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THE TRANSPORT OF PTEROYLGLUTAMIC ACID ACROSS THE SMALL INTESTINE OF THE RAT

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SUMMARY

1. The absorption of [^{3}H]pteroylglutamic acid was studied using the everted sac technique. Sacs prepared from the portion of the gut between 7 and 70 cm from the pylorus transported pteroylglutamic acid at similar rates, but sacs prepared from the more distal region of the small intestine were less effective in this respect.

2. Pteroylglutamic acid transported into the solution on the serosal side of jejunal sacs was present largely in an unaltered form.

3. When the pteroylglutamic acid on the mucosal side of the sac was varied over a range of $1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M, a saturatable process was shown to exist in the jejunum. A K_t for this process of $0.7 \cdot 10^{-6}$ M was obtained. Pteroylglutamic acid was concentrated by the tissues with respect to the mucosal solutions when it was initially present in the mucosal solution at $1 \cdot 10^{-7}$ M concentration.

4. With no initial concentration gradient of pteroylglutamic acid, there was no transport to the serosal side against an electrochemical gradient.

5. With a concentration of $1 \cdot 10^{-6}$ M pteroylglutamic acid on the mucosal side of the sac, glucose was found to stimulate pteroylglutamic acid transport and water transport in a parallel manner. In the absence of glucose, pteroylglutamic acid transport was appreciable.

6. At $1 \cdot 10^{-6}$ M pteroylglutamic acid concentration, transport varied with the pH value of the incubating solutions, the optimum pH for pteroylglutamic acid transport being 6.0 and that for water transport 7.0.

7. It is suggested that pteroylglutamic acid is transported by both passive diffusion and solvent drag with the water flow, when it is initially present on the mucosal side of the sac at $1 \cdot 10^{-6}$ M concentration.

INTRODUCTION

Folate is known to be readily absorbed in man and other mammalian species. Pteroylglutamic acid is only one component of the dietary folate but it has been used successfully in the treatment of folate deficiency. Many workers have studied the transport of pteroylglutamic acid and its derivatives in the small intestine, but the mechanism of its transport is still a matter of controversy. Several workers using the everted sac technique of WILSON AND WISEMAN[1] in the rat[2] and the hamster[3] or a technique involving intragastric administration in the rat[4], have suggested

that folic acid is absorbed by passive diffusion. Others, using everted sacs of rat intestine[5,6] or hamster intestine[7,8] have produced evidence to show that it is transported by an active process. A similar conclusion was reached from a study conducted *in vivo* in the rat[9]. It has also been suggested that pteroylglutamic acid is absorbed by an active process in man[10,11]. Indications have been obtained that pteroylglutamic acid undergoes metabolic change during absorption in the hamster[8] and in man[12], but this claim has been disputed for man[13].

In this study, the processes whereby pteroylglutamic acid is transported in the small intestine of the rat were examined and characterised and the metabolic fate of this vitamin was investigated. [^{3}H]Pteroylglutamic acid and the everted sac technique were used. The information gained in this study can be used to aid in the understanding of folate malabsorption found in clinical conditions such as coeliac disease and tropical sprue. Part of this work was presented to the British Society of Gastroenterology.[14]

MATERIALS AND METHODS

[^{3}H]Pteroylglutamic acid (potassium salt) was obtained from the Radiochemical Centre, Amersham, Great Britain. Solutions of [^{3}H]pteroylglutamic acid were freeze-dried for storage, and kept at —40° and used within a few weeks. The radioactively labelled compound was diluted with unlabelled pteroylglutamic acid (from Koch Light Laboratories, Colnbrook, England) to give the concentrations required. The purity of both the [^{3}H]pteroylglutamic acid and the unlabelled pteroylglutamic acid was verified by thin-layer chromatography as described below.

Preparation and incubation of everted sacs

Male rats, Wistar strain, weighing approx. 170 g were starved for 24 h and killed by a blow on the head. The entire length of intestine was removed and washed through with ice-cold saline until the washings were clear. The intestine was everted and sacs were prepared by the method of WILSON AND WISEMAN[1]. The sacs were taken from the jejunum, starting 7 cm from the pylorus, each sac being 7 cm in length. The sac was blotted on filter paper, weighed empty, and then filled with 0.4 ml of incubating solution and weighed again. The sac was dropped into 5 ml of incubating solution in a 25-ml conical flask and the flask gassed with O_2–CO_2 (95:5, by vol.) for 45 sec. The incubating solutions were Krebs bicarbonate saline containing 11 mM glucose or a modified phosphate buffer containing 0.11 M sodium phosphate buffer (pH 6.2), 35.7 mM NaCl, 5.5 mM KCl, 1.8 mM $MgSO_4$ and 11 mM glucose. When the latter solution was the incubation fluid, no pH change could be observed in the solutions after incubation. The flask was shaken for 30 min in a water bath at 37° and the sac removed, blotted and weighed. It was then opened and the contents allowed to drain into a test tube and the empty sac reweighed. The sac was then rinsed in distilled water and homogenised in a final volume of 3 ml distilled water.

