10-formylfolic acid were run simultaneously. Autoradiographs were prepared as previously described and exposed for 21 days. After development, the autoradiograms were analysed by means of the recording microdensitometer.

It was found that for each solvent system the major peak in the incubation solution co-chromatographed with the folic acid standard. However only a very low concentration of folic acid was detectable in the homogenised tissue supernatant and it was not the major component of this solution. Figures 3.27 and 3.28

are microdensitometer traces which illustrate the autoradiograms for the phosphate buffer and butanol-acetic acid chromatograms respectively. In each case the incubation medium trace is labelled "A" at the major peak (folic acid) and the tissue homogenate trace is labelled "B" at the major peak. Peak "B", the major component of the tissue supernatant, and peak "b" which had equivalent R_f values in the incubation medium, were identified as 5-methyltetrahydrofolic acid by comparison with standard compounds and previously reported R_f values (Beavon, 1973).

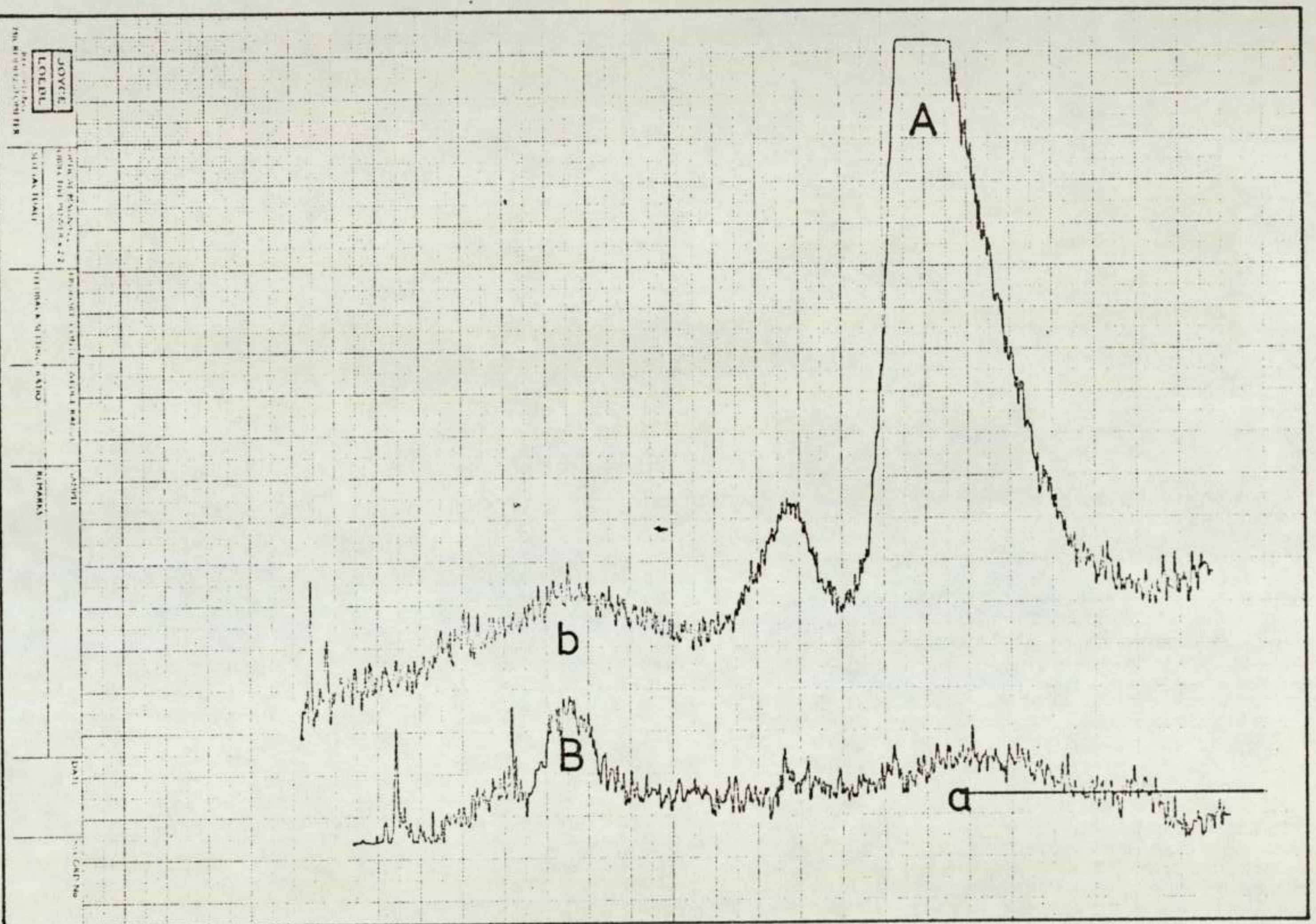


FIGURE 3.27. Microdensitometer traces for autoradiograms run in phosphate buffer. The incubation medium is labelled "A" at the major peak; the tissue homogenate is labelled "B".

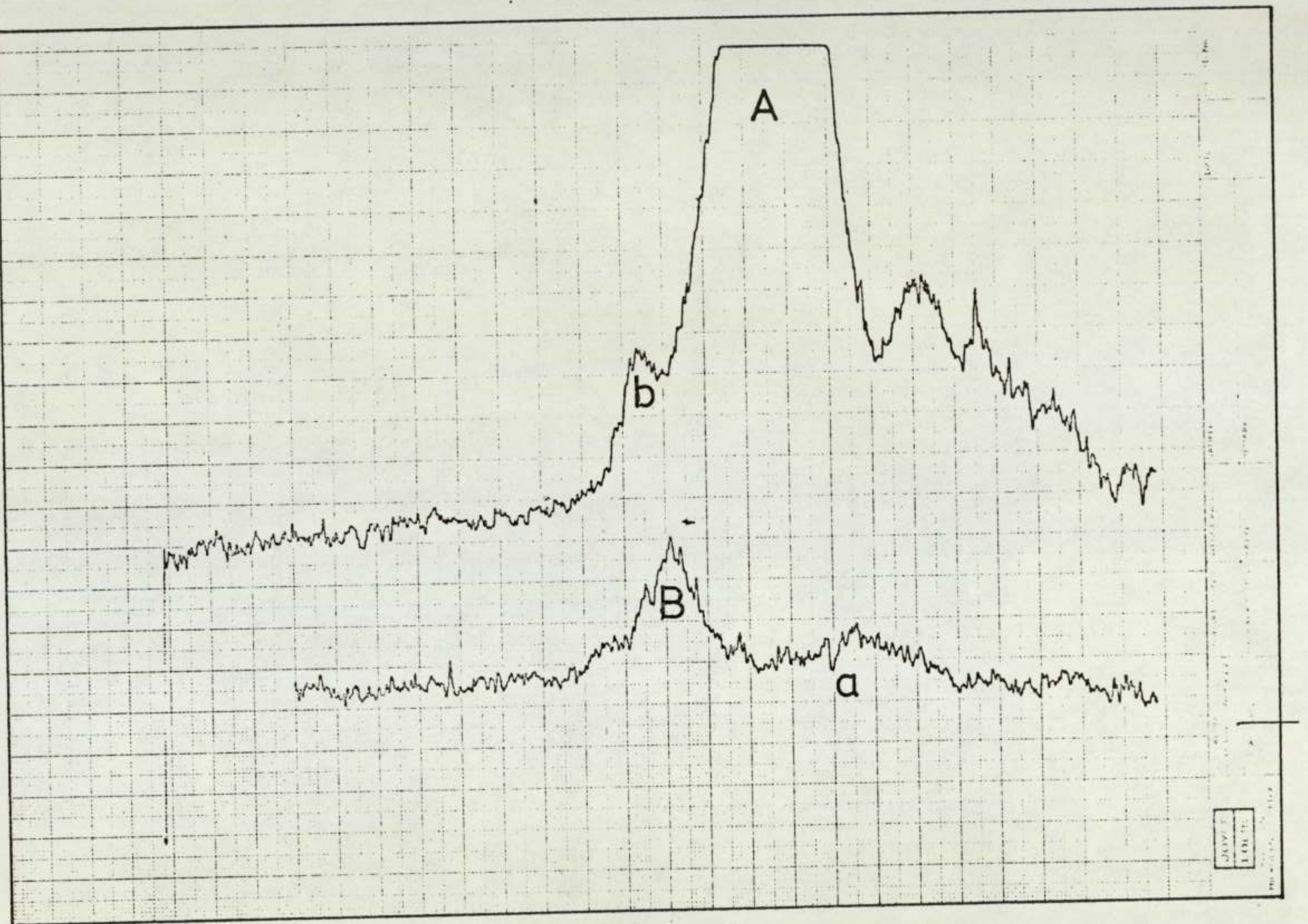


FIGURE 3.28 Microdensitometer traces for autoradiograms run in butanol-acetic acid. The incubation medium is labelled "A" at the major peak; the tissue homogenate is labelled "B".

xi) Isolated Cell Studies.

Isolated cells were prepared as described in Section 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. Examined under light microscopy, cell samples were found to contain a few isolated nuclei and some lymphocytes and micro-organisms. The author's general impression was that these cell preparations contained rather more mucus than is usually reported, though it is difficult to tell whether it is a feature of this particular preparation. In a trypan blue exclusion test, 60% of the cells were found to exclude the dye after 60 minutes at room temperature. The appearance of the cells under light microscopy is illustrated in Plates I. to VI.

Folic Acid Uptake.

The absorption of folic acid by isolated mucosal cells was studied using the isolation and incubation technique described in Section 2, Part 2. In a series of experiments carried out at 10^{-5}M it was never possible to observe any increase in the amount of folic acid associated with the isolated cells over an incubation period of 2 minutes to 30 minutes.
Figure 3.29 illustrates a typical experiment.
Cells were prepared from 2 rats as described above, and incubated in the standard incubation medium containing 10^{-5}M. ^{14}C folic acid (5.5 mci/mmol) under continuous oxygenation. Cells were removed by pipette at the

intervals shown and diluted with 10 mls. of ice-cold saline. Washing was terminated at wash 3 (Figure 3.30 and the sample was split into aliquots for assay. The first three points are the mean of 2 determinations, number 4 is the mean of 4.

For comparison, a similar experiment conducted with mucosal scrapes is also illustrated in Figure 3.29 Mucosal scrapes were prepared from two animals as described above, and were incubated under the same conditions as the isolated cells. Samples were removed at the times shown, washed in the same manner as the isolated cell samples, split into four batches which were assayed to give the values shown in Figure 3.29 and Table 3.23.

Figures 3.30 and 3.31 illustrate experiments designed to observe the "wash-out" of folic acid from the isolated cell preparation and compare it with the loss of inulin under the same conditions. In each case, isolated cells were prepared as usual from the jejunum of two rats. The cells were incubated for 10 minutes in the presence of 10^{-5}M. ^{14}C folic acid and ^{14}C labelled inulin. The concentration of labelled folic acid solution was adjusted to give approximately the same activity as in the inulin solution.

The purpose of the experiment was to observe the loss of folic acid by the preparation during the dilution and washing process, and compare it with the "wash-out" of inulin, a supposedly extracellular marker.

TABLE 3.23

ISOLATED CELLS.

Uptake of Folic Acid by Isolated Cells:
Comparison with Mucosal Scrapes.

	Folate Content (n.moles/gm dry wt.)	
Time (minutes)	Isolated Cells	Mucosal Scrapes
2	23.0 (2)*	11.4 (2)**
10	22.0 (2)*	46.8 (2)**
15	-	41.5 (2)**
20	20.30 (2)*	-
23	-	40.3 (2)**
30	19.70 (2)*	-
31	-	41.3 (2)**

Number of animals in brackets.

* Each animal value is derived as the mean of 2 assays.

** Each animal value is derived as the mean of 4 assays.

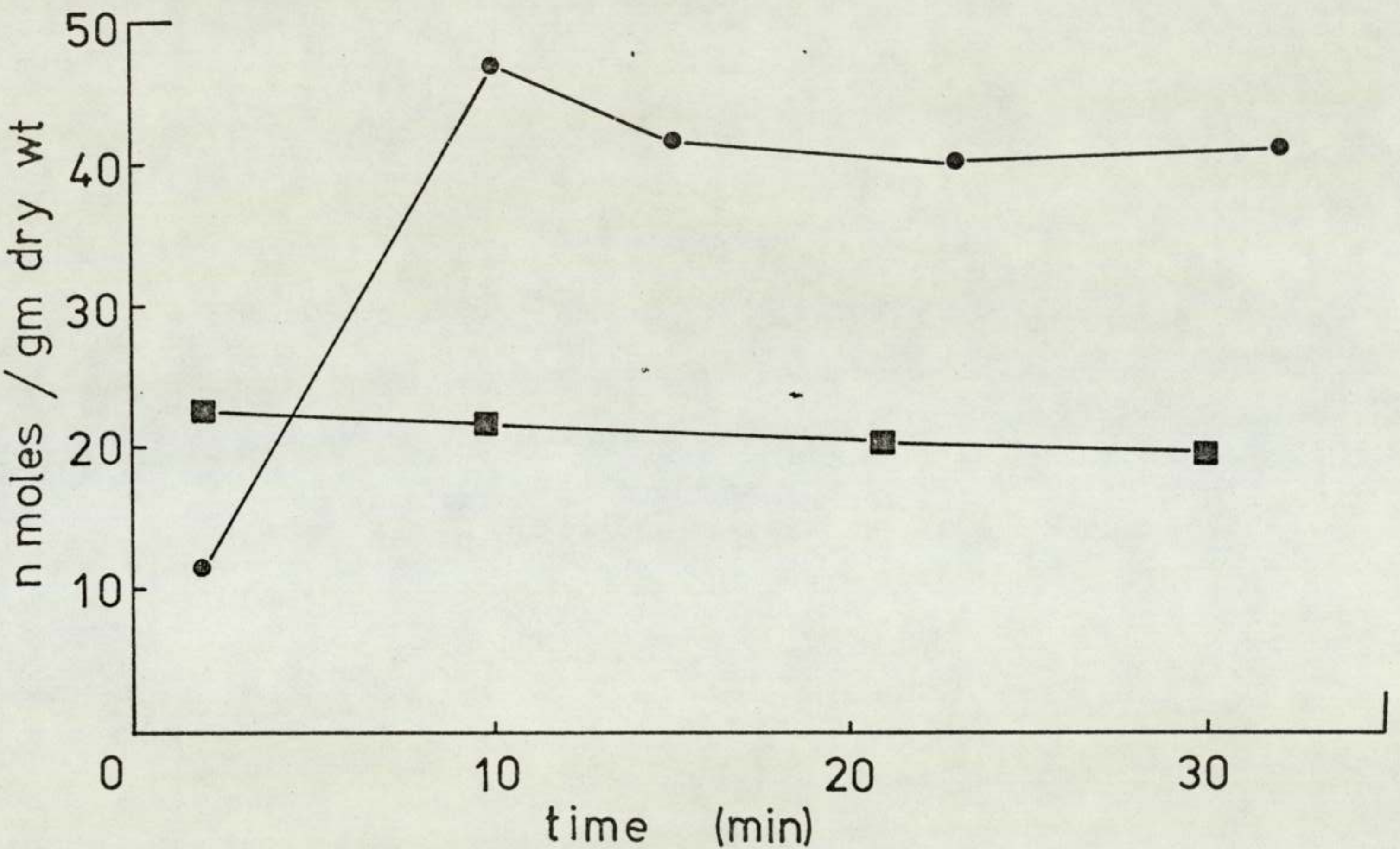
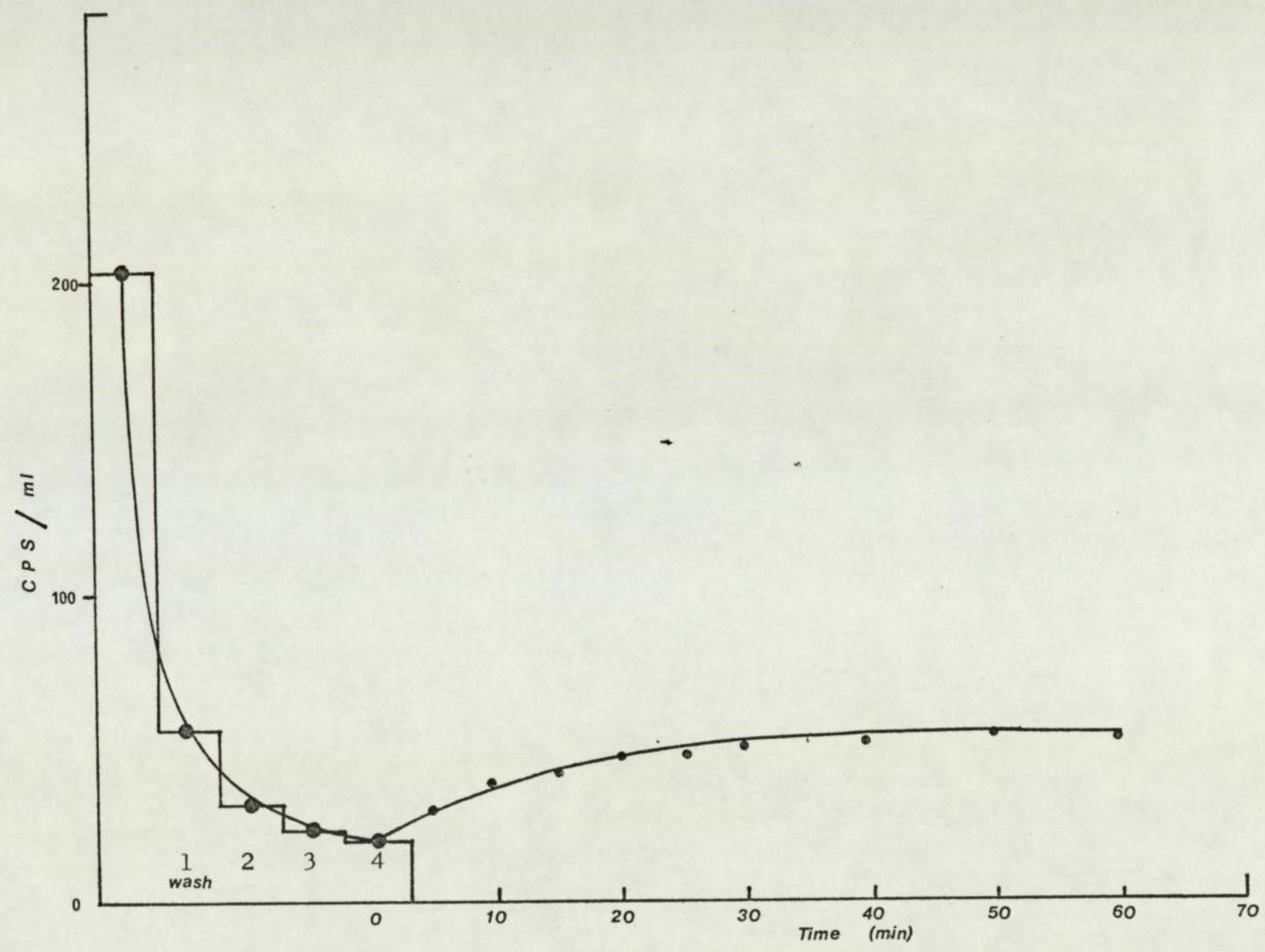


FIGURE 3.29 Uptake of 10^{-5}M. folic acid by isolated cells (■), and mucosal scrapes (•).

Figures 3.30 and 3.31 illustrate the activity of the supernatant solutions after the first dilution, and after spinning the cells down through four washes. The activity of the supernatant was determined at the intervals shown.

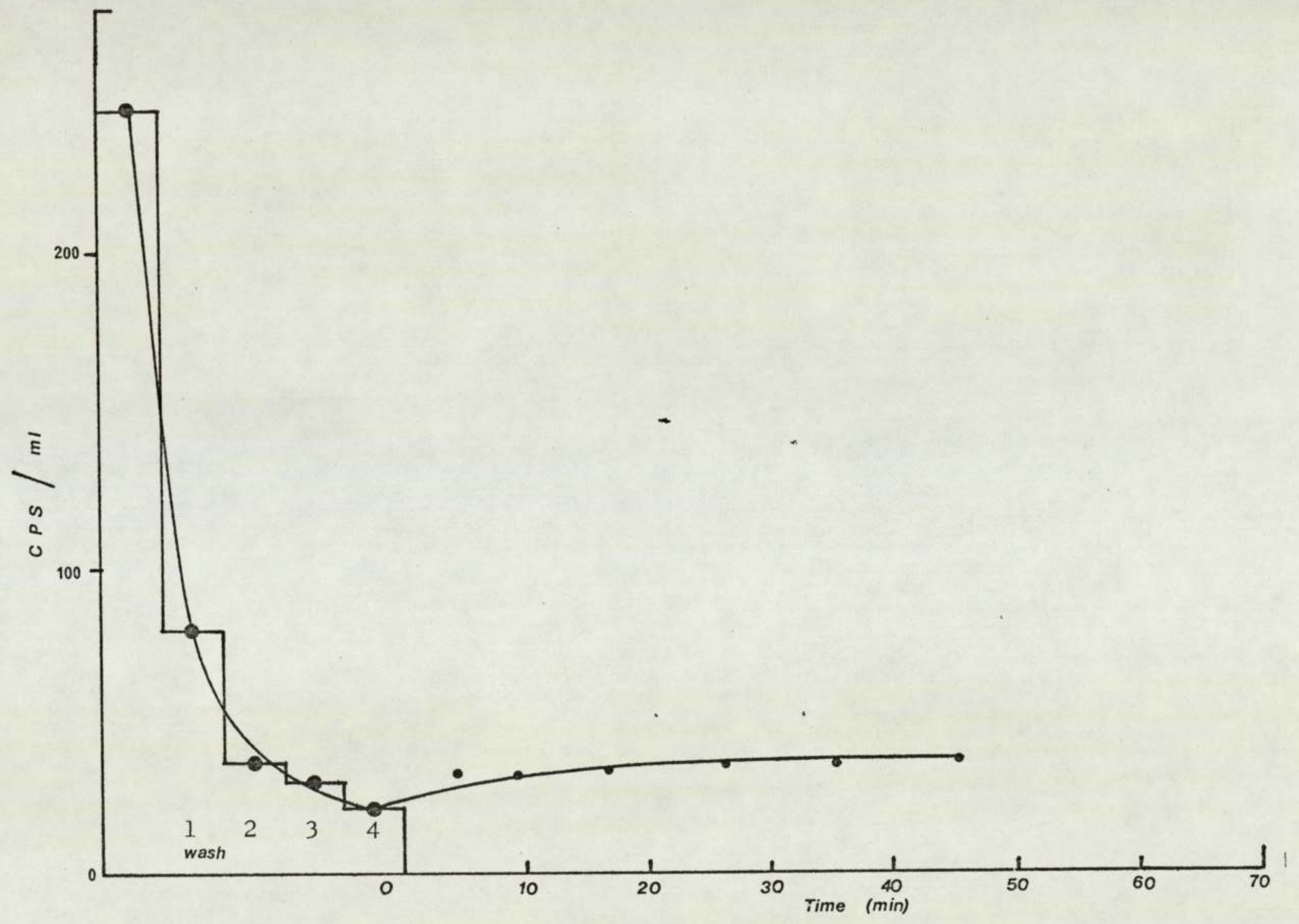
All quantitative results given in the text refer to cells assayed after the stage equivalent to wash 3 in Figure 3.30.

FIGURE 3.30.



Activity of the supernatant over isolated cells incubated for 10 mins. in the presence of folic acid.

FIGURE 3.31.



Activity of the supernatant over isolated cells incubated for 10 mins. in the presence of ^{14}C inulin.

Uptake of folic acid at 2 pH levels.

Cells were prepared from two animals in the usual way. The batch was divided into two samples which were then incubated in 10^{-6}M. ^{14}C folic acid (specific activity 55.3 mci/mmol) in phosphate buffered medium adjusted to pH 7.4 (control batch) and 5.0 (test batch). The cells were sampled at the intervals shown, and the activity of each sample was determined from duplicate aliquots. The final pH of the incubation solutions was determined, and is given in Figure 3.32. Each point is the mean of the two determinations.

TABLE 3.24

ISOLATED CELLS.

Time (minutes)	Folate Content of Isolated Cells. (n.moles/gm dry wt.)	
	pH 5.9*	pH 6.9*
7	2.3 (2)	2.0 (2)
16	5.6 (2)	4.0 (2)
33	7.5 (2)	5.0 (2)
42	7.1 (2)	-

* Final pH at the end of 45 minutes incubation.

Number of animals in brackets. Each animal value is derived as the mean of 2 assays.

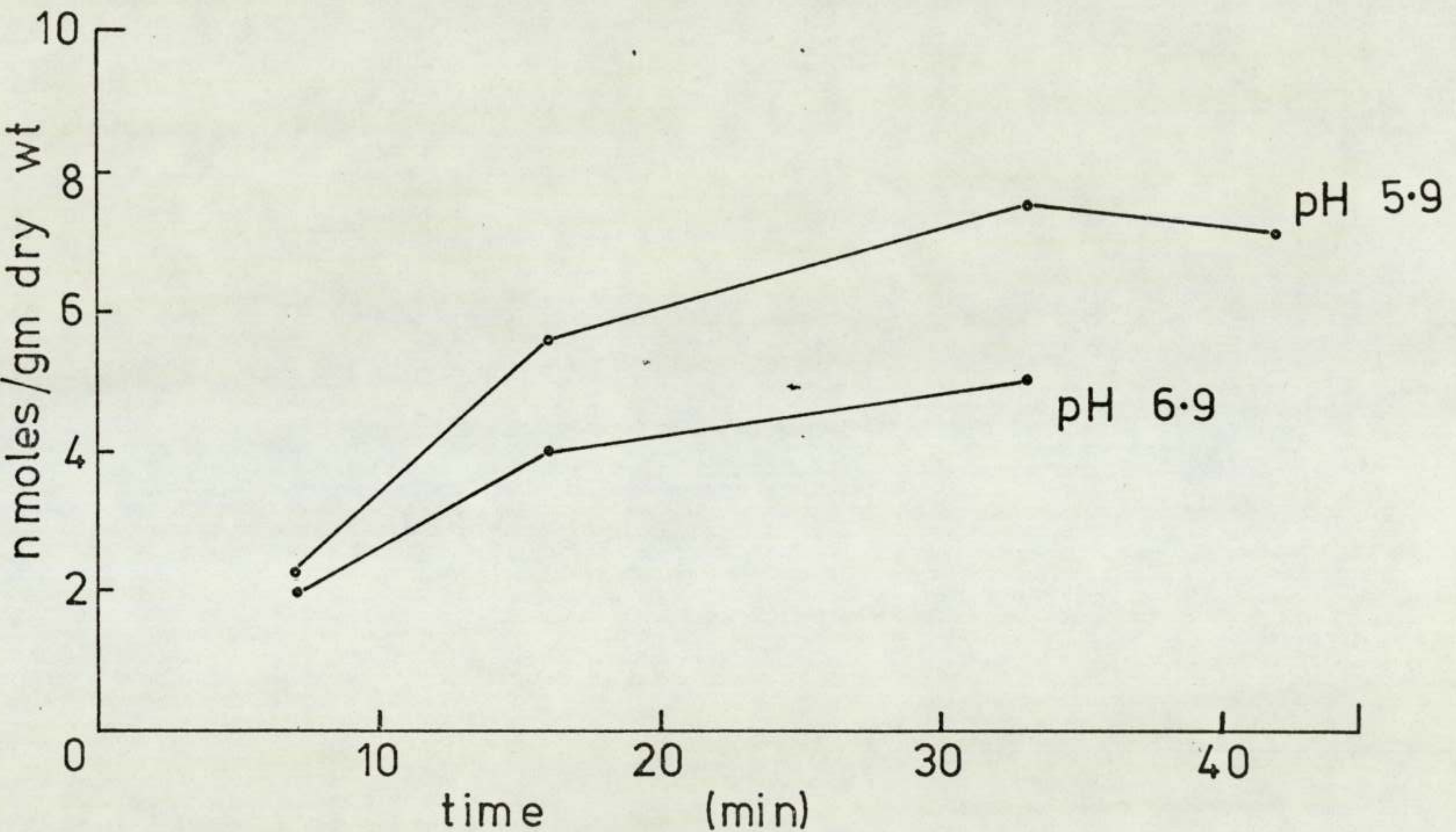


FIGURE 3.32. Uptake of folic acid (10^{-6}M) by isolated cells during incubation at two pH values. The values shown were determined at the end of the experiment.

PLATES

LEGEND.

PLATE I.

The tips of two adjacent villi of the upper jejunum of the rat. Section prepared from an everted ring maintained at $4^{o}C$ 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 visbile. X 200

PLATE II.

A single isolated absorptive cell of the jejunal mucosa. Cells were prepared by the hyaluronidase disaggregation technique and maintained at room temperature before fixing and staining with nuclear fast red. The intact brush border is visible on the left-hand extremity of the cell. The other two cells visible below and to the right are lymphocytes. X150

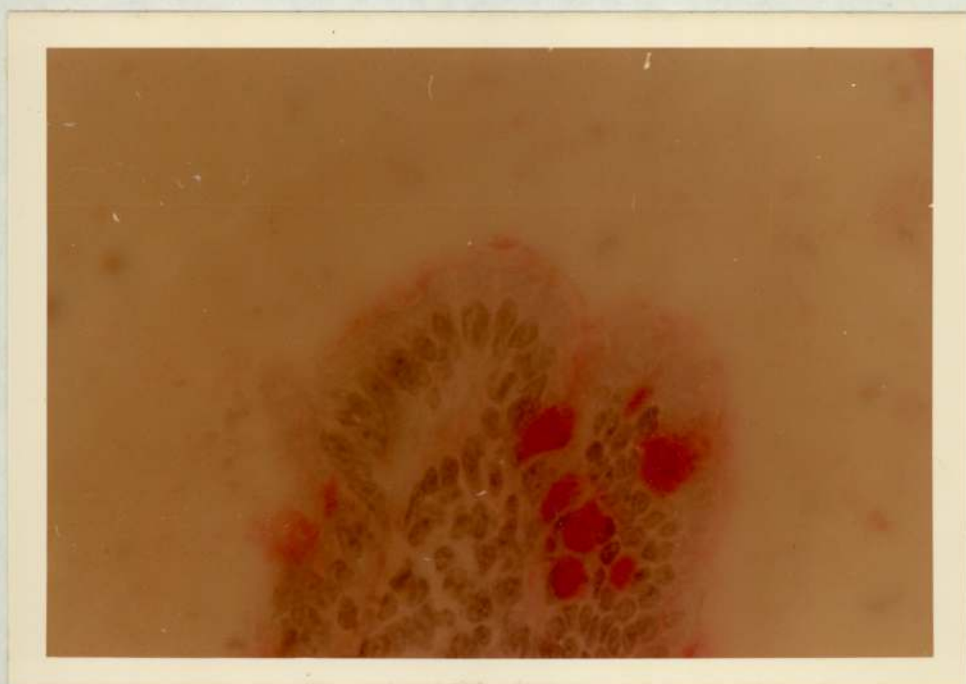


PLATE I.

PLATE II.

LEGEND.

PLATE III

Isolated mucosal cells obtained by hyaluronidase disaggregation and stained with periodic-acid Schiff stain. X100

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). X50

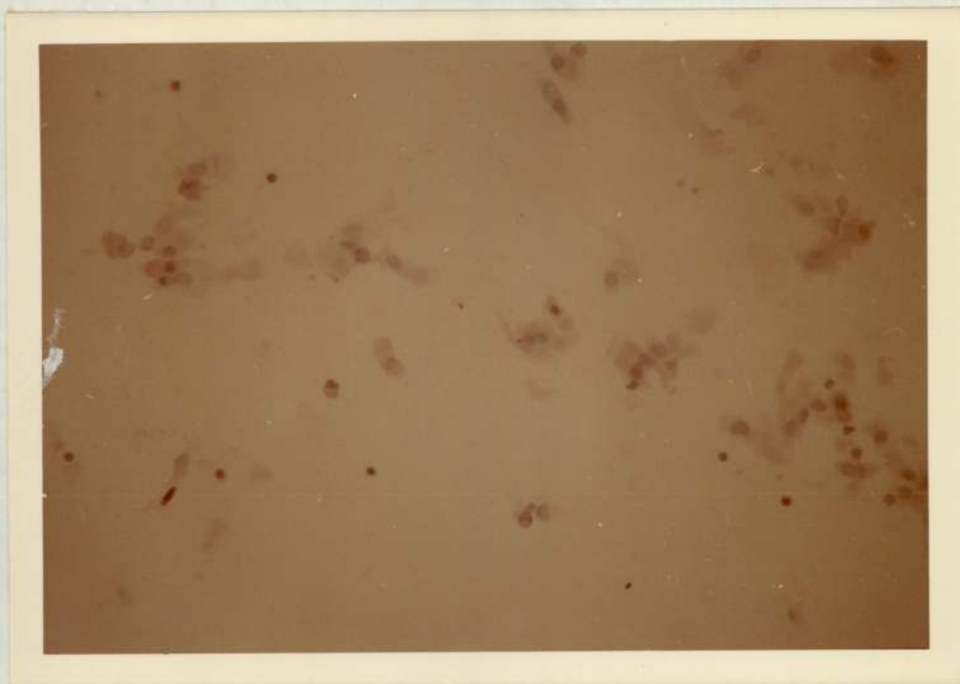


PLATE III.

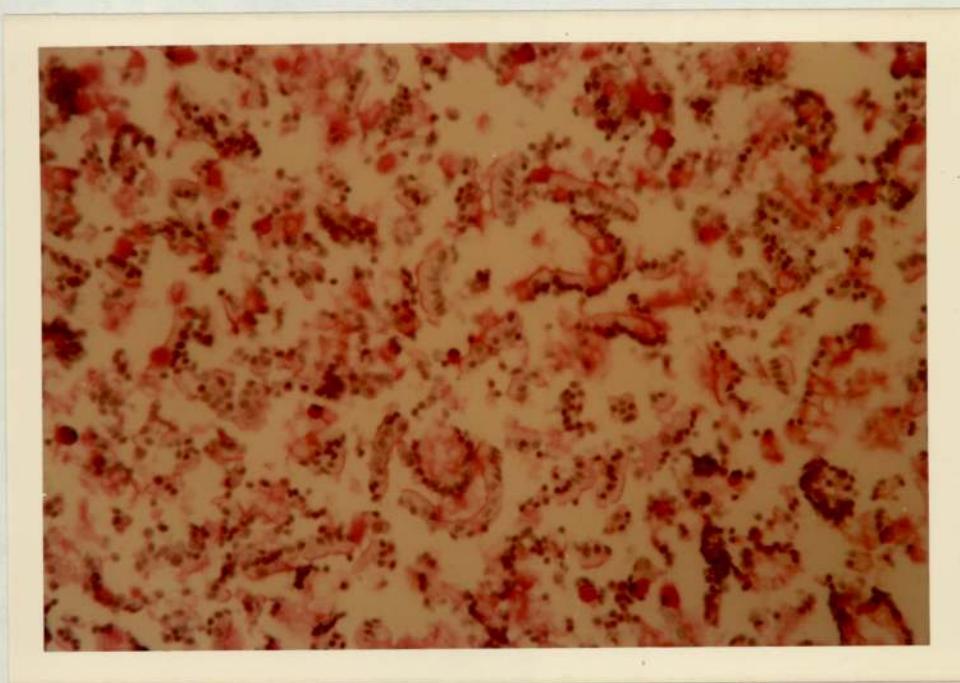


PLATE IV.

LEGEND.

PLATE V.

A group of isolated mucosal cells obtained by hyaluronidase disaggregation and stained with haemotoxylin-eosin. X 200

PLATE VI.

A group of isolated mucosal cells obtained by hyaluronidase disaggregation and stained with haemotoxylin-eosin. X150

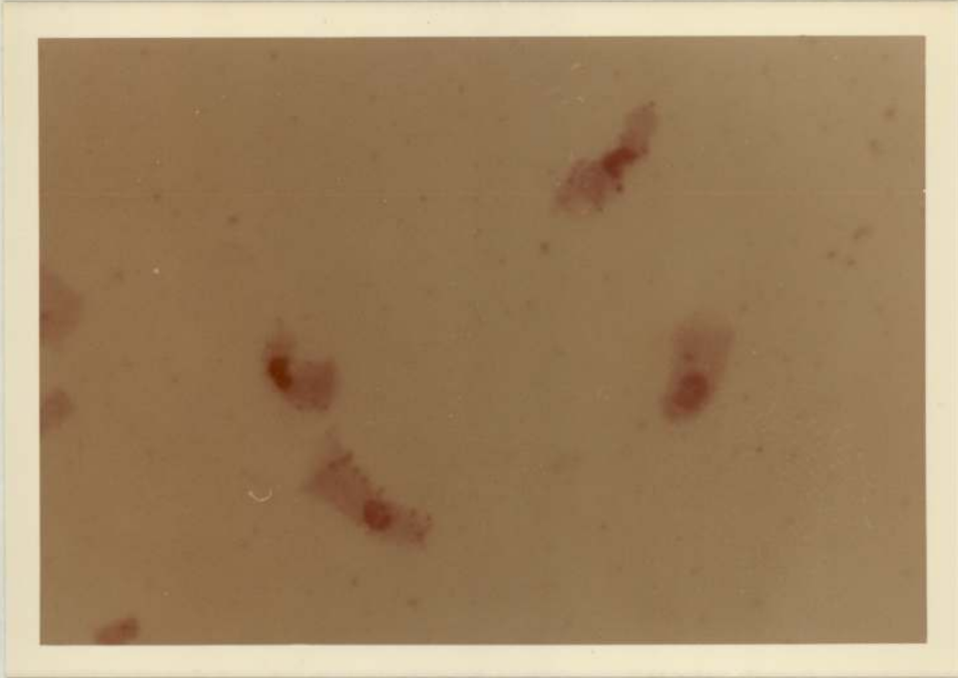


PLATE V.

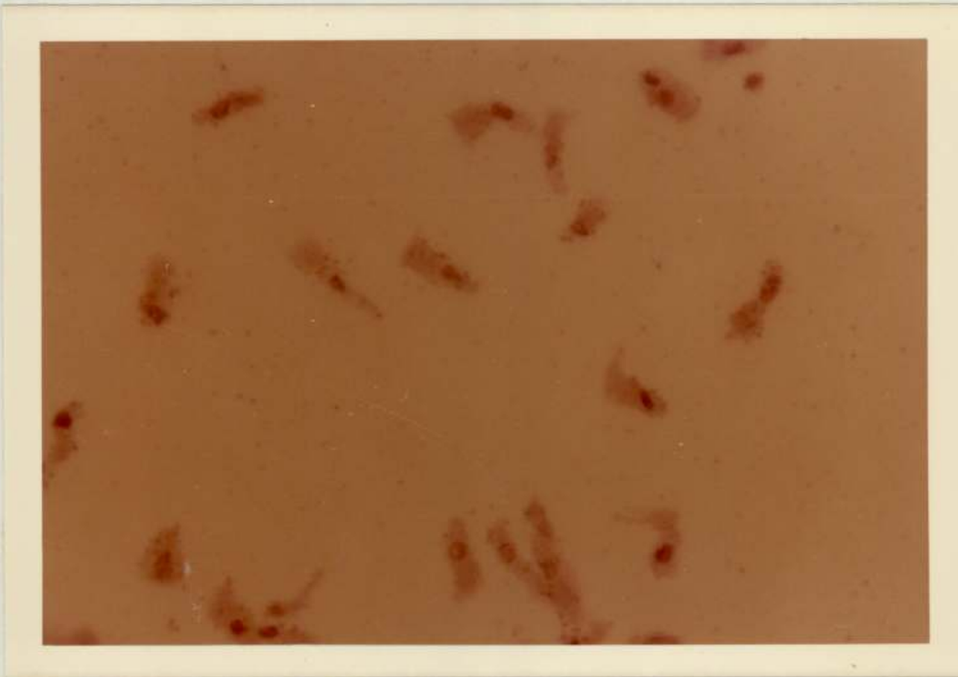


PLATE VI.

SECTION 4.

DISCUSSION AND CONCLUSIONS.

It is generally agreed that the cell membrane is a complex semi-fluid lipoidal structure representing a significant barrier to the diffusion of solutes present in the adjacent aqueous phases. Whereas substances with a substantial lipid solubility may cross the membrane passively, it is necessary to postulate the existence of specialised transport "channels" in order to account for the rapid movement of materials with low lipid solubility or for the transport of substances against an electrochemical gradient. Such transport channels are thought to entail structural modifications of the cell membrane, the behaviour of which can be predicted theoretically and tested experimentally. This discussion will be concerned with the possible interpretation of folic acid transport in terms of currently accepted models and it is appropriate to begin with a brief discussion of the theoretical concepts involved.

The following terminology will be used throughout this section. The term "passive diffusion" will be used to describe the movement of solutes by simple free diffusion without the intervention of specific transport mechanisms. The term "active transport" has acquired some ambiguity with use and following the lead of Wilbrandt and Rosenberg (1961) it will not be used in this discussion. Instead, the phrase "uphill transport" will be used to describe the movement of a solute against an electrochemical gradient and a transport system capable of such behaviour will be termed a "pump". Mediated transfer which does

not occur against a concentration gradient will be described as "equilibrating"; transport systems which do lead to uphill transport will be described as "accumulating". Finally, the term "carrier" will be taken to mean a hypothetical entity having the characteristics of a membrane component capable of reversibly binding a solute at one face of a membrane, conveying it across as a substrate-carrier complex, and releasing it into solution at the other face.

<u>Passive Diffusion.</u>

The movement of solute by simple diffusion from one region of a solution to another through a cross sectional area A, is directly proportional to the concentration gradient across that section. The rate of movement is described by Fick's Law and may be expressed thus: -

$$\frac{ds}{dt} = -DA \frac{dc}{dx} \quad \ldots\ldots\ldots\ldots\ldots \quad (4,a)$$

The differential $\frac{ds}{dt}$ is the rate of movement of S with time, $\frac{dc}{dx}$ is the concentration gradient at a point in A, and D is the diffusion coefficient.

In the case of diffusion across a membrane the equation can be simplified and expressed in experimental terms: -

$$\frac{ds}{dt} = \frac{DA}{a} \left(c'_{out} - c'_{in}\right) \quad \ldots\ldots\ldots \quad (4,b)$$

In equation 4,b the term $(c'_{out} - c'_{in})$ represents the difference in the concentration of a solute at the outer and inner surfaces of a membrane of thickness a. Since the concentrations in the membrane are not easily measured, they may be replaced by the concentrations in the aqueous phases immediately adjoining the membrane surfaces, c_{out} and c_{in}. By introducing the partition coefficient B: -

$$B = \frac{\text{concentration in the membrane}}{\text{concentration in aqueous phase}}$$

we obtain,

$$\frac{ds}{dt} = \frac{DAB}{a} (c_{out} - c_{in}) \quad \ldots . \quad (4,c)$$

$$= PA (c_{out} - c_{in}) \quad \ldots . \quad (4,d)$$

where $\frac{DB}{a} = P$, the permeability constant.

Thus the flux J, of a substance across a cell membrane is given by

$$J = P (c_{out} - c_{in}) \quad \ldots\ldots\ldots\ldots \quad (4,e)$$

so long as the diffusion of s in the aqueous phase is sufficiently rapid to maintain c_{out} and c_{in} constant.

It can easily be seen from equation 4,e that the diffusion of solute across a membrane will be

proportional to c_{out} while c_{in} remains constant, and a straight line relationship will be obtained for the rate of transfer of s as a function of c_{out}.

In order to pass into the membrane, the solute must overcome the potential energy barrier associated with passage from an aqueous to a lipid phase; this process will be relatively difficult for hydrophylic polar compounds having poor lipid solubility, and for such materials the rate of transport into the cell is likely to be negligible. Many naturally occurring substances would be thus barred from rapid incorporation into cells were it not for the existence of specialised transport mechanisms.

Carrier Transport.

The most frequently cited model for mediated membrane transfer is that based on the concept of carrier transport which is, as yet, a largely theoretical process thought of as occurring in the following sequence of events.

1. Substance binds reversibly to a component of the membrane termed the "carrier".

2. Movement of substrate-carrier complex occurs from one face of the membrane to the other.

3. The substrate unbinds and is released into the aqueous phase.

The rate of transport v of a substrate S may be expressed as follows: -

$$v = D\ (CS_{in} - CS_{out}) \quad . \quad . \quad . \quad . \quad . \quad (4,f)$$

where D is the permeability constant for the substrate carrier complex, the concentration of which is expressed as CS at the inner and outer faces of the membrane.
If the assumption is made that the diffusion constant for the carrier substrate complex is smaller than the rate constants for the binding reactions at either side of the membrane, and further, that the diffusion constants from free carrier and carrier-substrate complex are equal, then: -

$$v = DC_t \left(\frac{c_{out}}{c_{out} + K_m} - \frac{c_{in}}{c_{in} + K_m}\right) \quad . \quad . \quad (4,g)$$

where C_t represents the total carrier concentration and K_m is the dissociation constant of the carrier substrate complex. In this relationship, DC_t represents the maximum possible rate of transfer which will occur when the term in brackets equals 1. Replacing DC_t with V_{max} gives: -

$$v = V_{max} \left(\frac{c_{out}}{c_{out} + K_m} - \frac{c_{in}}{c_{in} + K_m}\right) \quad . \quad . \quad (4,h)$$

In situations where the second term in the brackets, representing the relative saturation of the carrier at the inner surface of the membrane, tends to zero, the relationship strongly resembles the equation for the Michaelis Menten relationship as given in the preceeding section (Equation 3,a page 104).

$$v = \frac{V_{max} \cdot c_{out}}{c_{out} + K_m} \quad . \ . \ . \ . \ . \ . \ . \ . \ . \ . \quad (4,i)$$

i) <u>Intestinal transport kinetics of folic acid.</u>

From the data of Section 3 Part 1 it is apparent that three component processes are to be distinguished in the overall transport of folic acid. Two effects contribute to the total tissue content at any one time, the rapid non-saturable uptake, and the time-dependent transport.

The rapid initial uptake of folic acid may itself be the sum effect of several processes all of which are complete within the first 2 minutes of incubation. It was noted that if the washing process was varied so that tissue was allowed to stand for a minute or more between saline flushes, a fall in the tissue content was recorded relative to samples which were not allowed to stand. This could represent back-diffusion of intracellular folate, or possibly diffusion

from an unstirred extra-cellular compartment such as the brush-border complex. If so, the initial unsaturable transport might represent material entrained within this layer. A second possibility is that the everted rings possess a surface array of binding sites capable of taking up folate from solution. Such binding might occur at sites within the brush border itself (Leslie & Rowe, 1973) or at intracellular sites accessible via damaged cells at the cut surface of the gut wall.

Both the tissue accumulation and the serosal transfer of folic acid are saturable processes. As has been discussed in Section 1, the general observation that some form of saturable uptake mechanism existed for folic acid has been made in the past, and the present observations confirm and quantify these reports. It is difficult to account for the findings of Halsted and Mezey (1972) that linear transport kinetics were observed from 5.7×10^{-8}M to 5.7×10^{-4}M. However it may be significant that their observations were made in the _in vivo_ perfused intestine. Winne (1973) has argued that in some situations the unstirred layer adjacent to the absorptive membrane may act as the rate limiting diffusion barrier and give rise to anomolously high values for K_m. Furthermore the perfused intestine is likely to be very poorly stirred relative to _in vitro_ preparations such as those used in the present study and it is possible that under these circumstances saturation kinetics may not be observed. A similar phenomenon might account

for the fact that Burgen and Goldberg (1962) reported a K_m of $4 \times 10^{-5}M$ for the uptake of folate from perfused *in vivo* loops, whereas, using *in vitro* techniques, Smith, Matty and Blair (1970) reported a value of $7.0 \times 10^{-7}M$, compared with values of $22.4 \times 10^{-7}M$ for the tissue accumulation, and $29.0 \times 10^{-7}M$ for the serosal transfer, obtained in the present study.

Comparison of the V_{max} values for the tissue uptake and the serosal transfer shows that whereas the accumulation of folic acid in the gut wall occurs at a maximum rate of 2.2 nmoles/gram dry weight per minute, the average value for the serosal transfer over a 30 minute period is 0.3 nmoles/gm dry wt. per minute at approximately the same mucosal concentration. This implies that the process by which absorbed folic acid is transferred from the gut wall into the serosal solution is several times slower than the rate of uptake into the tissue. This might result from the presence of a second major diffusion barrier within the gut wall, limiting the transport of folate which had entered the tissue, or it could result from a process of sequestration by which absorbed folate is removed from free solution within the tissue fluid and is no longer available for passive transfer. The observation that some form of accumulation of folic acid occurs within the intestinal wall *in vitro* is consistent with the previous reports of Smith, Matty and Blair (1970), Smith, (1973) and Selhub, Brin and Grossowicz (1973), all of whom have reported that the wall of the rat intestine was capable of accumulating

folate, and maintaining an apparent concentration higher than that of the mucosal and serosal solutions. Baugh *et al* (1971) reached a similar conclusion for dog intestine *in vivo*. It was reported that at the end of an experiment in which the absorption of pteroyl-diglutamate from the lumen was observed, 44% of a given dose of folate remained in the lumen, 1% was transferred into the serum, 8% was recovered as a soluble extract from the gut wall, and it was inferred that 48% was retained by the gut wall and was precipitated with the proteins. This strongly suggests that some form of protein binding has occurred during the absorption process, rather than an accumulation of folate at a high concentration in solution. Experiments in this laboratory (J.A. Blair and F. Staff, unpublished observations) indicate that between 5% and 10% of an oral dose of folic acid is retained by the gut wall 24 hours after administration, and that this percentage falls gradually over a period of days.

The observation that uptake by the gut wall is impaired at $0^{o}C$. might reflect some form of metabolic dependence or perhaps that the physical permeability of the plasma membrane is reduced at this temperature. This could arise as the result of a change in the molecular structure of the membrane at low temperatures. Such an effect has been postulated to account for the sudden change in the activation energy of glucose exchange-diffusion in erythrocytes which occurs at

20°C. (Lacko, Wittke & Geck, 1973). It is suggested that a phase change occurs in the lipid fraction of the membrane such that below the transition temperature the constituent molecules become highly ordered; this interpretation is supported by spin label studies on sarcoplasmic membranes which undergo a phase transition at 22°C. (Eletr & Inesi, 1972).

Q_{10} values of 2 for the accumulation process at either end of the concentration range observed are rather high for a diffusion controlled absorption and are at the lower end of the scale which could be regarded as compatible with an enzyme mediated process. This is a simplification of doubtful value however. Diffusion of a poorly lipid soluble compound such as folic acid might well have a high activation energy associated with it, particularly if diffusion of an ionised species were involved, as in this case there would be a considerable energy barrier to be overcome for the anion to enter the negatively charged cell membrane.

The characteristics of the transport process that have been examined so far are compatible with an uptake mechanism mediated by a carrier transfer system, and followed by some form of uptake within the gut wall. Mere compatability with such a simple model does not, however, constitute proof. In the next part of this section another possible uptake mechanism is considered in some detail. This will be followed by a full discussion of the experiments undertaken in order to

decide between the various possibilities.

An alternative explanation for the saturable transport kinetics of folic acid uptake may 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 was originally developed to account for the permeability characteristics of ionisable drugs. As was discussed at the beginning of this section, the membrane permeability of a substance for which no specific transport system exists is largely governed by its lipid solubility. This is consistent with the concept of a cell membrane as a lipoidal diffusion barrier separating two aqueous phases. Passive diffusion of a solute from one aqueous compartment to the other will be governed by its ability to enter the lipid phase and this ability is reflected by the substance's oil/water partition coefficient. Schanker _et al_ (1958) pointed out that whereas large polar molecules are relatively hydrophilic and have very low lipid solubility, the same molecule in the neutral form would be likely to have a much increased affinity for the lipid phase, and a correspondingly higher membrane permeability. In the physiological situation, the rate of passive absorption for an acidic or a basic drug would be dependent upon its dissociation constant, as this would govern its degree of ionisation in the luminal fluid.

To test the general validity of the concept of non-ionic diffusion Schanker _et al_ (1958) used an _in vivo_ perfusion technique to observe the rate of uptake of a series of drugs with different pKa values, from the entire small intestine of the rat. The buffered perfusate was infused at a pH of 7.2 and was found to have a pH of 6.6 when it emerged from the ileum. In general it was confirmed that drugs which were present mainly as the neutral species in the intraluminal fluid were absorbed much more readily than those which were present primarily in the ionised form. As a general rule it was found that acidic drugs which had a pKa less than 3 were poorly absorbed, as were basic drugs with a pKa greater than 8. The absorption of very strong acids and bases was described as imperceptible.

Certain departures from the general pattern were demonstrated; for example, species such as barbital and thiopental, which have very similar pKa values, exhibited markedly different absorption rates. This was thought to be due to other factors affecting the membrane solubility besides the dissociation constant of a species, and did not conflict with the general principle. Of greater importance was the observation that certain substances such as salicylic acid, which is strongly ionised at the intraluminal pH and was present as the neutral species at a ratio of 1 to 4000 with the ionised form, still manifested a relatively rapid absorption.

In subsequent work (Hogben _et al_, 1959) it was

shown that by varying the luminal pH, the rate of uptake of various species could be made to vary in a manner qualitatively predictable in terms of the non-ionic diffusion principle. However, the steady-state concentration ratios developed between the plasma and the lumen implied a "virtual" pH for the lumen of 5.3 when the observed pH was 6.6. The calculated value for the "virtual" pH shifted only slightly with changes in the measured pH of bulk fluid. To account for these anomalies it was postulated that a layer of relatively acidic fluid existed at the mucosal surface. This pH "microclimate" was conceived of as a fluid compartment 1 to 2 pH units more acidic than the bulk phase and only partially sensitive to gross changes in the pH of the bulk luminal fluid (Hogben, 1960).

A striking demonstration of the importance of micro-environmental effects in biochemical systems has been provided with the aid of enzymes immobilised in artificial membranes. Goldstein, Levin and Katchalski (1964) argued that the behaviour of a natural enzyme *in situ* must be influenced by the local concentrations of substrate and products, which, in turn, are controlled by the enzyme's activity and the physical conditions in its vicinity. The reality of this idea was demonstrated by means of a water-insoluble trypsin derivative bound to a maleic acid-ethylene copolymer. It was shown that the behaviour of the enzyme could be related to local pH effects induced by the presence of

multiple anionic groups in the co-polymer matrix.

A more elaborate model was later developed in which the enzyme papain was bound within an artificial collodion membrane. The resulting complex consisted of a collodion layer 200-300 μ thick containing 280 Å pores, within which was adsorbed a monomolecular layer of the enzyme. The permeability of the membrane was such that the sucrose permeability coefficient was about 54% of that in aqueous solution (Goldman et al, 1965).

In a comparative study the pH-dependence of the enzyme activity with respect to the substrate benzoylarginine ethyl ester was found to differ significantly from that of the crystalline enzyme, the activity being low at neutral pH values, which were optimal for the free enzyme, and anomolously high at alkaline pH. This behaviour was attributed to the existence of a microclimate within the membrane matrix which had an ambient pH several units more acidic than that of the bulk phase. It was postulated that the conditions in the immediate vicinity of the enzyme, and hence the activity of the enzyme itself, were the result of a steady state equilibrium established for the influx of substrate and the efflux of the products of hydrolysis, the steady state concentration of protons being higher than that in the external solution. It was later established that the behaviour of the enzyme-membrane complex was quantitatively predictable on this model. Furthermore it was possible to restore the enzyme's behaviour to normal by buffering the solution

sufficiently strongly, and, after suitable modification of the membrane, it was possible to observe the colour of an infused indicator and thereby confirm the existence of a relatively low pH within the matrix (Goldman _et al_, 1965; Goldman et al, 1968; Goldman, Kedem & Katchalski, 1968). The authors concluded that wherever acid is produced by the reactions of an immobilised enzyme the local pH may be expected to differ from that of the bulk phase, this conclusion has since been supported by other workers, utilising a different enzyme-polymer complex (Axen, Myrin & Janson, 1970).

It has been repeatedly observed that some mechanism exists in the upper small intestine of the rat and man which causes the acidification of neutral or alkaline buffers in the lumen (McRobert, 1928; Ponz & Larralde, 1950; Parsons, 1956; Wilson & Kazyak, 1957; Blair, Lucas & Matty, 1974; Lucas, 1974). It has been demonstrated that the acidification mechanism involves production of protons at the mucosal surface, and that this process is reduced by metabolic inhibitors and stimulated by the addition of ATP to the mucosal solution (Lucas, 1974; Blair, Lucas & Matty, 1974). On the basis of these observations it has been proposed that acidification occurs as a consequence of ATP hydrolysis at some site within the brush-border complex and that this provides a mechanism for the input of protons necessary to maintain a region of localised acidity at the absorptive surface of the cell (Blair, Lucas & Matty, 1972).

The surface specialisation of the intestinal absorptive cell will be discussed in more detail in a subsequent part of this section. At this stage it is only necessary to point out that the luminal surface of the mucosal cell is a complex structure composed of microvilli which are themselves covered with a fibrous muco-polysaccharide structure termed the glycocalyx, fuzz, or perhaps more elegantly, the enteric surface coat (Ito, 1965). The importance of this zone as the microenvironment in which the process of exchange between the extracellular and the intracellular medium takes place is only just being realised but the significance of its intense enzyme activity and as yet unknown physical conditions has been emphasised in several recent reviews (Parsons & Boyd, 1972; Ugolev, 1972).

A further feature of the mucosal surface which is of relevance here is the probable existence of unstirred layers in association with biological membranes (Dainty & House, 1966). There is as yet little direct experimental evidence as to the characteristics of unstirred layers in the intestine, although their importance as potential rate limiting factors in intestinal transport has been frequently commented upon (Dietschy, Sallee & Wilson, 1971) and Winne (1973) has described their quantitative effects theoretically. It seems very likely that the complex surface structure of the intestinal cell

would be such as to maintain a poorly stirred fluid compartment, and that this would present an effective diffusion barrier to substances diffusing outward from the glycocalyx.

Evidence for local variations in pH at membranes is still scarce but an interesting observation by Caldwell (1958) suggests the existence of a microclimate at the surface of an *in vitro* crab muscle fibre preparation. During an investigation of the intracellular pH of muscle and nerve cells, Caldwell observed that the pH of the bathing solution in the immediate vicinity of leg muscle fibres of *Carcinus maenas* was about 0.6 of a pH unit more acidic than the bulk saline when the fibres were soaked in standard crab Ringer. It was suggested that the effect was due to the release of buffering materials by the cell surface, or to surface acidification as a side effect of metabolism.

The conclusions to be drawn from these considerations can, in the absence of direct experimental observation, only be tentative. But in view of the fact that the conditions suitable for the maintenance of an acidic microclimate of the type postulated by Hogben *et al* (1959) appear to exist, it is of interest to consider the behaviour of folic acid in such a region. For convenience the micro-climate will be referred to at times rather as if it were a discrete compartment with fixed boundaries and rather rigid conditions. This is merely a simplification; it would probably be more realistic to think

in terms of successive fluid lamina, the physical and chemical properties of which tend more to the conditions of the bulk mucosal phase with increasing distance from the cell membrane. The hydrogen ion concentration would be likely to increase more or less exponentially as the membrane is approached, and the pH value which is of interest is that immediately adjacent to the water-lipid phase interface.

The relative dissociation of an acid or base at a given pH is calculated from the pKa value of the group, or groups, which undergo ionisation. The pKa value is the negative logarithm of the dissociation constant, and corresponds numerically to the pH value for 50% dissociation. The situation for folic acid is somewhat complex as there are three groups of relevance to this argument, the alpha and gamma carboxyl groups of the glutamate moiety, and the nitrogen atom at position 10 which gains a proton at low pH levels. Figure 4.1 illustrates the relative concentrations of the neutral molecule, the protonated cation, and the mono and dianion over a range of pHs from 2.0 to 8.0. The pKa values used in the calculation were obtained from the literature, and are tabulated below together with their sources. Table 4.2. contains the percentage concentration of neutral species over the same pH range.

Clearly, if the usual assumption is made that only the neutral species will be capable of crossing the lipoid membrane, passive diffusion of folic acid only becomes possible in an aqueous medium with a pH less

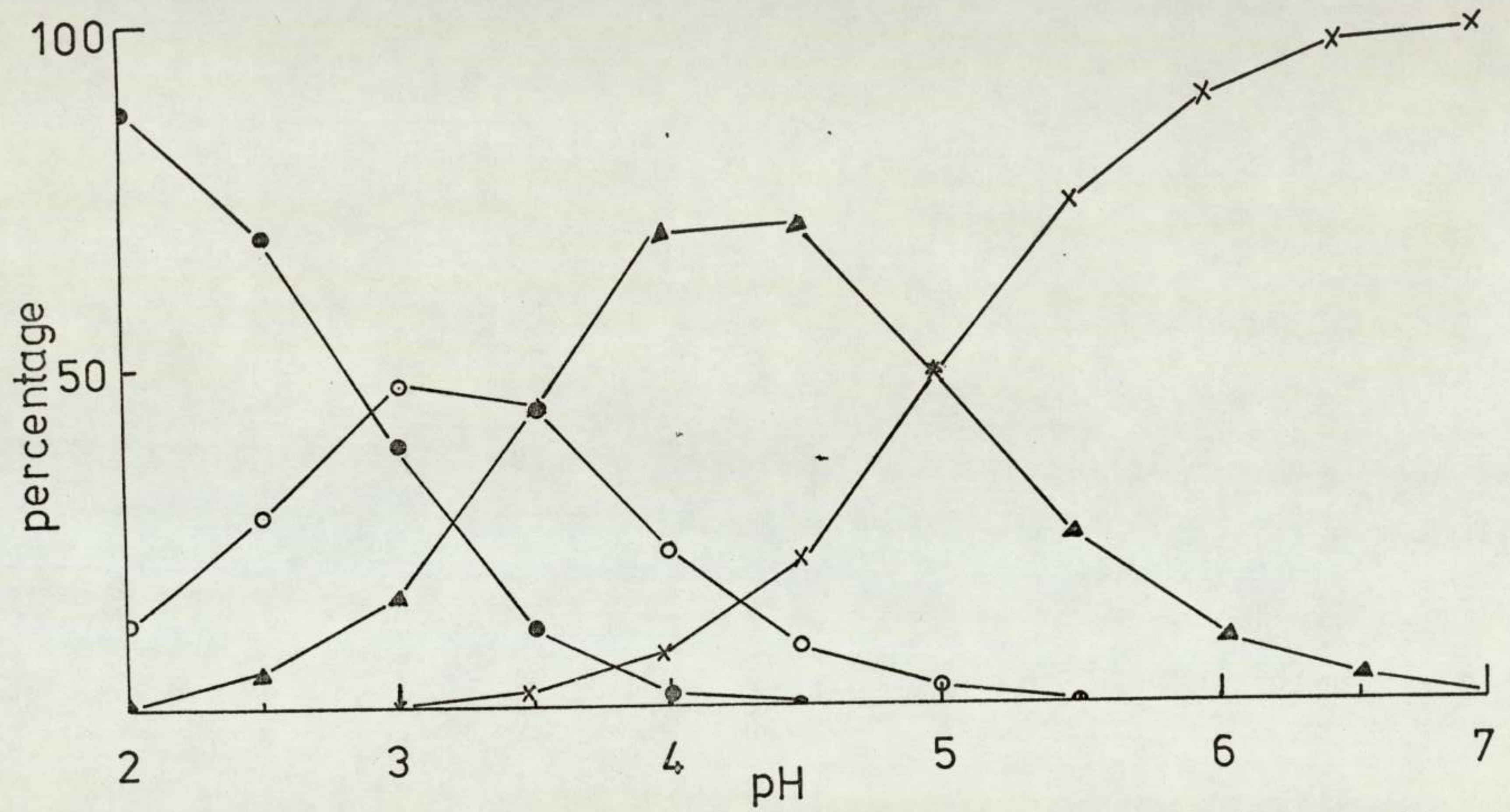


FIGURE 4.1. Relative concentrations of neutral and ionised species at acidic pH values.

(x) = dianion; (▲) = monoanion; (o) = neutral molecule; (•) = cation.

TABLE 4.1.

Ionising Group	pKa	Reference
alpha carboxyl group	3.5	Kallen & Jencks, 1966; Albert, 1952.
gamma carboxyl group	5.0	ditto
N 10	2.9	Osborn, Talbert & Hunnekens, 1960.

TABLE 4.2.

Relative concentrations of neutral species and monoanion at acidic pH values.

pH	% neutral species	% monoanion
2.0	11.1	0.3
2.5	27.6	2.7
3.0	47.3	14.9
3.5	43.8	43.8
4.0	21.9	69.3
4.5	7.0	70.4
5.0	1.5	49.2
5.5	0.2	23.9
6.0	0.0	9.0
6.5	0.0	3.0
7.0	0.0	0.9
7.5	0.0	0.3

than 5. Between 4 and 3 however, appreciable quantities of neutral form are present and about 45% of dissolved folic acid should be available for non-ionic diffusion over the pH range 3 to 3.5.

Intestinal absorption of folic acid by non-ionic diffusion is thus rendered feasible if the immediate aqueous microenvironment at the water/lipid interphase has a pH of 4.5 to 3.5. A further consequence of this microclimate is revealed by the pH dependent solubility curve for folic acid. Biamonte and Schneller (1951) measured the solubility of folic acid over a range of pHs in sodium phosphate-citric acid buffer at a temperature of 30°C. The solubility was shown to be essentially the same at a given pH, over the range 25 - 45°C. The data of Table 4.3 shows that the solubility of folic acid is dependent on pH, and falls very steeply below pH 6 from approximately 3 mg. per ml to less than 2 mg. litre at pH 3. (4.4 mg. per litre = 10^{-5}M). This result suggests a quite different interpretation for the uptake kinetics graphs 3.4 and 3.6.

Assuming that folic acid crosses the mucosal membrane by passive diffusion of the neutral species, the initial rate of transfer will follow a simple linear relationship;

$$V \alpha \text{ Concentration}$$

As the bulk concentration is raised, the microclimate phase will come into equilibrium with it until the

TABLE 4.3.

pH-dependent solubility of folic acid.

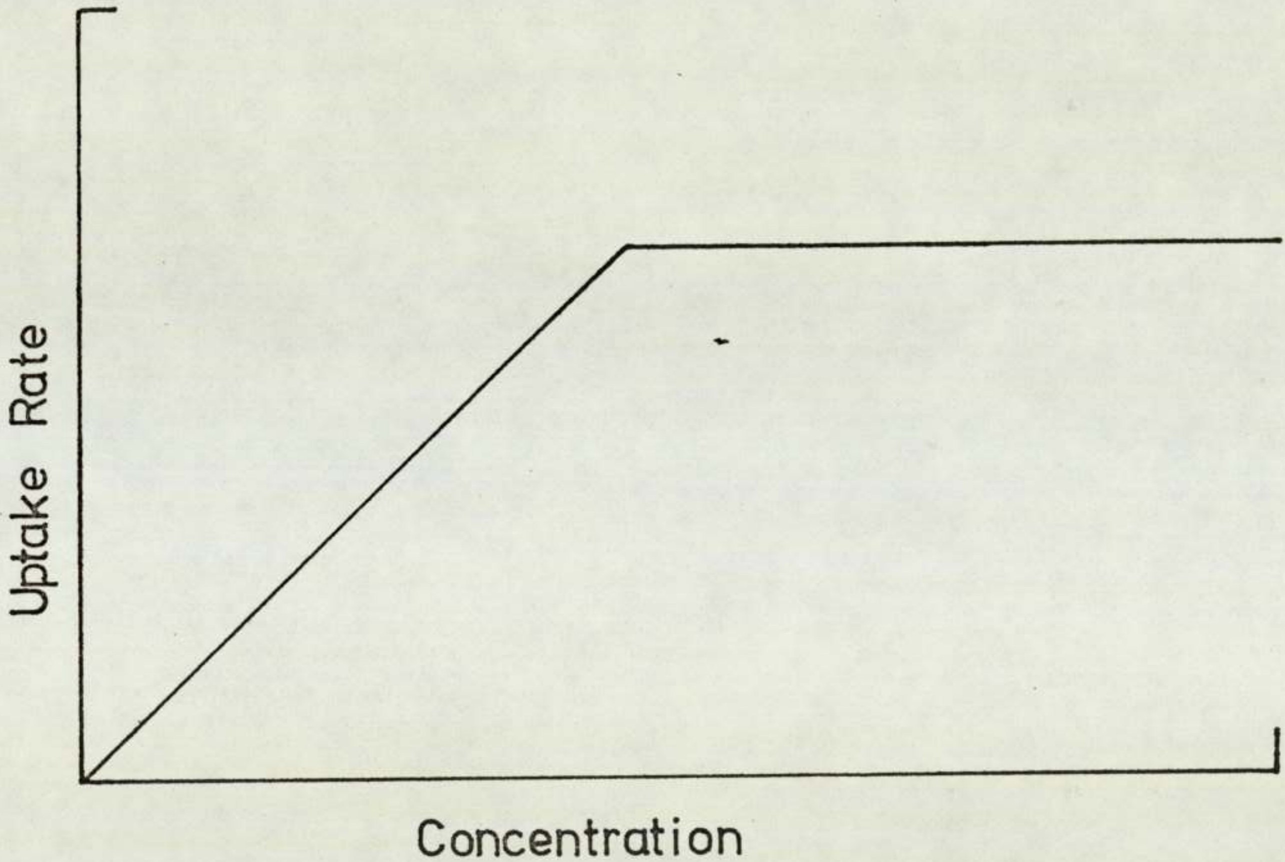
pH	Solubility
3	1 - 1.5 mg/litre
4	8 mg/litre
5	80 mg/litre
6	2 mg/ml.

limiting solubility of the microclimate is achieved. Thereafter, raising the bulk concentration will not increase the concentration of diffusable species at the mucosal surface and no further increase in the rate of transport will be observed. On this model, Figure 3.4, which represents the rate of tissue accumulation of folic acid at increasing mucosal concentrations, could be looked upon as an approximation to the form shown in Figure 4.2. which is a diagramatic representation of the argument.

In Figure 4.3. the data of Figure 3.4 has been replotted by calculating the least squares line of best fit ($r = 0.98$) for the first 5 values. This line has been extrapolated to meet the horizontal which is drawn through the mean of the two data points at higher concentrations. From the point of intersection a theoretical limiting concentration of 3×10^{-6}M is obtained, and this corresponds to a hypothetical pH in the microclimate of about 3.

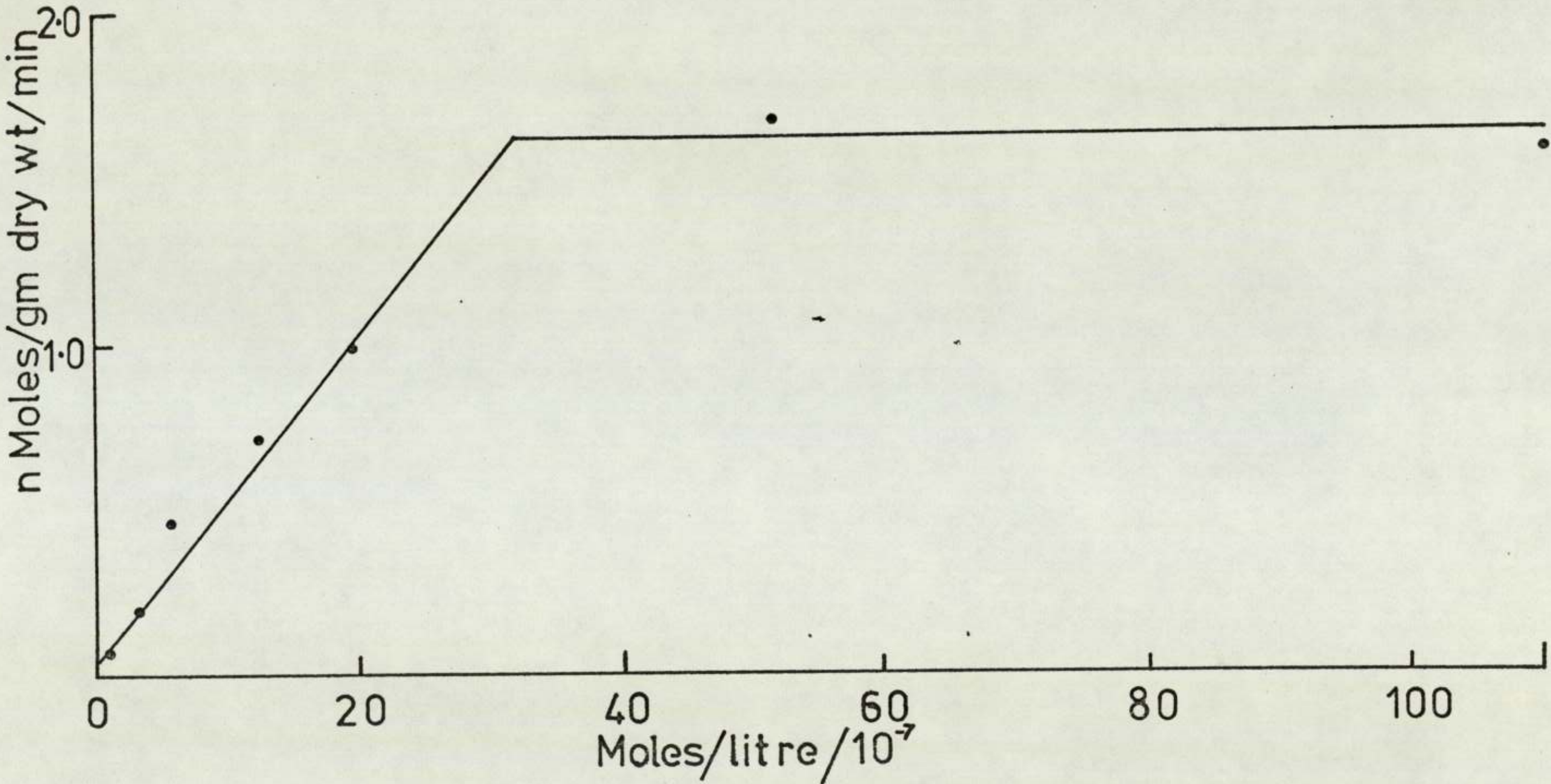
In summary then, a hypothetical microclimate with a pH in the region of 3-4 would facilitate the absorption of folic acid by a process of non-ionic diffusion but would lead in theory to a saturable transport process with kinetics similar to those observed in the present study.

The discussion so far has rested on the assumption that the absorptive membrane of the intestinal mucosal cell is completely impermeable to



<u>FIGURE 4.2.</u> Theoretical form of rate-concentration graph for passive transport via a saturable mucosal microclimate.

FIGURE 4.3.



Rate of uptake of folic acid by everted rings of rat jejunum (the data of Fig. 3.4, page 96).

organic anions; as was pointed out by Hogben et al (1959) in their original analysis, this may not be so. A number of subsequent reports have confirmed that salicylic acid and other acidic drugs have unexpectedly high rates of absorption and it has been proposed that at least some organic anions of relatively high molecular weight have an appreciable membrane permeability (Nogami & Matsuzawa, 1961; Kunze, Rehbock & Vogt, 1972).

A recent study by Ochsenfahrt and Winne (1974 a,b) indicates that a number of drugs including benzoic acid and salicylic acid diffuse across the intestinal barrier at least partially as the ionised form. Furthermore, from their analysis of solvent-drag effects, these authors conclude that the interaction of drug and water molecules is most likely to occur within the lipid phase of the membrane, rather than within water filled pores or channels.

Returning to the folates, and the influence of pH on their membrane permeability, the interesting work of Hakala (1965) on the transport mechanism of methotrexate in the ascites tumour cell may be of considerable importance. Hakala demonstrated that though the uptake of methotrexate by these cells appeared to be a passive diffusion process, an energy-dependent mechanism, susceptible to inhibition by DNP, was responsible for efflux. It was shown that the passive influx of methotrexate was very slow, and this

was thought to be due both to the low lipid solubility of the compound, and to the fact that at neutral pH it was present as the ionised form. Since the cell carries a net negative charge (Terayama, 1962) the very low permeability of methotrexate could be accounted for as the combined result of low lipid solubility and the electrostatic repulsion exerted by the like charges on the molecule and on the cell surface. As has been shown above, a reduction in the pH of the medium would lead to decreased ionisation of the molecule, and as Hakala suggested, an increased uptake by the cells. This was found to be the case. The uptake increased sharply when the pH of the incubation medium was reduced below 6, so that the rate of influx at pH 5.4 was twice that at pH 7.

The ionising groups on the methotrexate molecule which are of relevance over the pH ranges considered are the alpha and gamma carboxyl groups of the glutamate moiety for which Hakala suggested pKa values of 3.76 and 4.83 respectively. The curves in Figure 4.1 which were calculated on the basis of assumed pKa values of 3.5 and 5.0 probably represent a good approximation to those to be expected for methotrexate over the pH range from 7 to 5. It can be seen that though it seems unlikely that appreciable quantities of the neutral molecule appear above pH 5, the observed increase in the permeability of methotrexate as the pH is reduced from 7 to 5.4 can be

accounted for if the cell membrane is permeable to the monoanion. The potential energy barrier posed by the negatively charged membrane is likely to be considerably lower with respect to the manoanion as compared to the dianion. Consequently, reducing the pH below approximately 6 would lead to an increasingly higher concentration of ions with sufficient energy to enter the lipid phase and diffuse into the cell.

Assuming that these ideas are applicable to the intestinal cell it can be seen that if folic acid is passively absorbed as the monoanion it should have an appreciable permeability if the immediate extra-cellular environment of the absorbing membrane has a pH less than 6. An artificial adjustment to a higher pH value, or an impairment of the cells' ability to maintain an acidic microclimate at the brush-border would be likely to result in a reduction in uptake. It must be noted that though a microclimate pH with an ambient pH of between 5 and 6 would account for the rapid absorption of the folate monoanion, such a pH would not give rise to the low solubility postulated as an explanation for the saturation kinetics of folate absorption and this would suggest the presence of some other saturable step in the overall mechanism.

A number of studies have indicated that folic acid transport in the rat and man is influenced by the pH of the bulk mucosal phase. Although no direct evidence is yet available as to the local pH in the

surface layer, gross alterations in the pH of the bulk phase may well reflect the changes occurring at the mucosal surface.

In an *in vitro* study using everted sacs of rat jejunum, Smith, Matty and Blair (1970) demonstrated that folic acid uptake was stimulated when the bulk phase pH was adjusted to values less than 7. The optimal mucosal solution pH was found to lie between 5.5 and 6.5. In man, it has been shown that the simultaneous administration of sodium bicarbonate and folic acid resulted in an impairment of folic acid uptake (Benn *et al*, 1971). In the same study it was also demonstrated by intraluminal pH measurements that 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 folic acid. A further observation, that patients on long-term phenytoin therapy also had an unusually alkaline jejunal pH, prompted the suggestion that the folate malabsorption often observed in such patients could be accounted for by a drug-induced increase in the jejunal pH, and it was demonstrated that a 10 day course of anti-convulsant therapy resulted in impaired folate absorption in otherwise normal subjects. Elsborg (1970) has shown that phenytoin only causes malabsorption of folic acid in the rat if it raises the pH of the intestinal contents. However, Perry

and Chanarin (1972) have disputed both the observation that the anti-convulsant phenytoin reduces folate uptake, and that simultaneous administration of sodium bicarbonate and folic acid results in impaired folate absorption.

Benn *et al* (1971) suggested that the folate malabsorption of coeliac disease might also be connected with the elevated intra-jejunal pH which has been demonstrated in this condition (Benn & Cooke, 1971). This suggestion is supported, albeit indirectly, by the observations of Mattila, Jussila and Takki (1973) who reported that the drug sulphafurazole (pKa 4.8) was poorly absorbed by patients suffering from intestinal villous atrophy. This they suggest may be due to a decrease in the lipid solubility of the drug caused by a pathological increase in intestinal alkalinity.

The reverse effect for folic acid, that is, an increase in uptake associated with an increase in the acidity of the jejunum caused by acute pancreatitis has recently been described by I. H. Rosenberg (personal communication, 1974). In a patient with a jejunal pH of 5, 90% of a small oral dose was absorbed, as compared with 60% in normal subjects. Administration of sodium bicarbonate increased the jejunal pH to 7, and decreased the intestinal absorption to 30%. In a normal subject, sodium bicarbonate increased the jejunal pH from 6.5 to 8, and decreased the per cent absorption from 60% to 20%.

Having now established the kinetics of folic acid absorption, reviewed the evidence for its possible

connection with jejunal acidification, and discussed the mechanism by which this might occur, there now follows a detailed discussion of the experimental tests suggested by the hypotheses outlined above.

The observation of saturation kinetics is usually taken as firm evidence for the existence of a carrier-mediated transport mechanism. In order to test the alternative hypothesis, a series of experiments were carried out to determine whether or not folic acid absorption could be shown to manifest some of the other principal criteria of carrier-mediated mechanisms, namely competitive inhibition and counter transport. Such an approach can be looked upon as an attempt to refute the "saturable microclimate" hypothesis, since if it could be established that the characteristics of folate absorption were compatible with carrier mediation then the alternative explanation would become redundant. The experiments with isolated cells were, on the other hand, undertaken in an attempt to verify the prediction that if passive absorption is responsible for folate uptake, then by reducing the pH of the solution in contact with the absorptive surface of the cell the influx of folic acid should be increased.

The experiment with metabolic inhibitors which is discussed first was carried out in order to try and resolve some contradictory reports in the earlier literature.

ii) Metabolic Inhibitors.

The inducement of anaerobic conditions in the incubation medium and the use of 2,4 dinitrophenol as an uncoupler of oxidative phosphorylation are familiar methods of inhibiting aerobic metabolism in *in vitro* preparations (Sanford, 1967). Both methods lead to decreased intracellular levels of ATP, and have been used in the past to block energy dependent processes in the intestine (Friedhandler & Quastel, 1955).

In the present study, it was demonstrated that inhibition of aerobic metabolism led to a decrease in the tissue accumulation and the serosal transfer of folic acid relative to that of control sacs from the same animals incubated under normal conditions. The general inhibition of energy dependent membrane transport processes was confirmed by the fact that serosal water uptake, a phenomenon generally thought to depend on the activity of solute pumps, was suppressed in the control sacs.

These observations contradict those of Turner and Hughes (1962) who tested a range of metabolic inhibitors, including DNP, for their possible effect on the intestinal transport of folic acid and other vitamins in *in vitro* preparations, and concluded that a slight enhancement of serosal transfer was the only effect detectable. Yoshino (1968) also concluded that DNP was without effect on the absorption of folic acid from perfused *in vivo* loops. In a more recent

investigation however, Kesavan and Noronha (1971) showed that the serosal transfer of folate derived from polyglutamates was inhibited *in vitro* by the presence of 0.5 mM DNP. It was also shown that whole body X-irradiation caused a similar suppression of folate uptake *in vivo* and *in vitro*, and that this could be restored by the addition of ATP to the mucosal bathing medium *in vitro*. An interesting aspect of these observations was that deconjugated folate appeared to accumulate at, or in, the jejunal mucosa of irradiated rats during the first hour of the experiment. The precise site of this accumulation is not made clear however.

The general conclusion to be drawn from the findings of the present study is that some aspect of the intestinal absorption of folate in the rat is dependent upon the energy derived from aerobic metabolism, though it is clear that this dependence is not absolute. The difficulty in interpreting this type of observation lies in deciding precisely where the input of metabolic energy occurs, and what is the functional relationship between the expenditure of energy and the translocation of solute.

It is at least feasible that the passage of folate across the mucosal membrane is mediated by means of energy dependent pumps. It has been shown (Wilbrandt & Rosenberg, 1961) that the transport of solute against an electrochemical gradient requires a supply of energy,

and a commonly suggested mechanism involves the flow of solute across the membrane in combination with some entity the movement of which occurs as the direct result of a chemical reaction. It has not been proven that folic acid is accumulated against an osmotic gradient under the conditions of the present study, though it is possible that such accumulation does occur at some site within the gut wall. The fact that transport does not entirely cease with the removal of aerobic energy does not rule out such a hypothesis as it is conceivable that energy derived from anaerobic metabolism might be available to power such a pump, or that a carrier mediated mechanism might be capable of equilibration in the complete absence of metabolic energy.

Another possibility is that under normal conditions a substantial enhancement of passive folate diffusion occurs as the result of solvent drag. In the test sacs in this study the normal water transfer is inhibited to the extent that a slight loss of water occurs from the serosal solution, and the observed depression in folate transfer may represent the removal of the solvent drag component. It has been recognised since the observations of Fisher (1955) that the mucosal transfer of water does enhance the passage of poorly absorbed compounds such as urea. It seems unlikely that a large charged molecule such as folic acid could be conveyed through membrane pores. However the recent report of Ochsenfahrt and Winne (1974,b) on the contribution of

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, and that interaction between water molecules and transported solute occurs within the lipid matrix.

As a third possibility it is suggested that the present results are consistent with the hypothesis that a microclimate exists at the surface of the mucosa with a pH such that appreciable concentrations of diffusable folate are available for passive influx. Such a pH microclimate would be dependent upon normal cell metabolism, as the input of protons is presumably derived from the normal acidification mechanism of the upper intestine (Blair, Matty & Lucas, 1972). Lucas (1974) has shown that the acidification mechanism of jejunal sacs is inhibited by anaerobic conditions and by DNP. Furthermore, an increase in acidification may be elicited by the addition of ATP to the mucosal medium. Conditions in which the production of protons at the mucosal surface is inhibited would be expected to lead to a rise in the pH of the surface layer and a corresponding decrease in the concentration of permeable species at the surface of the epithelial cell.

Kesavan and Noronha (1971) observed that a 700R whole-body dose of X-radiation resulted in decreased oxygen uptake by the intestine and a suppression of oxidative phosphorylation, as well as a 43% reduction

in the uptake of folates. Since the folate transport could be restored by the addition of mucosal ATP they suggested that the effect of radiation was to disrupt the normal energy dependent transport system by blocking the input of ATP. These results seem consistent with the hypothesis that ATP hydrolysis is the system responsible for acidification of the jejunum (Lucas, 1974), and that an impairment of this process leads to conditions unfavourable for folate influx.

iii) Competitive Inhibition.

The object of this study was to test for the existence of a carrier mediated step in the absorption of folic acid by everted sacs, using the concept of competitive inhibition. The material selected as a potential inhibitor, 10 formyl folic acid, is stable under the experimental conditions, and has no known toxic effects on the intestine. Little is known directly about its intestinal absorption but since a high proportion of dietary folates are 10 formyl derivatives it is probable that they are absorbed in significant amounts from the intestine (Butterworth, Santini & Frommeyer, 1963; Santini, Brewster & Butterworth, 1964).

The work of Leslie and Rowe (1972) demonstrated the existence of a brush-border protein with an affinity for folic acid and some folate derivatives. As has been mentioned before, one could interpret this as

evidence for the existence of a membrane carrier system at the mucosal surface, and if this were so an inhibition of folate absorption by intact tissue might be observed in the presence of another compound with some affinity for the carrier. The binding site detected by Leslie and Rowe was shown to have an equal affinity for folic acid and tetrahydrofolic acid, but the addition of the 10 formyl group at position 10 of tetrahydrofolic acid reduced the affinity to some extent.

The data of Section 2, Part IV indicates that 10-formylfolic acid at a mucosal concentration of 10^{-5}M has no significant effect on the serosal transfer of folic acid at a concentration of 10^{-6}M. On the other hand the tissue accumulation of folic acid is reduced by about 36% under these conditions. These observations provide a striking parallel to those recently reported on the effect of high concentrations of methotrexate on folic acid transport _in vitro_. Selhub, Brin and Grossowicz (1973) studied the tissue accumulation, serosal transfer, and metabolism of folic acid using everted sacs incubated in the presence and absence of methotrexate. It was shown that at low concentrations, methotrexate effectively blocked the reduction of folic acid, presumably by inhibition of DHF reductase, but had no observable effect on transport. At relatively high concentrations the tissue accumulation of folate was reduced by 40%, but the serosal transfer was unchanged. In the other study, Olinger, Bertino and Binder (1973)

demonstrated that methotrexate had no effect on the initial mucosal influx into everted sacs, although again the tissue accumulation was reduced. Furthermore, a significant increase in the serosal transfer of folic acid was observed in the presence of methotrexate.

It must be concluded from these earlier observations, and from the results of the present study, that some form of folate accumulation takes place within the gut wall, and that this is susceptible to competition by methotrexate and 10 formyl folic acid. However, the observation that the serosal transfer of folic acid is not reduced under these conditions implies that this process is functionally separate from the tissue uptake mechanism. This is explicable if the site of competitive inhibition is located not at the "input membrane" of the brush-border, but at some site of accumulation within the gut wall. On this interpretation it is envisaged that folic acid enters and leaves the gut wall by passive diffusion, but is concentrated within the tissue. The material entering via the mucosal surface may be thought of as entering two pools, an aqueous phase available for serosal transfer, and a sequestered pool which might comprise material taken up onto binding proteins, or accumulated by energy dependent processes within intra-cellular vacuoles or intercellular spaces. Competition for binding sites or competitive inhibition of specific transfer mechanisms at the intracellular accumulation sites would then account for the diminished tissue uptake

observed in the present studies, but the serosal transfer, being a passive process, would remain proportional to the mucosal concentration and would therefore not be reduced.

The presence of a carrier mediated step leading to the serosal transfer of folic acid cannot be ruled out but if it exists it must have a lower affinity for both methotrexate and 10 formyl folic acid than the site or sites involved in tissue accumulation. The observation by Olinger, Bertino and Binder (1973) that the serosal transfer of folic acid was enhanced by the presence of methotrexate in the mucosal medium might indicate competitive stimulation of folate transfer at a carrier site, but since no information is provided as to the amount of methotrexate appearing serosally in these experiments, it is difficult to speculate any further.

iv) Stimulated Efflux Studies.

The purpose of the experiments described in Section 3, Part V. was to look for the possible existence of a carrier mediated step in the transport of folic acid across the mucosal surface by means of an exchange diffusion test. Rosenberg and Wilbrandt (1957) showed that if a carrier transported substrate is equilibrated across a membrane, transitory "uphill" efflux of the substrate may be elicited by adding a

second substrate with an affinity for the carrier to the external medium. This is brought about because the appearance of the second substrate creates an inwardly directed gradient of substrate (2)-carrier complex, and in consequence an outwardly directed gradient of free carrier at the inner surface of the membrane. This assymetrical distribution of free carrier favours net efflux of substrate (1) until equilibration of substrate (2) has been achieved. 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). A similar experiment was conducted by Saloman, Allums and Smith (1961), in which the exit of D-xylose from the mucosal epithelium of isolated *in vitro* guinea pig intestine was stimulated by the addition of D-glucose to the mucosal solution.

In the present studies, identical jejunal sacs were preloaded with labelled folic acid and then re-incubated in the presence and absence of various folates in the mucosal solution. Sacs incubated in folate free medium were used as controls against which the "stimulated efflux" from the test sacs was measured. To summarise the results, a high external concentration of unlabelled folic acid induced a significant increase in the rate of efflux of labelled folic acid, as did methotrexate, and 10-formyl folic acid. Pteroic acid would not induce an efflux rate above that of control

sacs, and pteroyl-D-glutamic acid induced a small increase in the rate of efflux which was not shown to be statistically significant in the present study. An interesting feature of the efflux phenomenon is that it appears to be associated primarily with the upper intestine. No stimulation of efflux could be induced within the most distal 10 cms. of the ileum. This is the region shown to have a low rate of transport compared to the rest of the small intestine (Smith, Matty & Blair, 1970); the low serosal transfer noted in ileac sacs compared to jejunal sacs after 60 minutes of "efflux incubation" is compatible with this report.

It is tempting to ascribe the present results to the presence of a carrier mediated transport system at the mucosal surface having a relatively high affinity for pteroyl-L-glutamic acid, 10-formylfolic acid and methotrexate, a reduced affinity for pteroyl-D-glutamic acid, and low affinity for pteroic acid. Alternatively if absorbed folic acid is sequestered within the cell in some way it is possible that the intracellular labelled folate is displaced from accumulation sites by a high concentration of unlabelled folate which has entered the cell by passive diffusion. Such displacement could occur by competition for physical binding sites, or alternatively, by a counter-transport effect at the surface of intracellular vesicles or intercellular spaces.

A strong indication that the stimulated efflux

effect occurs as a result of displacement from binding proteins is provided by the observation that though folic acid is the major component of the material which appears in the mucosal solution after displacement, the major folate which is obtained by homogenisation and extraction with distilled water is probably 5-methyltetrahydrofolic acid, the concentration of folic acid being very low. Since the displaced material amounts to about 40% of the calculated tissue content, this implies that only a small proportion of the folic acid which enters the gut wall remains free in solution. The fact that a substantial concentration of 5-methyltetrahydrofolic acid is extractable from the tissue is not surprising since this appears to be the first metabolic product derived from folic acid by the intestine (Olinger, Bertino & Binder, 1973).

Two further observations have a bearing on the interpretation of this work. The first may be looked upon as a simple inversion of the initial experiment in which preloaded sacs were exposed to a high mucosal concentration of folic acid. A single large everted sac was preloaded as usual, but at the termination of the preloading incubation it was drained and cut in two to provide a test sac and a control sac. The control sac was filled with folate free medium in the usual way; the test sac was filled with a solution containing 10^{-5}M unlabelled folic acid, equivalent to the mucosal medium used in the efflux

experiments. It was reasoned that since folic acid is able to diffuse from the serosal to the mucosal surface of the intestine, the test sac should differ from the control sac in having a relatively high concentration of unlabelled folic acid on the serosal side of the mucosal membrane. Assuming that the folate enters the epithelial cells via their serosal poles, competition for hypothetical carriers should occur between labelled and unlabelled folic acid at the serosal surface of the "input membrane", and the efflux of labelled folate should be depressed relative to the control sac. If on the other hand displacement from intracellular binding sites is responsible for the efflux effect, this should occur whatever the route by which unlabelled folic acid enters the cell and efflux from the test sac should be stimulated as usual. Inspection of Figure 3.24 indicates that when the level of efflux radioactivity is corrected for the small difference in the dry weight of the sacs a definite stimulation of efflux from the test sac is seen.

In another study, control and test jejunal sacs were prepared and preloaded as before with a view to comparing the serosal transfer of sacs re-incubated in the presence and absence of unlabelled folic acid. The preloading incubation in these experiments was restricted to ten minutes in order to reduce the amount of serosal transfer occurring before exposure of the mucosal surface to the cold folic acid. In Figure 3.23

the serosal transfer of test sacs is compared with paired control sacs from the same animals, as is the mucosal concentration of labelled folic acid at 30 minutes.

The object of this second experiment was to examine the "symmetry" of the efflux process. If the stimulated mucosal efflux arises as a result of exchange diffusion, this should have the effect of depleting free intracellular folate, thereby reducing the amount available for transfer into the serosal solution. A depletion in total serosal transfer relative to the control sacs should therefore be observed. On the other hand, if the action of cold folate is to displace labelled folate from intracellular accumulation sites, more folate should be available for serosal transfer and the passive movement should be "symmetrical" and detectable as a slight increase in total serosal transfer. It can be seen that in every case there is a small decrease in the total serosal transfer by the test sacs.

It is suggested that the observations on the competitive inhibition effects of 10-formylfolic acid in which the total tissue accumulation is reduced in the presence of 10:1 molar ratio of 10-formylfolic acid to folic acid while the serosal transfer remains unaffected, coupled with the report of Olinger et al, (1973) that methotrexate has no effect on the initial mucosal uptake rate of folic acid, are incompatible with the existence of a carrier-mediated folic acid transfer process at the mucosal surface having a high affinity

for these compounds. One would not want to come to quantitative conclusions on the basis of the single experiment using a high serosal concentration of unlabelled folic acid especially as the concentration of folic acid appearing at the serosal surface of the epithelium is unknown. However the simple qualitative observation that the efflux was stimulated under these conditions rather than reduced is also incompatible with a simple counter transport model for the efflux process. The most likely explanation then is that stimulated efflux is the result of competitive displacement of labelled folic acid by unlabelled folic acid at some site or sites within the gut wall.

However, this last conclusion must not remain unqualified. The observation that the serosal transfer of folic acid is reduced under the test conditions is hard to explain in terms of exclusively passive unmediated transmural transport. Although the site of competition need not necessarily be located at the mucosal surface it is hard to escape the conclusion that the presence of unlabelled folic acid has either reduced the concentration of labelled folate available for serosal transfer, or that competition for a transfer channel has occurred at some site, possibly at the serosal pole of the mucosal epithelial cells. If this is so then it is perhaps appropriate to envisage an overall folate transport process involving passive influx at the mucosal pole of the epithelial cell, followed by a process of

intracellular sequestration at binding or accumulation sites. The transfer of folate into the serosal compartment may be partly mediated by a specific transport step though this remains conjectural.

It is of interest to compare the present findings with what little is known about transport processes in other normal mammalian cells, and to discuss the possible identity of the intracellular binding sites.

Johns, Sperti and Burgen (1961) have pointed out the rapidity with which an injected dose of ^{3}H-folic acid is taken up by the tissues of man, and have shown that this absorbed dose may be displaced into the plasma by a subsequent dose of unlabelled folic acid. In view of the fact that methotrexate and p-aminobenzoylglutamate did not appear to be accumulated by cells it was suggested that the cellular uptake mechanism was a highly specific process dependent on the presence of an intact pteridine ring. Later work (Johns & Plenderleith, 1963), has compared a range of folate derivatives and related compounds for their ability to displace an absorbed dose of labelled folic acid. It was concluded that only those compounds having an intact unsubstituted pteridine moiety were effective displacers. The authors discussed the two possible mechanisms of displacement, competition for binding sites or counter-transport, and concluded that since it was very likely that a carrier system existed in order to account for the rapid saturable transport of folate into cells, counter transport was the more likely

mechanism of the two. An interesting additional observation (Johns, Plenderleith & Hutchinson, 1963) was that though methotrexate was a strong competitor for transport into human cells, it was only weakly effective in displacing a preloaded dose. The authors' interpretation was that a specific transport mechanism existed for folate uptake by cells, and that methotrexate had a high affinity for binding to the carrier system but was not itself transported into the cells. Thus although it acted as an efficient competitive inhibitor, penetration was poor and counter-transport could not be elicited.

It can be seen that the present results and several other literature reports suggest that the uptake of folic acid by the intestine differs significantly from the process in other cells. It seems established that methotrexate does not act as a competitive inhibitor for the overall transmural movement of folic acid *in vitro* (Olinger, Bertino & Binder, 1973; Selhub, Brin & Grossowicz, 1973) whereas from the present observations it is clearly an effective displacer of preloaded folic acid in the mucosal epithelium. In contrast, pteroic acid, which is able to displace folic acid from human tissues is completely ineffective at the mucosal surface.

Whatever the functional situation of the binding site which has been detected in the present studies it would appear that the benzoylglutamate moiety is of considerable importance in the binding process. In view of the fact that an appreciable serosal transfer

of pteroic acid occurs, it is probable that its failure to elicit an efflux of folic acid reflects a low affinity for the binding sites. No transport studies have been undertaken with pteroyl-D-glutamic acid, however it should be noted that this material has less than 10% of the microbiological activity of pteroyl-L-glutamic acid, and this might well be the result of a reduced affinity for DHF-reductase (J. A. Blair & K. Ratanasthien, unpublished observations).

The observation that preloaded 5-methyltetrahydrofolate is not displaced by folic acid is interesting, as it would indicate, in the case of exchange diffusion, that the folic acid carrier had no affinity for 5-methyltetrahydrofolate. From a teleological viewpoint it would appear odd that a substance such as folic acid, which occurs naturally in only small amounts, should be transported by a carrier system which had no affinity for a commonly occurring natural folate. On the other hand it has been reported that 5-methyltetrahydrofolate has unsaturable uptake kinetics over a wide range of concentrations *in vitro*, and shows no other sign of mediated uptake (Strum *et al*, 1971; J.A. Blair & A. Razzaque, unpublished observations). Reduction and methylation appears to be the normal metabolic fate of folic acid entering the liver cell, and of at least some of the folate absorbed by the intestine (Corrocher, Bhuyan & Hoffbrand, 1972; Olinger, Bertino & Binder, 1973). It seems perfectly feasible that the normal site of 5-methyltetrahydrofolate within

the intestinal cell might be a binding site unavailable to folic acid or other unreduced forms.

It is not possible at present to positively identify the accumulation sites but a number of possibilities as to the nature of the intracellular binding proteins can be mentioned. One obvious candidate is dihydrofolate reductase which is presumably the site at which reduction of folic acid prior to methylation occurs during intestinal uptake (Olinger, Bertino & Binder, 1973). It has been demonstrated by means of autoradiography of cells labelled with ^{3}H methotrexate that DHF reductase is concentrated in the apical cytoplasm of the mucosal cells (Darzynkiewicz et al, 1966). Methotrexate has an affinity for this enzyme many times that of folic acid and pteroic acid has an affinity many times less (Blakley, 1969). This is an obvious site of accumulation for newly transported folate, but in view of the relatively small amounts of folic acid which are methylated during transport (Olinger, Bertino & Binder, 1973), and the fact that whereas small amounts of methotrexate have been found to suppress this metabolism, much higher concentrations are required to abolish the accumulation of folic acid by the gut wall (Selhub, Brin & Grossowicz, 1973), it seems unlikely that DHF reductase is the major site of tissue storage.

Goresky, Watanabe and Johns (1963) have pointed out that some aspects of the transport kinetics of folic acid during its reabsorption by the renal tubules are analogous to the handling of the dye bromosulphophthalein

(BSP) by the liver. It is interesting to note in this context that the uptake and storage of BSP, bilirubin, and other organic anions by liver cells is controlled in part by two cytoplasmic proteins, designated Y and Z (Levi, Gatmaitan & Arias, 1969; Reyes _et al_, 1971). It has been shown that these proteins are to be found in tissues which extract bilirubin and BSP from the plasma, and they have been shown to bind these anions both _in vitro_ and _in vivo_. Furthermore, drugs and physiological conditions which cause a proliferation of the X and Y proteins lead to a concomitant increase in the initial uptake rate and intracellular storage of BSP and bilirubin. In view of the fact that it has been shown that Z is present in the small intestinal mucosa of mammals it is very interesting to note that both Y and Z, derived from mammalian liver, have recently been shown to bind folates _in vitro_ (Corrocher _et al_, 1974). The apparent importance of Y and Z proteins in the uptake and storage of organic anions in the liver must make Z a possible candidate for a similar function in the intestine and this possibility seems well worthy of further study.

v) _Isolated Cell Studies._

Before discussing the results obtained in the present study, using intestinal cells isolated by the hyaluronidase disaggregation technique, it will be useful to briefly review the other methods currently available

for the preparation of such cells.

The earliest methods employed in the study of isolated mucosal constituents involved fairly unsophisticated techniques of mechanical disruption which nevertheless produced material useful for *in vitro* studies. The "mucosal scrape" preparation of Dickens and Weil-Malherbe (1941) was originally used to investigate the *in vitro* aerobic metabolism of the intestinal mucosa, and later, the synthesis of purines from labelled precursors (Paterson & Zbarsky, 1955, 1958). Crane and Mandelstam (1960) considered its usefulness as a basic preparation for transport studies and reported that both it, and the "isolated villi" preparation derived from it by further mechanical fragmentation, were capable of energy dependent transport of sugars. Homogenisation of mucosal scrapes formed the basis for the preparation of epithelial cell "ghosts" by Clark and Porteus (1965) but these cells do not appear to be suitable for transport studies.

Other more recent techniques have also employed mechanical disruption as a means of isolating the mucosal cells, though in such a way as to produce true single cell suspensions free from contamination by other cells and tissue fragments. The method of Sjostrand (1968) involves a very rapid separation of the cells from the underlying tissue by the application of gentle mechanical pressure to the surface of a rapidly rotating everted intestine. Sjostrand reports that the resulting cells

have very little contamination by other material or by mucus, and displayed extremely good morphology. Furthermore, the appearance of the cells as judged by light microscopy remained largely unchanged after 48 hours in a refrigerator. A second method of mechanical isolation, that of Harrison and Webster (1969) involving low amplitude high frequency vibration, is also reported to give healthy viable cells. An interesting feature of this technique is that by harvesting the cells at intervals during the vibration period, cells of decreasing maturity can be collected from progressively lower sections of the villi. Both these methods have been compared by Iemhoff et al (1970) who concluded that though the cells obtained by the method of Sjostrand (1968) showed signs of considerable morphological and physiological damage, those obtained by the vibration technique were morphologically intact and had metabolic characteristics comparable to those of intact intestine.

Dissolution by chemical treatment is perhaps the most common method currently employed for the isolation of mucosal cells, and a variety of agents are available. A trypsin and pancreatin mixture appears to have been successfully used by Harrer and Reilly (1964) to produce a viable preparation uncontaminated by bacteria or tissue fragments, and suitable for metabolic studies (Stern & Reilly, 1965). Later work by this group describes a satisfactory method of isolation employing sodium citrate; the cells so obtained were evidently

capable of energy dependent glucose accumulation (Stern & Jensen, 1966).

Huang (1965) has described a method of isolation using lysozyme but this technique has been criticised on the grounds that the long period of incubation involved was likely to have led to cell damage (Stern & Jensen, 1966; Perris, 1966).

Hyaluronidase has been employed by a number of workers who have described cell preparations having apparently satisfactory metabolism and transport characteristics (Perris, 1966; Kimmich, 1970). The use of this technique by Leslie and Rowe (1972) for the study of folic acid transport suggested its further investigation in the present study.

Evans _et al_, (1971) have described an isolation technique using the chelating agent EDTA which is thought to disaggregate the cells from the basement membrane by a process involving chelation of metal ions. The cells produced by this technique are rapidly obtained and are of good functional and morphological appearance (Evans, 1974; personal communication).

In the present studies it was never possible to detect any increase with time in the concentration of folic acid associated with the isolated cells when the incubation solution contained 10^{-5}M folic acid. Figure 3.29 shows a representative experiment. It can be seen that uptake of folic acid is complete within 2 minutes and at each subsequent sample time there is a

small decrease in the folate content. These results are in agreement with the findings of Leslie and Rowe (1972) who reported that in similar experiments no increase in the amount of folate associated with the cells as a function of time could be demonstrated over the nanomolar to the millimolar concentration range. The present results appear to be only approximately in quantitative agreement with those of Leslie and Rowe who reported that at 1.85 x 10^{-5}M their cells bound 16.0 pmoles/mg of protein as compared with 23.0 pmoles/mgm dry weight for the two minute sample in the experiment in Figure 3.29.

In figures 3.30 and 3.31 the activity of the supernatant solutions during the first dilution of the cell samples and at four subsequent washes is compared for folic acid and ^{14}C labelled inulin, a molecule usually assumed to be incapable of penetrating viable cell membranes. It can be seen that the "wash-out" of radioactivity from the samples is very similar for both folic acid and inulin and this was taken as confirmation that the association of folic acid with the cells was primarily extracellular.

Two interpretations of these results appear feasible. It is possible that the normal in vivo route of folic acid absorption is not, as has been assumed so far in this discussion, transcellular at all, but involves an extracellular pathway via pores and intercellular spaces. This conclusion is challenged however by the work of Momtazi and Herbert (1973) who have used isolated

cells prepared by the vibration technique (Harrison & Webster, 1969; Webster & Harrison, 1969). In the work of Momtazi and Herbert isolated cells were incubated for 60 minutes under a variety of conditions and the total uptake of folic acid expressed as picograms per 10^6 cells was recorded. Unfortunately the report does not contain any information as to the time dependence of the accumulation and in view of their method of expressing their results direct quantitative comparison is very difficult. However these authors have shown that folate accumulation by their cells was linear with concentration, sensitive to glucose, and enhanced in jejunal cells compared with cells from the ileum. In view of the previous report by these workers that suspensions of isolated cells obtained by mechanical disaggregation of guinea pig intestinal mucosa were capable of accumulating folic acid for long periods, it seems very likely that cells obtained by such methods exhibit true intracellular accumulation, and the failure to do so of the cells prepared by the hyaluronidase incubation technique is an indication of some form of functional lesion.

This conclusion is strengthened by a comparison of the behaviour of the mucosal scrape preparation with the cells used in the present study. From Figure 3.29 it can be seen that whereas the isolated cell preparation exhibits a rapid initial accumulation with no measurable time dependence, the mucosal scrapes appear to accumulate folate over the first ten minutes or so of the incubation,

and then reach a plateau value which is about double that of isolated cells. This is a further indication that the isolation technique used in the present study has impaired the folate absorptive capacity of the cells, and it is in strong contrast to the observation of Momtazi and Herbert (1973) who reported that mucosal scrape preparations were significantly inferior to vibration-obtained isolated cells in their ability to absorb and accumulate folic acid.

The question now arises as to the nature of the functional lesion affecting folic acid transport in these isolated cells. It is unlikely that the component cells of a specialised structure such as the mucosal epithelium can undergo the fairly drastic procedures involved in any isolation technique without some degree of physiological damage taking place. It has been shown for example that isolated cells prepared from mucosal scrapes, though they may be capable of complex metabolism for useful periods of time, show a rapid loss of RNA and DNA during incubation. (Stewart & Zbarsky, 1963). So great was the loss that after two hours of incubation at $37^{o}C$ only 25% of the RNA and 1.2% of the DNA content determined immediately before incubation was still present. This gross loss of nuclear material may however simply reflect the fact that the cells prepared by this method are of poor integrity, as the authors reported that by 2 hours most of the cells were visibly broken when examined by light microscopy.

The enzyme trypsin, which is commonly used in cell isolation techniques and has been employed in the

preparation of isolated mucosal cells (Harrer, Stern & Reilly, 1964) has long been known to disturb the integrity of cell membranes, the principal effect being a rapid loss of glycopeptides. Incubation of human erythrocytes with trypsin, for example, causes the loss of glycopeptides containing up to 50% of the sialic acid of the intact cell (Winzler *et al*, 1967). Not surprisingly, these surface effects seem to lead to significant changes in the overall physiological status of the cell. Mallucci, Wells and Young (1972) showed that treatment with low doses of trypsin brought about a very rapid increase in the mass and volume of cultured cells, presumably by a direct effect on surface receptors. Disturbances in the membrane transport of non-electrolytes have been demonstrated in alveolar macrophage cells after trypsin treatment (Min-Fu Tsan *et al*, 1973). The primary effect was a fall in the intracellular amino acid pool, which led to decreased exchange diffusion of lysine.

With the proviso in mind that any isolation technique involving treatment of the cells with proteolytic enzymes is likely to lead to physiological changes, it seems safe to conclude that hyaluronidase treated cells are in many respects functionally similar to other preparations. The fact that such cells are capable of energy dependent sugar, electrolyte, and amino acid transport strongly implies that at least some of the specific transport systems of the membrane must remain intact (Kimmich, 1970; Reiser & Christiansen,

1971; Leslie & Rowe, 1972).

A clue to the nature of the defect induced by the hyaluronidase isolation technique may be provided by the observation of Leslie and Rowe (1972) that no glycoproteins or lipoproteins were detected in the analysis of the brush-borders isolated from their cell preparations. This strongly suggests that some structural derangement of the brush border membranes has occurred though it is not clear from their report at what stage the loss of glycoproteins took place.

It seemed very probable that the absence of glycoproteins and lipoproteins in the membrane analysis of the hyaluronidase treated cells of Leslie and Rowe indicated the removal of the glycocalyx or enteric cell coat structure associated with the microvilli of the brush-border. The surface coat of the mucosal epithelial cell, extensively studied by Ito and other workers (Ito, 1965, 1969; Mukherjee & Williams, 1967) is a polysaccharide-rich layer, varying in prominence from species to species, forming a continuous barrier between the tips of the microvilli and the bulk fluid of the intestinal lumen. It is not entirely clear to what extent the surface coat is distinct from the mucus secretions of the epithelial goblet cells. Ito (1969) and Mukherjee and Williams (1967) have stressed the fact that the surface coat is a product of the individual cell to which it is attached, rather than an adsorbed "blanket" covering the epithelium as a whole. Other workers however make a

clear distinction between the surface glycoprotein layer which is a feature of all cell membranes, and the unusually thick layer of the intestine, which may be derived from free polysaccharide of the epithelial mucus secretions (Parsons & Subjeck, 1972). In any case, hyaluronidase which acts specifically on linkages in the glycosamino glycan structure, would be expected to disrupt the intrinsic glycoproteins of the cell membrane (Kennedy, 1973).

It would seem from the observations of Ito (1965) that the surface layer of cat intestine is an unusually stable structure in comparison with the glycoprotein coats of other cells. Ito subjected everted sacs and pieces of minced mucosa to incubation with a variety of proteolytic and mucolytic agents, including trypsin and hyaluronidase, and concluded from electron micrographs that none had any discernible effect on the adherence of the layer. He concluded that the surface layer remained intact while the cell was viable but that procedures such as isolation of brush borders, or the natural process of cell extrusion from the tips of the villi resulted in its loss. It seems feasible however that the long period of hyaluronidase incubation employed in the present technique; coupled with disruption of the epithelial structure and the subsequent saline washing procedure, might lead to some disruption of the surface layer and adherent enteric mucus. The histological studies described in Section 3 were undertaken in order to investigate this issue.

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 associated with the brush-borders of the columnar cells is quite clear, as are the very prominent mucus-rich goblet cells. Ito (1965) has used a similar technique for the optical resolution of the prominent surface layer present on cat intestinal epithelial cells. However, in view of the fact that the surface layer in rat is somewhat smaller than its counterpart in cat and human intestine it would perhaps be rash to conclude that the fine pink line discernible at the extremities of the brush-borders in Plate I. is identical with the structures revealed by electron microscopy. The point is made however that insofar as everted material reflects the situation in the normal *in vivo* intestine, a relatively thick polysaccharide layer is a feature of the functioning absorptive cells.

Plate III is a cell preparation prepared by the hyaluronidase disaggregation technique and stained with PAS as before. The absence of stainable polysaccharide material at the brush-border is obvious, and it is quite clear that the isolation technique has resulted in a marked alteration in the gross surface properties of the brush-border. The complete absence of PAS staining material argues strongly that the enzyme digestion process has resulted in a loss of surface

polysaccharides and glycoproteins, and this is consistent with the observations of Leslie and Rowe (1973) on the protein constituents of brush borders prepared from similar cells.

Such a major breakdown in the enteric surface coat is perhaps surprising in view of Ito's (1965) observations on the effects of proteolytic and lipolytic agents, but 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. In this respect it is interesting to compare 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. The very striking difference in the brush-borders of these cells is obvious and it must be concluded that the two isolation techniques provide cells with widely differing surface characteristics. It should be noted that the cells in Plate IV occur as small fragments of epithelium rather than individual cells. This effect is also observed with the EDTA isolation technique and may be due to the fact that the chelating effect brings about a detachment of the cells from the basement membrane but has little effect on the intercellular lateral junctions. It seems feasible that the survival of the surface layer in the cells isolated by the citrate technique is due in part to the partial preservation of the epithelial integrity.

It has been suggested earlier in this discussion that the passage of folic acid into the mucosal cell occurs by passive diffusion, and that the permeability depends upon the degree of ionisation of the molecule, this in turn depending upon the pH of the aqueous phase in contact with the brush-border membrane. It has been recognised for some time that the immediate environment of the cell membrane was, in all likelihood, influenced by the presence of a glycoprotein surface coat, and Bennet (1962) who first used the name "glycocalyx" as a general term for all polysaccharide cell coats, has suggested that it may act in a manner analogous to a stationary chromatography phase, selectively binding ions and controlling the properties of the entrained fluid at the surface of the plasma membrane. In the present instance it is suggested that one of the functions of the surface coat is to maintain a region of low pH within the brush-border, and that this has the effect of increasing the concentration of permeable folate available for diffusion. If this were so, the absence of a thick surface coat would mean that the fluid layer in contact with the brush border membrane would tend toward the conditions of the bulk phase, particularly in an isolated cell preparation in which unstirred layer effects are at a minimum (Winne, 1973).

To test this hypothesis, an experiment was performed in which the uptake of folic acid by a homogeneous cell preparation was compared in incubation media having acidic and near neutral pHs. To increase

the sensitivity of the experiment the incubation was carried out using a 10^{-6}M concentration of ^{14}C folic acid, at the maximum available specific activity, 55.3 mci/mmol. It can be seen from Figure 3.32 that the folate content of the cells incubated at an initial pH of 5, and a final pH of 5.9 is greater at each sample time than the activity of identical samples incubated at pH 6.9. Furthermore, the difference in content appears to increase with time, indicating a more rapid rate of uptake by the cells incubated at a low pH. It must be noted that in this experiment a small increase in the activity of the control cells is detectable, in contrast to earlier experiments conducted at a higher concentration in which only the rapid binding effect was observed. This may be due to some difference in the condition of the cells in this later experiment, though every effort was made to ensure continuity of technique. Alternatively it may be that the use of a higher specific activity folic acid solution has enhanced the sensitivity of the method.

At pH 7, about 1% of the folic acid in the control solution is in the form of the monoanion, the remainder being dianion. However, in the test solution, at the commencement of incubation at pH 5, about 1.5% of the folic acid was in the neutral form, and about 50% as the monoanion; at the final pH, 5.9, about 9% of the total folic acid was present as the monoanion.

Assuming that in this particular cell preparation the pH of the bulk phase accurately reflects the pH at the

cell membrane, and that the neutral molecule and the mono-anion are substantially more permeable than the dianion, the present results can be accounted for as passive permeation, and the pH effect is similar to that observed by Hakala (1965, a, b) in her description of the uptake of methotrexate by the Ascites tumour cell.

vi) General Conclusions and Suggestions for Further Work.

We arrive then at a working hypothesis for folic acid transport which entails a process of passive absorption linked, nevertheless, to the metabolic activity of the intestine. To briefly recapitulate, it is proposed that folic acid is transferred as the neutral species, and probably to some extent as the mono-anion, throughout the small intestine. Diffusion across the mucosal surface is followed, in the jejunum at least, by sequestration or binding within the gut wall, and is enhanced by the acidification mechanism of the upper intestine. The probable existence of an unstirred pH "microclimate" at the jejunal surface favours the presence of the permeable folate species in this region, but imposes an upper limit on the rate of transport of folic acid because of the low solubility of the compound at low pH.

In their discussion of the behaviour of enzymes bound to insoluble co-polymers, Goldstein, Levin and Katchalski (1964) suggested that it might be possible to utilise enzymes as molecular "probes" to determine some of the characteristics of a given microenvironment. On the same principle, if a "pH microclimate" of the type discussed in this work is shown to exist it may be appropriate, in retrospect, to look upon folic acid as a "transport probe" indicating the specialised pH conditions obtaining at the surface of the jejunal mucosa. Furthermore it should be possible to utilise other folates,

having different dissociation properties and correspondingly predictable transport characteristics. For example, tetrahydrobiopterin has ionising groups such that the concentration of neutral species is reduced at low pH; the rate of transport of this compound should therefore show the opposite trend to that of folic acid in response to alterations in intestinal acidity.

The studies with isolated cells reported in this work bring to light important differences in the physiological behaviour and morphological appearance of isolated cells prepared by various techniques. If the microclimate model is correct it should be possible to correlate the persistence of the polysaccharide layer at the brush-border with the ability of the cells to absorb folic acid. Comparison of the folic acid uptake characteristics of a range of isolated cell preparations should therefore provide evidence for, or against, the present hypothesis, and in turn provide new information on the functional characteristics of these various preparations.

Such indirect studies must of course be accompanied by a direct measurement of the pH in the fluid layers immediately adjoining the jejunal mucosa; such studies, utilising pH microelectrodes, are currently in hand in this department (J.A. Blair & M.L. Lucas, 1974, personal communication).

During the writing of this thesis a new publication has appeared describing folic acid transport

in the everted sac and the perfused intestine of the rat (Elsborg, 1974). Surprisingly enough, this author has not observed a saturable absorption, nor any difference in the rate of uptake in the jejunum compared with the ileum and in consequence he concludes that folic acid absorption is a passive process in the rat, though the probable importance of the luminal pH is also recognised. The fact that saturable transport was not observed in this study underlines the importance which must be attached to the type of technique employed to observe the phenomena of intestinal transport. Elsborg has used an *in vivo* perfusion technique to observe the disappearance of folic acid from the luminal fluid, a similar technique to that employed by Halsted and Mezey (1973) who also failed to observe a saturable absorption. The anomoly may be due to the effect of poor luminal stirring in this type of preparation, or to the failure to distinguish between the saturable, time-dependent absorption, and the rapid uptake which was not saturable at the concentrations employed in the present study.

It has been suggested that the saturable transport kinetics for folic acid may be adequately explained if the absorption occurs through an extracellular aqueous layer having a pH low enough as to limit the solubility of the material to between 10^{-6}M and 10^{-5}M and it has been argued that no firm evidence has been obtained for the presence of a carrier-mediated step at the brush-border membrane. Nevertheless, the observation that the serosal transfer

of preloaded folic acid is inhibited by a high mucosal concentration of unlabelled folic acid could 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 and Ventura, 1972). It would be wrong therefore to rule out entirely the possible existence of a carrier-mediated component at some stage in the overall transfer, and it would be interesting to employ a new approach to the problem, possibly using a new preparation.

In vitro preparations of amphibian intestine have come into use in recent years (Parsons & Prichard, 1968) and appear to offer certain advantages over mammalian tissues. Fox and Hogben (1974) have recently reported that a significant net mucosal-serosal flux is observed for nicotinic acid across sheets of bullfrog intestine in vitro and this they ascribe to the presence of an active transport system for this compound. These workers point out that Turner and Hughes (1963), Turner (1959), and Spencer and Bow (1964) all failed to report this phenomenon in rat intestine, and they suggest that this may be because isotope equilibrium cannot be achieved in mammalian preparation in the relatively short incubation times available. 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. Whether or not this argument is correct remains to be seen but it would be interesting

to examine folate transport in a similar preparation.

It has been shown that folic acid is not accumulated in a free form within the fluid of the gut wall and it has been argued that it is probably bound to intracellular proteins, although this might conceivably be preceded by active uptake into intracellular vacuoles. The problem of the intracellular site might be best investigated by means of high resolution autoradiography at the microscopic level. By using sections of gut wall and, assuming that a suitable preparation is developed, isolated cells, at incubation periods from a few seconds upwards, it should be possible to determine the areas of labelled folate accumulation within the mucosal cell, the intact epithelium, and the submucosal layers. Such an approach has already been employed by Darzynkiewicz et al (1966) who observed that labelled methotrexate became localised in the apical cytoplasm of mouse intestinal cells. The authors suggested that the methotrexate binding sites were to be accounted for as DHF-reductase but an obvious corollary of a detailed investigation of the intracellular localisation of folate in the gut is the isolation and rigourous identification of labelled cell constituents.

It is hoped that these studies will focus attention on the importance of intestinal pH levels in man, and that the enteric surface coat in health and disease will receive increased attention. The work on intestinal pH levels in adult coeliac disease and the probable connection between the increased alkalinity of the jejunum

and the reduced mucosal ATPase levels in this disease, have been mentioned (Benn *et al*, 1971). It was also stated that reduced folate uptake had been reported in ulcerative colitis, a disease normally thought of as being exclusively associated with the large intestine (Franklin & Rosenberg, 1973). This posed a problem since folate transport is apparently localised in the proximal region of the small intestine; this may be resolved however by a recent report which describes various histological and chemical changes in the jejunal mucosa of patients suffering from ulcerative colitis, including reduced levels of acid and alkaline ATPases in a large proportion of cases (Binder, Soltoft & Gudmand-Hoyer, 1974).

Finally there is perhaps a danger of taking an over-simplified view of folic acid transport in the intestine. It must not be forgotten that the gut is an active site for folic acid metabolism (Olinger, Bertino & Binder, 1973; Brown, Davidson & Scott, 1974) and as has been shown, it appears to function as a folate storage site for short periods at least. The mucosa is extremely active in terms of cell proliferation, and is therefore very susceptible to the effects of folate antagonists (Zamchek, 1960; Jacobson, 1954). Furthermore, folic acid itself exerts a therapeutic effect on the symptoms of tropical sprue, and it has been shown that both folate malabsorption and D-xylose malabsorption, themselves side-effects of anti-convulsant megaloblastic anaemia, can be reversed by the oral administration of folic acid (Reynolds *et al*, 1965).

It has even been claimed that folic acid is able to prevent phenobarbitone-induced D-xylose malabsorption *in vitro* though the mechanism remains unexplained (Matthews, 1966).

It appears then that a complex inter-relationship may exist between folate metabolism and folate absorption, which may in turn have a long-term influence on the absorptive capacity of the intestine for other materials. If this is so it may be important for future research to look upon folate transport as but one aspect of the overall physiology of the mucosal cell rather than a solitary process to be studied in isolation.

APPENDIX I.

Method of animal sacrifice:
effect on water transport *in vitro.*

The transport of isotonic fluid across the mucosal surface is a feature of viable _in vitro_ intestinal preparations. The physiological mechanism of fluid uptake is not fully understood, but is generally thought to occur as a corollary of active solute transport at the mucosal surface.

According to the "standing osmotic gradient" model (Curran, 1965; Diamond, 1971), the active accumulation of solute sets up a local osmotic gradient within some epithelial compartment, leading in turn to the osmotic absorption of water and an elevated hydrostatic pressure which becomes the driving force for the transport of water and solute into the serosal compartment. The anatomical basis of this process is not, as yet, fully resolved, but both the microvilli of the brush border, and the intracellular channels of the mucosal epithelium have been suggested as sites for the establishment of standing concentration gradients in the intestine (Diamond & Bossert, 1967; Jackson & Cassidy, 1970). Water absorption gives rise to an increase in the serosal volume (serosal transfer) and an increase in the wet weight of the tissue (gut fluid uptake). According to Jackson and Cassidy (1970), gut fluid uptake is localised to the epithelial cells which expand longitudinally during absorption.

Whatever the details of fluid uptake by the _in vitro_ intestine, there seems to be little doubt that

it occurs as a consequence of energy dependent transport processes. Gut fluid uptake is known to be dependent upon the presence of metabolic substrates (Jackson & Cassidy, 1970) and it appears to be stimulated in parallel with transmural potential difference and sodium fluxes in fasted rats (Beck & Dinda, 1973). In the present study, serosal fluid transfer was found to be significantly reduced in the presence of DNP and under anaerobic conditions (see Section 3).

Levine *et al* (1970) raised the possibility that the use of anaesthetic may affect the viability of *in vitro* tissues prepared from the sacrificed animal, and suggested that the gut fluid uptake in their preparations may have been reduced thereby. Whatever the validity of Levine and her co-workers' critique of the everted sac, the questions raised are interesting and during the development of the techniques used in this study it was decided that fluid transport would provide a convenient means of assessing the energy dependent transport activity of the mucosal epithelium, and would be used to decide on the best method of animal sacrifice.

Methods.

The animals used in this study, the method of preparing the everted sacs, the incubation routine and the assessment of fluid uptake were as described in detail in Section 2.

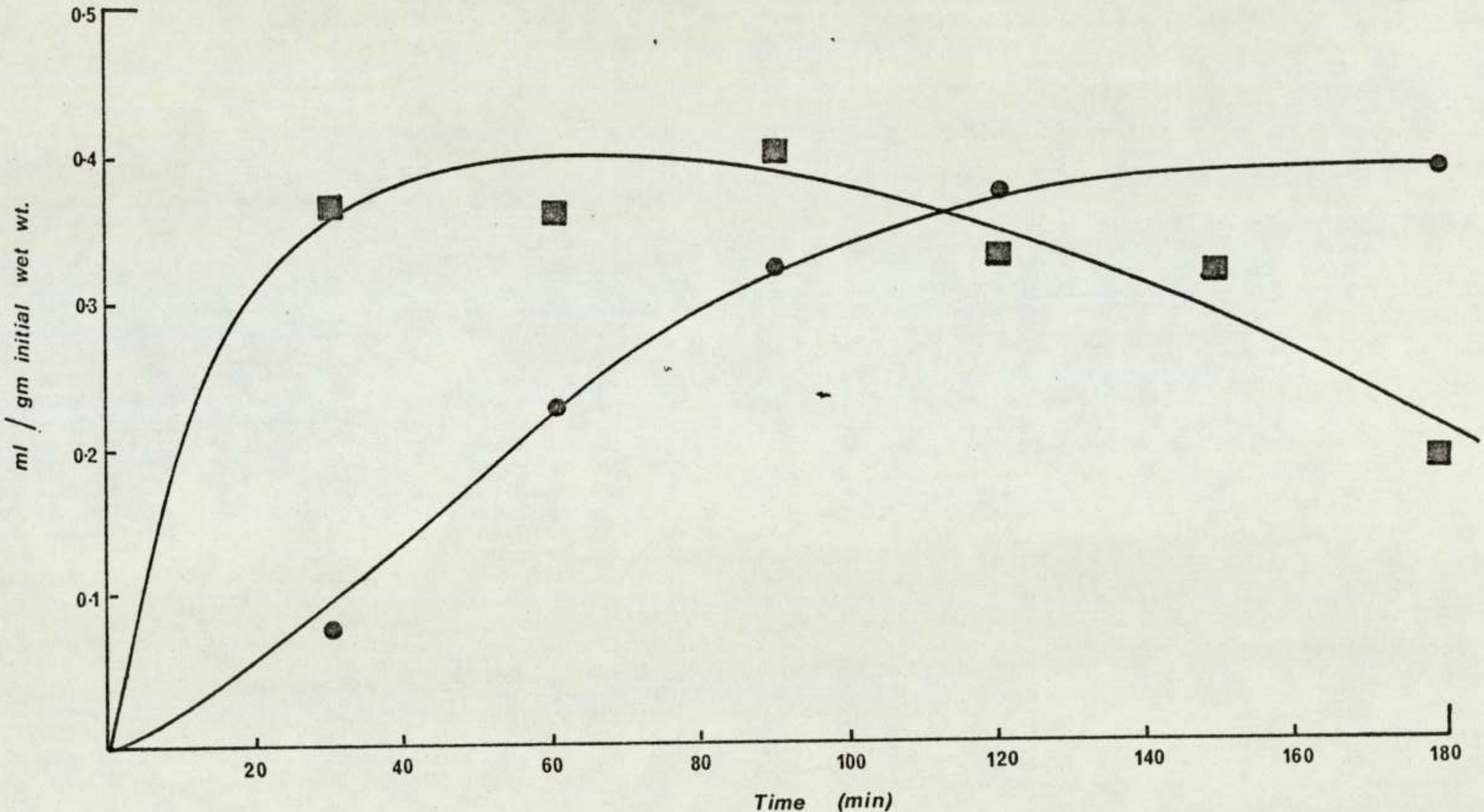
Animals were killed by two methods: -

1. Etherisation. The animals were placed in a glass vacuum desiccator, the lower section of which contained a quantity of cotton wool soaked in di-ethyl ether. As soon as the animal lost consciousness it was removed from the killing-jar, the intestine was exsected as previously described, and the animal was killed by decapitation.
2. Stunning. The rat was placed gently on the surface of a laboratory stool with a minimum of disturbance. After a few seconds the animal was 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. Animals which were unduly excited or alarmed were not used in these experiments.

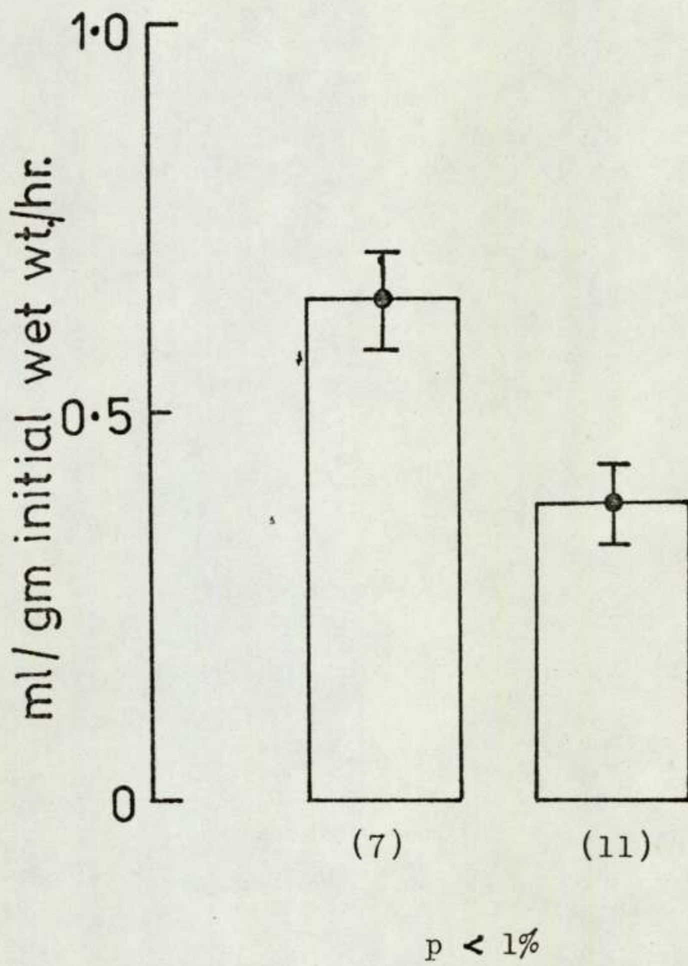
Results.

Figure I.1. shows the time course of gut fluid uptake and serosal transfer for animals killed by stunning and decapitation. It can be seen that serosal transfer continues for two hours before reaching a constant value. Gut fluid uptake levels out after 1 hour and declines slowly after 90 minutes.

Figure I.2. is a comparison of the total fluid transport of jejunal sacs from animals sacrificed by the two methods. Each column represents the mean and standard error of the number of animals given in



<u>FIGURE I.1.</u> Time course of gut fluid uptake (■) and serosal fluid transfer (●) in everted sacs derived from animals killed by stunning and decapitation.



$p < 1\%$

FIGURE I.2. Comparative rates of total fluid transport by everted sacs from animals sacrificed by stunning and decapitation (A) and by ether anaesthesia (B).

parentheses. A value for a single animal was the average value of four sacs.

Discussion.

From these results it was concluded that everted sacs prepared from etherised animals had a significantly lower fluid transport capacity than similar sacs prepared from stunned and decapitated rats.

There does not appear to have been any detailed study published on the effects of factors such as environment, handling and the mode of sacrifice on the behaviour of subsequently prepared *in vitro* intestinal preparations, but in view of their importance this is regrettable. Personal discussions with physiologists indicate a variety of opinions on the best method of killing the animal, but these seem usually to be based on unpublished observations or personal preference. The use of anaesthetics is often justified on the grounds that stunning and decapitation induces severe shock and that this gives rise to low blood flow and consequent anoxia during the period prior to the perfusion of the excised intestine. Under anaesthesia, the intestine may be perfused with the blood flow still intact, a method consistent with the recommendation of Fisher and Parsons (1949). Against this, however, must be set the fact that anaesthetics 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.

Just what these toxic effects are likely to be can be inferred to some degree from various authors' observations. The anaesthetic effect of ether is thought to be connected with the suppression of oxidative metabolism in the brain and Taylor (1963) has noted a significant reduction in glycolysis in everted sacs prepared from animals sacrificed with ether. Matthews (1966) has observed that the transport of D-Xylose is inhibited *in vitro* by phenobarbitone, and pre-treatment with this substance has been shown to cause enhancement of iron uptake, and hypertrophy of mucosal smooth endoplasmic reticulum in the rat (Thomas et al, 1972).

A brief survey of the literature demonstrates that both methods of killing have been used extensively by many authors in a variety of *in vitro* intestinal studies. In view of the results of this short study it was concluded that the use of ether probably results in a suppression of at least some of the energy dependent transport processes of the jejunum and, in consequence, stunning and decapitation was chosen as the method of killing to be used in the remainder of the project. It is suggested, however, that a detailed study with a view to arriving at an optimum set of conditions for the preparation of *in vitro* intestinal material would be of very great service to intestinal physiology.

APPENDIX II.

Chemical behaviour of folic acid
during physiological incubation.

Autoradiographic analysis was used to check the level of impurity in the labelled folic acid, to test the stability of the compound during incubation under physiological conditions, and to observe the presence of any metabolites which might appear during incubation in the presence of intestinal tissue.

Method.

Everted rings of jejunum were prepared as described in Section 2 from the upper intestine of a single rat. All the material was transferred to a 25 ml. Erlenmeyer flask containing 10 ml. of the standard incubation medium to which had been added 10^{-5}M. ^{14}C-labelled (54.3 mci/mmol) folic acid. The flask was then incubated with shaking at 37° for 60 minutes under a continuous jet of 5% CO_2 in O_2. A similar sample of incubation medium was set up and incubated under identical conditions in the absence of tissue.

Three solutions were analysed by thin layer chromatography:

I) ^{14}C labelled medium before incubation.

II) ^{14}C labelled medium after incubation with tissue.

III) ^{14}C labelled medium after incubation in the absence of tissue.

Thin layer chromatograms were run on cellulose plates (MN 300, 0.1 mm) in 3 solvent systems (Section 2, Page 83, solvents 1, 2 and 3). Standard solutions of unlabelled folic acid, 10-formylfolic acid and 5-methyltetrahydrofolic acid were prepared and run at the same time,

using fluorescent indicator plates where necessary to locate absorbing spots. The developed autoradiograms gave conspicuous folic acid spots and other fainter ones in some cases. Some of the traces were assessed quantitatively using a Joyce-Loebl recording micro-densitometer.

Discussion.

Folic acid was found to be the principal component in all the solutions. A radioactive impurity was found to be present in the ^{14}C labelled folic acid as supplied by the manufacturers and this is illustrated in Figure II.I (solution I, solvent 2 at peak B). The impurity accounts for about 2% of the total radioactivity in the sample and is within the manufacturer's specification. Inspection of Figure II.2 which is a similar analysis of solution III reveals no increase in the concentration of impurity A and it may be concluded that folic acid undergoes no degradation during 60 minutes incubation under physiological conditions in the absence of tissue.

Figure II.3 illustrates the equivalent analysis of the medium after 60 minutes of incubation in the presence of tissue. A series of new spots are visible, representing metabolic products of folic acid: the R_f values of these compounds are given in Table II.1.

An interesting feature is that spot B, which was present as an impurity in the original solution,

has increased in intensity relative to spot A, folic acid. This may merely reflect the fact that absorption of fluid has concentrated B in the bathing medium and increased its concentration relative to folic acid which is preferentially absorbed by the tissue. Alternatively, if B is a breakdown product of folic acid, the result of cleavage at the 9-10 position giving a pteridene and the free aromatic amine, then it is perhaps feasible that this reaction would be accelerated by the presence of riboflavin and thiamine which have been reported to have this effect (Biamonte & Schneller, 1951) and which, having leached out of the gut wall, might be expected to be present in the bathing solution at about 10^{-8}M. (Turner & Hughes, 1961).

Three new peaks are visible in Figure II.3 and their R_f values are given in Table II.1. together with the R_f values for the other solvent systems. No positive identification of these compounds is possible. It can be seen from the relative intensity of the peaks in Figure II.3 that the metabolites are present at a very low concentration, and it would require a very careful study using many more solvent systems than the three used in the present experiment to arrive at a definitive analysis. From the reports of other workers however, it seems likely that the metabolites appearing in the incubation medium are reduced and substituted derivatives of folic acid (Olinger, Bertino & Binder, 1973; Selhub, Brin & Grossowicz, 1973). Comparison with the R_f values of Beavon (1973), and the unlabelled marker compound, suggests that 5-methyltetrahydrofolic acid is a likely candidate for the compound at peak C in Figure II.3.

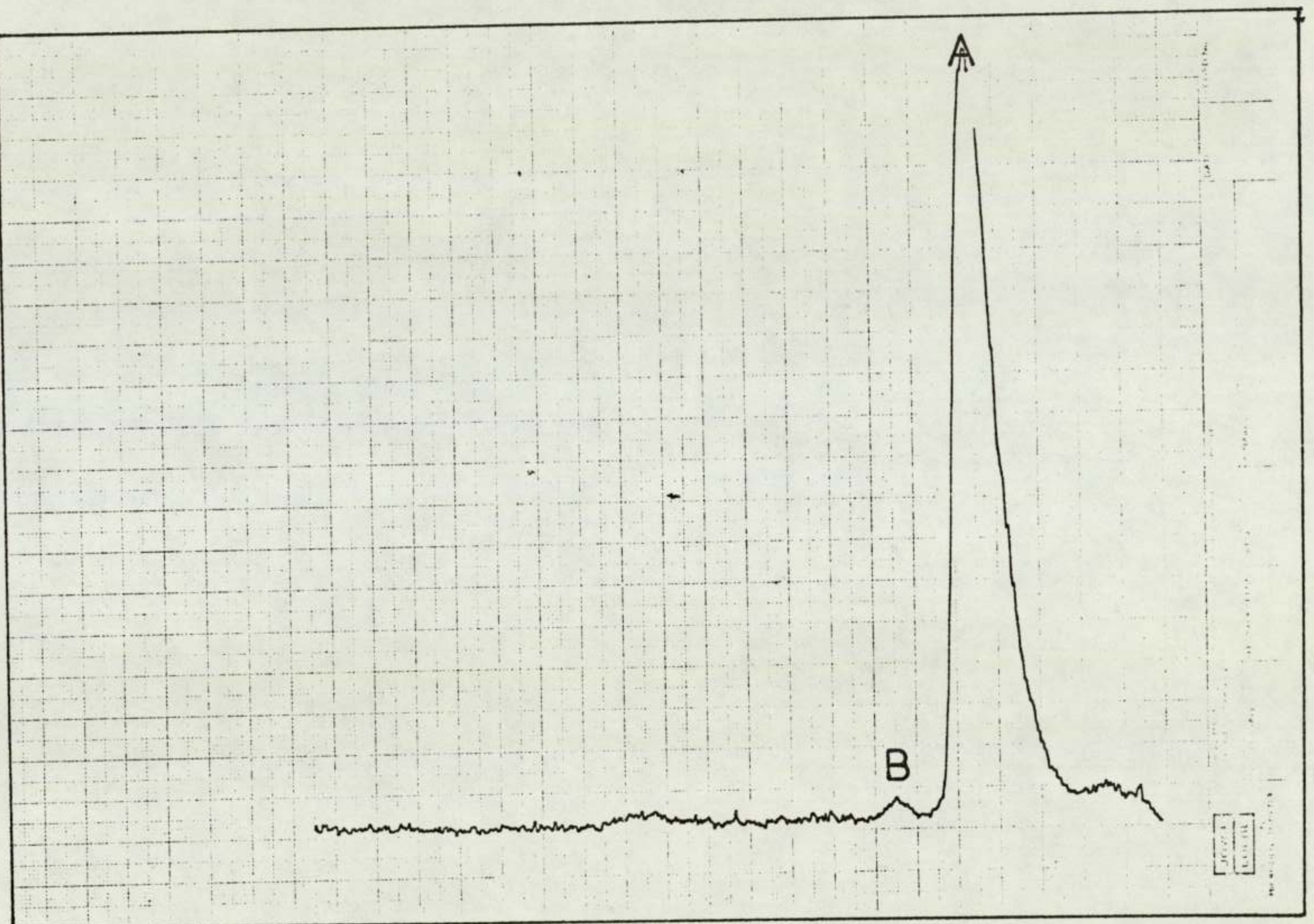


FIGURE II.1. Microdensitometer trace: folic acid before incubation without tissue, t.l.c. run in propanol-ammonia (Solvent 2).

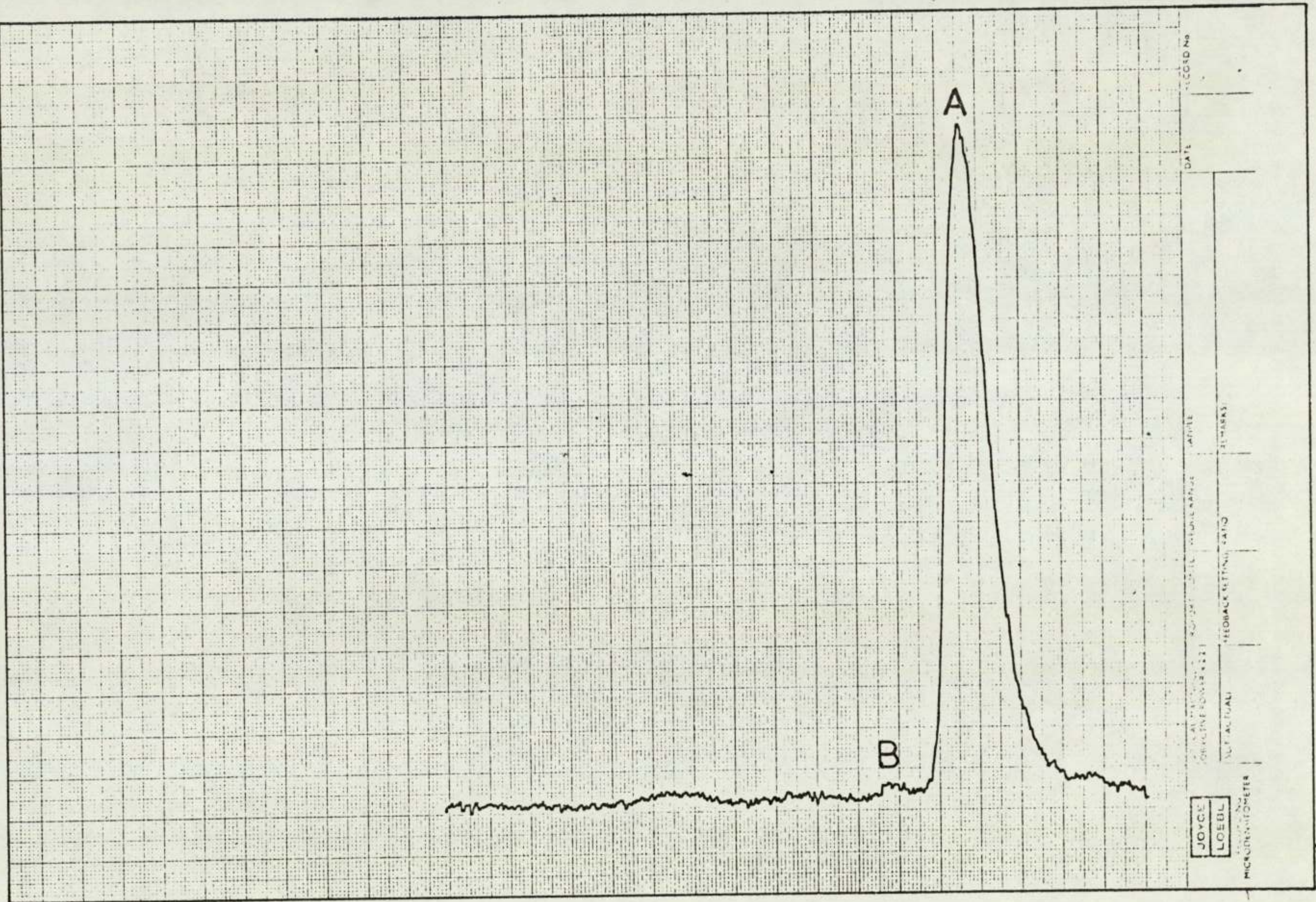


FIGURE II.2. Microdensitometer trace: folic acid after incubation without tissue, t.l.c. run in propanol-ammonia (Solvent 2).

A
B
C
D
E

FIGURE II.3. Microdensitometer trace: folic acid after incubation with tissue, t.l.c. run in propanol-ammonia (Solvent 2).

TABLE II.1.

Solvent System (Section 2, Page 83)	R_f Value Solution III	Beavon 1973 5-Methyl THF.
1	0.4 (folic acid)	
	0.65	
	0.68	0.68
2	0.2 (folic acid)	
	0.3	
	0.43	
	0.55	0.55
	0.68	
3	0.48 (folic acid)	
	0.58	
	0.85	0.85

APPENDIX III.

In vitro viability; transmural potential and oxygen uptake.

i) 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.

During the development of the techniques described in Section 2 a series of potential difference measurements were made, principally in order to compare the present techniques with those of other workers and thus ensure that the treatment of the tissue, particularly the eversion process, did not result in undue damage.

Methods

The technique used in the present study was similar in principle to that of Barry et al (1964) though using a simplified apparatus.

Cannulated everted sacs were prepared as described in Section 2. The mucosal solution was continuously gassed as usual, with 5% CO_2 in O_2. The potential difference was led away from either side of the gut wall by means of polythene tubes containing

KCl/Agar jell. The potential measurements were made on a Vibron voltmeter via calomel half cells. The usual procedure was to prepare a single 10 cm. sac from the upper jejunum of each animal and record the highest stable potential obtained during a 20 minute incubation period; in some experiments the incubation period was extended to 1 hour. A few observations were made using sections of non-everted intestine. All determinations were carried out using the standard incubation medium containing 28 mM glucose.

Results.

The average transmural potential difference obtained from the upper jejunum of 8 rats in this study was 4.9 mv $\pm$ s.e.m. 0.3 mv. Potentials of this order were maintained for periods of at least 60 minutes. In two experiments the transmural potential difference developed by non-everted sacs was measured, there appeared to be no significant difference in the magnitude of the potential developed.

Discussion.

Barry *et al* (1964) reported that the potential difference developed by the upper jejunum was somewhat lower than that observed in the mid-intestine, quoting a value of 5.6 mv. $\pm$ s.e.m. 0.3 mv. in the presence of glucose. The value of 4.9 obtained in the present study is comparable to that reported by Barry *et al*, and could be maintained for incubation periods of at

least 60 minutes. This suggests that the functional integrity of the mucosal epithelium in the everted intestine used in the present study is satisfactory, at least in comparison to that prepared by these other workers.

ii) Oxygen Consumption.

The oxygen consumption of the everted rings and the isolated mucosal cells was measured to provide a further index of functional viability.

Method.

Oxygen consumption was assessed using the Warburg Manometer. The tissues (everted rings or isolated cells) were prepared as described in Section 2, Part 2. Equivalent samples of material from a single animal together with 3.5 mls. of the standard incubation medium, were placed in two standard Warburg flasks which had been accurately calibrated beforehand. The two test manometers and a thermobarometer were set up in the incubator at $37^{o}C$ and equilibrated for 10 minutes under pure oxygen. The flasks were then sealed and shaken for the duration of the experiment at 100 oscillations per minute; simultaneous experimental and thermobarometric readings were taken at 5 minute intervals during the course of the incubation. At the end of the experiment the tissue samples were removed from the flasks and dried overnight at $80^{o}C$ to constant weight. The calibration of the manometers and

flasks, the details of the incubation procedure and the calculation of the results were as recommended by Umbreit Burris and Stauffer (1951).

Results.

The rate of oxygen consumption by everted rings and isolated mucosal cells was as shown in Table IV.1. below. The rate of oxygen uptake was approximately linear over the periods shown.

TABLE III.1.

In Vitro Oxygen Consumption.

	QO_2 (µl/mg dry wt. hr.)	Duration. (min)
Everted jejunal rings	9.5 (3)	90
Isolated jejunal mucosal cells	3.5 (3)	50

Number of animals in brackets.

These results indicate that the tissue preparations maintain aerobic metabolism for periods well in excess of the experimental incubation times of this study.

APPENDIX IV.

Intrinsic folate.

A short study was undertaken in order to measure the levels of endogenous folate appearing in the mucosal and serosal solutions during the incubation of everted sacs.

Method.

Everted sacs were prepared as usual. Incubations were carried out for 60 minutes in the standard incubation medium under the usual conditions. At the end of the incubation the sacs were drained, and the mucosal and serosal solutions, after the addition of 2 mg./ml. of ascorbic acid to prevent oxidation of the folates, were analysed by means of microbiological assay.

Results.

Table IV.1. contains data for the total concentration of microbiologically active folate present in the serosal and mucosal solutions, together with the concentrations of *P. cerevisiae* and *S. faecalis* active material.

Discussion.

Clearly there is a significant concentration of folate in the intestinal tissue, and this is released into the medium during incubations *in vitro*. It should be noted that though the total efflux is approximately

the same on either side of the gut wall, the concentration developed in the serosal solution is several times higher than that in the mucosal side. This is of considerable importance in any study of intestinal folate metabolism and transport carried out at low concentrations, and using microbiological assay since, as has been repeatedly pointed out (Leeming, Portman-Graham & Blair, 1972; Blair & Beavon, 1973; Blair _et al_, 1973; Elsborg, 1974) it may lead to confusion as to the origin, identity and concentration of the transported material.

TABLE IV.1.

Endogenous Folate Levels.

	Folate Concentrations (ng/ml ± s.e.m.)		
	Total Folate	*P. cerevisiae*	*S. faecalis*
Serosal Solution (3)	24.3 ± 2.0	5.1 ± 0.6	8.3 ± 1.2
Mucosal Solution (3)	3.7 ± 0.3	0.6 ± 0.05	1.4 ± 0.05

Number of animals in brackets. Each animal value is derived as a mean of 2 sacs.

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J. Physiol. (1974), **236**, *pp.* 653–661
With 5 text-figures
Printed in Great Britain

ABSORPTION OF FOLIC ACID BY EVERTED SEGMENTS OF RAT JEJUNUM

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(*Received 9 August 1973*)

SUMMARY

1. Everted rings of rat intestine were used to study the initial uptake rate of folic acid at various concentrations and incubation temperatures *in vitro*.

2. Folic acid was accumulated linearly for periods of at least 30 min.

3. The initial uptake rate was found to reach a constant value as the concentration of folic acid in the incubation medium was increased above 5×10^{-6} M.

4. Reducing the temperature of incubation from 37 to 27° C gave small Q_{10} values at either end of the concentration range.

5. Transfer of folic acid into the serosal compartment of everted sacs was shown to undergo a rate reduction in the same concentration range.

6. A mechanism for folic acid transport is suggested in which folic acid is converted to the neutral species at the mucosal surface in an acid microclimate.

INTRODUCTION

The mechanism for the transport of folic acid across the jejunum has not yet been established. Many studies have been reported (quoted in Smith, Matty & Blair, 1970), some of them conflicting and much controversy has ensued. Smith *et al.* using rats (1970) observed an elevated concentration within the tissue, and that the total transport of folic acid across the mucosal membrane was a saturable process, saturation occurring between 10^{-6} and 10^{-5} M concentration. More recently Smith (1973) has suggested that in the rat there is a rate limiting uptake into the mucosa and a separate passive transport into the serosal fluid. Halsted & Mezey (1972) have claimed that the mucosal uptake of folic acid in the rat is a linear function of concentration from $5{\cdot}7 \times 10^{-8}$ to $5{\cdot}7 \times 10^{-4}$ M.

The present studies were undertaken to provide more information on

folic acid uptake. It was hoped by using everted intestine segments, short incubation times, and by measuring the initial rates of uptake, to obtain more precise kinetic data than hitherto.

METHODS

The animals used were male Wistar rats between 180 and 210 g weight. The five rats in each experimental group were used on the same day, having been starved for about 18 hr overnight to clear the upper intestine of faecal material.

Animals were killed by a single blow on the head followed by immediate decapitation with scissors. The abdomen was opened and the entire small intestine was removed and transferred to a beaker of oxygenated saline, 0·9 % NaCl, at 0–4° C. The lumen was flushed through with 50 ml. of the same solution, and the intestine was everted according to the method of Wilson & Wiseman (1954). The first 2–3 cm of jejunum were discarded, and the next 40 cm of upper intestine were cut, using scissors on a cooled glass plate, into rings or segments about 2 mm in length.

The segments were incubated in Krebs phosphate Ringer containing glucose (28 mM) and appropriate concentrations of cold and [2-^{14}C] folic acid. The material from a single rat was randomized by gentle swirling, and then divided amongst 5, 25 ml., conical flasks each containing 5·0 ml. of incubation medium, and gassed continuously with 5 % CO_2 in O_2. The flasks were maintained at 37° C in a Griffin 100 series water-bath shaking at 100 oscillations per minute.

Samples were removed from the bath at appropriate intervals, washed with approximately 100 ml. chilled saline in a small Hirsch funnel, gently blotted, and transferred to filter-paper buckets of known weight. Each sample was then freeze-dried to constant weight and assayed by a modification of the method of Kalberer & Rutschmann (1961). Each bucket was completely burnt in O_2, and the radioactive CO_2 thus liberated was absorbed in 14 % mono-ethanolamine in methanol solution, and counted in toluene scintillator (2,5-diphenyloxazole (4 g) and 1,4-di-[2-(5 phenyloxazolyl)]-benzene (100 mg) dissolved in 1 l. A.R. toluene) in a Nuclear Enterprises scintillation spectrometer. Absolute activities were determined using ^{14}C hexadecane as an internal standard.

For serosal transfer studies the upper jejunum was cut into six sacs of 6 cm length, each of which was filled with 0·5 ml. of the Krebs Ringer–Phosphate buffer containing 28 mM glucose. The sacs were incubated for periods of 30 min in similar solutions to those used in the tissue accumulation studies. After incubation the sacs were washed and drained, and 100 μl. aliquots of the serosal solution were counted directly in liquid scintillator.

[2^{14}-C]folic acid and ^{14}C hexadecane were obtained from the Radio-Chemical Centre, Amersham. Scintillation grade toluene, 2,5-diphenyloxazole PPO, and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) were obtained from Koch Light Laboratories. Aqueous solutions were counted in NE 220 scintillator from Nuclear Enterprises Ltd.

Tissue viability

The physiological condition of the material was assessed by several means. Everted sacs, prepared by a similar initial method, were incubated in the absence of folic acid, and their ability to transport water was observed, as well as their ability to support a glucose stimulated transmural potential difference.

The rate of O_2 consumption by the segments was observed using the Ringer-phosphate medium under 100 % O_2 in a Warburg manometer.

RESULTS

Everted sacs from seven animals were found to transport water at a rate of 0·64 ml./g wet wt.h ± S.D. 0·16. Similar sacs, cut from the proximal jejunum of eight animals were found to support mean transmural potentials of 4·9 mV ± S.D. 0·85 in the presence of 28 mM glucose, for periods of at least 60 min. O_2 consumption by segments was found to be nearly linear over 90 min at a rate of 9·5 μl./mg dry wt.hr.

The radioactivity of the segments was used to calculate the absolute rate of accumulation, and this was found to be linear for at least 30 min. Fig. 1 shows the result of a typical experiment. Folic acid content, expressed as n-mole/g dry wt., is plotted as a function of time. The external folic acid concentration in this experiment was $1{\cdot}2 \times 10^{-6}$ M. Each point represents the mean of five experiments, and errors are expressed as S.E.

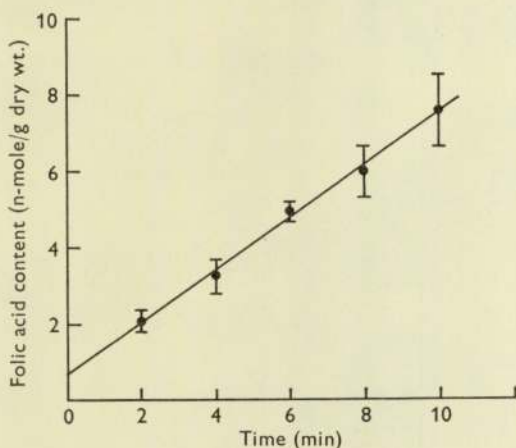

Fig. 1. Rate of accumulation of folic acid by isolated segments of rat intestine at a concentration of $1{\cdot}2 \times 10^{-6}$ M. Each point represents the mean and S.E. of five determinations.

of the mean. Fig. 2 shows the results of similar experiments performed over a range of external folic acid concentrations. The rates of uptake were determined by the line of best fit, calculated by the least-squares method, and no result was accepted which did not give a correlation coefficient of better than 0·98.

In another set of experiments, incubations were performed at 27° C at concentrations of 10^{-5} M and $0{\cdot}8 \times 10^{-7}$ M, and at 0° C at 10^{-5} M. The results are given below. Although folic acid was accumulated at 0° C, the results were found to be erratic and gave a poor fit to a straight line.

Folic acid concn. (M)	Temp. °C	Uptake of folic acid n-mole/g dry wt. min
1×10^{-5}	27	0·85
$0{\cdot}8 \times 10^{-7}$	27	0·02

At concentrations of 1×10^{-5} M and $0{\cdot}8 \times 10^{-7}$ we have obtained a Q_{10} value of 2·0.

The results of our observations on serosal transfer are given in Fig. 3. The movement of the folic acid into the serosal solution is expressed as n-mole/g dry wt. of sac. 30 min. Each point represents the mean of several observations and the appropriate value is given beside each point together with the S.E. of the mean.

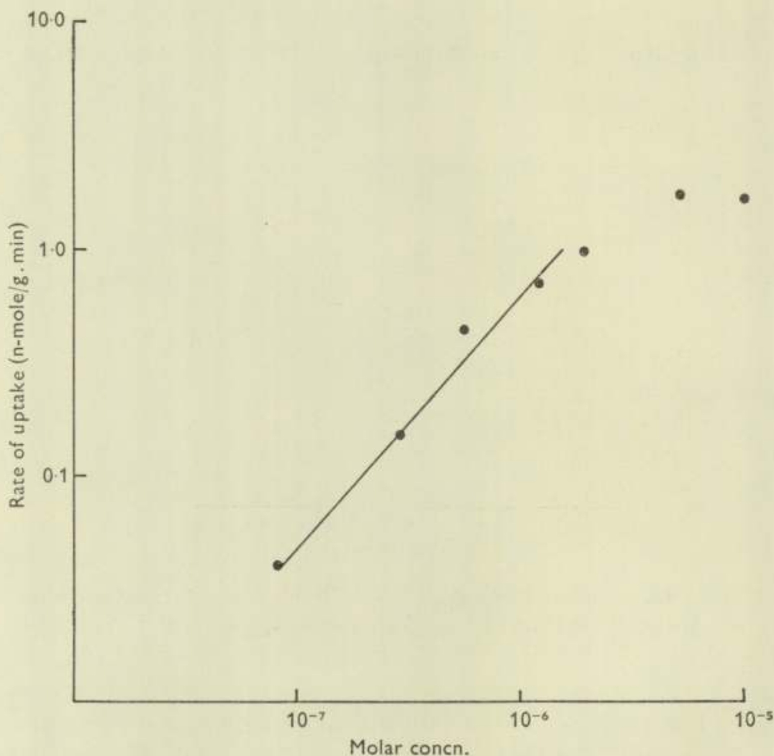

Fig. 2. Variation of initial uptake rate with concentration of folic acid.

DISCUSSION

Our results describe the accumulation of folic acid during the early stages of incubation when the metabolic status of the preparation approximates to that of the *in vivo* intestine. We have shown that during the

period when the tissue seems to be physiologically viable, folic acid is accumulated linearly for at least 30 min, and we have studied the way this initial rate of uptake varies with the external concentration.

It can be seen that the initial uptake rate increases linearly with concentration up to about 5×10^{-6} M, but at higher concentrations the increase in rate is sharply depressed and becomes 0 at 10^{-5} M, indicating saturable transport. That this rate change is a characteristic of the overall transport process is demonstrated by our observations on everted sacs, which show a marked reduction in serosal accumulation at similar concentrations.

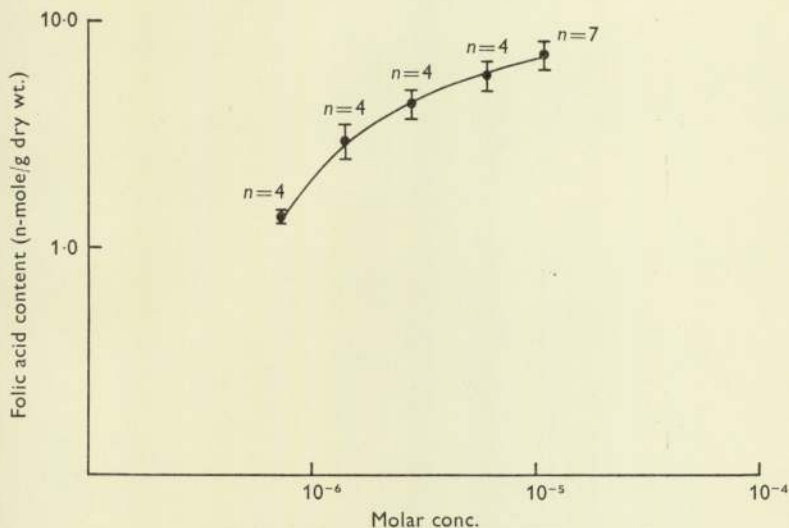

Fig. 3. Rate of serosal accumulation by everted sacs as a function of mucosal concentration. Each point represents the total serosal transfer after 30 min, expressed in terms of dry weight of tissue.

In general, these results confirm earlier *in vitro* studies carried out in this department (Smith *et al.* 1970) which showed that the accumulation of folic acid by everted sacs was saturable at mucosal concentrations between 10^{-6} and 10^{-5} M. Burgen & Goldberg (1962) also demonstrated a saturable uptake, although at a rather higher concentration, using an *in vivo* technique. Workers who have failed to report this saturation effect (Turner & Hughes, 1962; Yoshino, 1967; Hepner, Booth, Cowan, Hoffbrand & Mollin, 1968) seem to have worked in the low concentration range where we also have observed linear uptake.

We have attempted to fit our data to Michaelis–Menten kinetics, but both the Lineweaver–Burke plot (see Fig. 4) and the Hofstree–Edey plot (Dowd & Riggs, 1965) give a poor fit (Fig. 4). Our temperature data give

low Q_{10} values which seem to argue against an enzymatic or carrier process being directly involved in the initial transport step.

Although Burgen & Goldberg (1962) have claimed to show unequivocal evidence for active transport, this conclusion has never been confirmed. Indeed Leslie & Rowe (1972) have shown that isolated epithelial cells which are capable of actively accumulating leucine, show no uptake of folic acid other than a rapid binding effect. Our problem of interpretation then, is to reconcile the existence of a saturable uptake mechanism in whole tissue, with the lack of any of the other indications of an active carrier process.

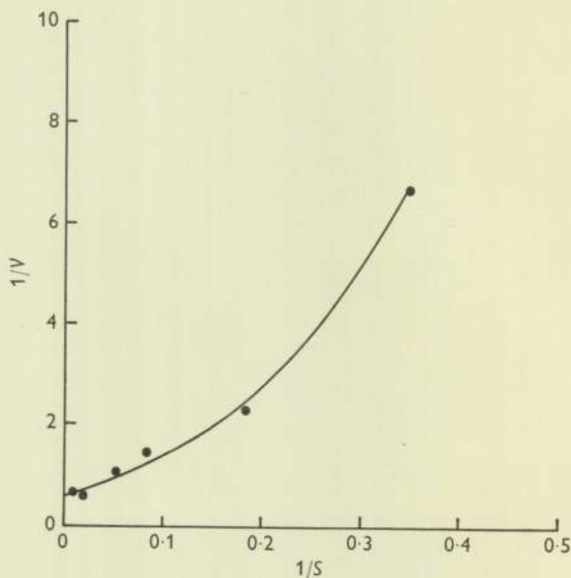

Fig. 4. Double reciprocal plot of folic acid accumulation rate ($\frac{1}{V}$) versus concentration ($\frac{1}{S}$) illustrating the deviation from a linear relationship.

Schanker and his co-workers Schanker, Tocco, Brodie & Hogben (1958) demonstrated that the rate of absorption of a drug *in vivo* is related to its dissociation constant, and that acid drugs are transported in the unionized form. Further work showed that the rapid absorption of a species such as salicyclic acid, which is strongly ionized at normal intestinal pH, could be explained by the presence of a region of relatively high acidity at the mucosal surface in which the neutral molecule predominated. They suggested that such a 'microclimate' would have a pH about 2 units more acid than the bulk intestinal medium and would vary only slightly, in response to gross changes in the bulk phase.

Folic acid is known to occur as the neutral species only at low pH.

A solution at pH 2·8 will consist wholly of undissociated molecules, whereas at pH 5 only 1% of the species will remain unionized. Assuming the rate of transport to be proportional to the concentration of neutral species at the mucosal surface, and that diffusion across the lipoidal membrane is the rate-limiting step, then we would expect a linear increase in absorption rate in response to increased concentration, until the neutral species reached saturation solubility in the microclimate region. Such a relationship might follow the idealized form shown in Fig. 5. From our data, the maximum rate of uptake seems to occur at about 5×10^{-6} M and from the solubility data of Biamonte & Schneller (1951) this would suggest a local pH of between 3 and 4.

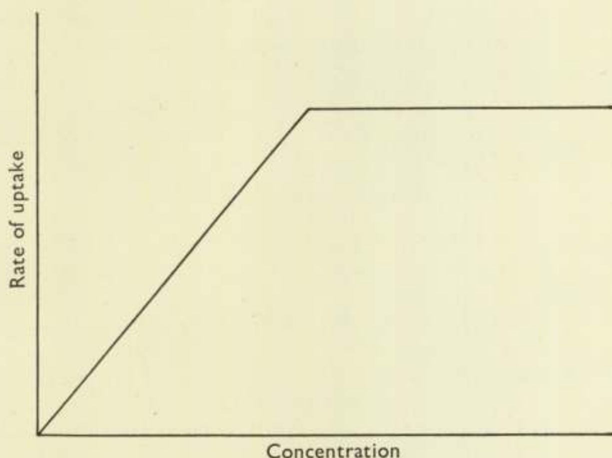

Fig. 5. Theoretical form of rate–concentration graph for passive transport via a saturable mucosal microclimate.

The existence of unstirred layers at the surface of membranes has been widely accepted since the work of Dainty (Dainty & House, 1966), and the intestinal epithelial cell is known to possess a specialized surface structure, the glycocalyx, which as Parson & Boyds (1973) have recently pointed out, is the immediate microenvironment in which transport is initiated, and which is almost certain to display highly specialized conditions. That the rat jejenum has a mechanism for the acidification of alkaline buffers, both *in vivo* and *in vitro*, has been known for some time (Pouz & Larralde, 1950; Foerster, Erdlenbruch & Mehnert, 1967; Wilson, 1953, 1954) and Blair, Lucas & Matty (1972) have suggested that this is due to the activity of mucosal ATPases. The maintenance of such an acid microclimate would be dependent upon the metabolic health of the intestine and, no doubt, the actual pH value would vary with the experimental conditions. This may well account for the observed unreliability of our data at 0° C, and for the

lack of agreement amongst various workers as to the saturation concentration of folic acid transport.

Other work, presently in preparation in this department, will confirm the findings of Strum and his co-workers (Strum, Nixon, Bertino & Binder, 1971) that the transport of 5-methyltetrahydrofolic acid is not a saturable process, and will show that this accords with the solubility and dissociation characteristics of this compound at physiological pH values.

We conclude then that our results can be well accounted for by the existence of an acid microclimate at the mucosal surface of the jejunum which allows folic acid to be absorbed passively as the neutral species. We feel this observation will do much to resolve the current controversy in the folate transport field, as well as provide a valuable indication of conditions prevailing in the immediate vicinity of so many other transport processes.

We wish to thank The Wellcome Trust for the scholarship which supported Mr Johnson in this work.

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