Determination of pteroylglutamic acid

Aliquots of the serosal solution, the mucosal solution or the homogenate were treated with trichloroacetic acid (final concentration 5 %) and the solution centrifuged.

Aliquots of the supernatant liquid were taken for determination of radioactivity in a liquid scintillation counter. Recoveries of pteroylglutamic acid in these experiments were greater than 95 %. Results are expressed as nmoles pteroylglutamic acid transported (into the tissues and the serosal solution) per g initial wet weight of sac.

Analysis of pteroylglutamic acid solutions and transported folate

Solutions containing folate were analysed by thin-layer chromatography on MN 300 cellulose powder (Machery Nagel & Co., 516 Düren, Germany). The solvent systems used were *n*-propanol–1 % aqueous ammonia (200:100, by vol.) and 0.1 M phosphate buffer (pH 7.0) containing 1 % redistilled mercaptoethanol. Folate solutions to be analysed were concentrated by freeze-drying, and unlabelled pteroylglutamic acid added as a carrier. The separations were carried out in the dark at 8°. The radioactive spots were detected with a Desaga thin-layer scanner. Quantitative estimations were made by eluting the radioactive material with 10 % ammonia and counting aliquots of the eluate in a liquid scintillation counter.

Measurement of the electrical potential across the jejunum

The electrical potential difference across the wall of the everted sac was measured by a modification of the method of BARRY *et al.*[15]. The everted sac was tied at one end and the other end was tied over the neck of a polythene funnel (4 cm diameter). The sac was filled with 0.4 ml of incubating solution and suspended in a test tube full of the same solution. Polythene tubes (1.5 mm diameter) containing 4 % agar in saturated KCl acted as salt bridges which led to calomel half cells which were connected with a Dynacap pH meter. One tube was inserted down the neck of the funnel into the sac and the other was introduced into the mucosal solution through a hole in the side of the funnel. Another polythene tube dipped into the mucosal solution through a hole in the side of the funnel and was used for gassing the mucosal solution with O_2–CO_2 (95:5, by vol.). The test tube was incubated in a water bath at 37° and the potential difference measured at 5-min intervals.

Viability of everted sac preparations

The viability of the everted sac preparations in the incubation solutions used was checked by measuring the potential difference across them and their respiration after incubation for 1 h in the incubating solutions. Measurements of the potential difference for sacs incubated in Krebs bicarbonate saline containing 11 mM glucose gave values of 3.2 ± 0.5 mV (5 sacs) and measurements for sacs incubated in the modified phosphate buffer gave values of 5.5 ± 0.5 mV (6 sacs). Respiration after incubation in these solutions for 1 h was followed using a Warburg manometer. Q_{O_2} values for sacs incubated in the Krebs bicarbonate and the phosphate buffer were 4.7 ± 0.3 (18 sacs) and 4.7 ± 0.3 (6 sacs), respectively. Values are given with the standard error of the mean.

RESULTS

Site of pteroylglutamic acid transport in the small intestine

The small intestine between 7 and 84 cm from the pylorus was examined in 6 rats for site of pteroylglutamic acid transport. The odd numbered intestinal

sacs (7 cm in length) were taken from 3 rats and the even numbered sacs from 3 rats. The order in which the sacs were incubated was randomised so that any variation in the rate of transport with distance from the pylorus could not be ascribed to the effect of keeping the sacs for different time periods prior to incubation. It has been shown by several workers[16–19] that the pH of solutions introduced into the lumen of the small intestine tends to alter until a value is reached which is characteristic of the part of the small intestine under investigation. Since the transport of pteroylglutamic acid varies with the pH of the bathing solutions, a solution was chosen for these experiments where the pH change during incubation was negligible. This solution was 0.11 M sodium phosphate buffer (pH 6.2) containing 35.7 mM NaCl, 5.5 mM KCl, 1.8 mM $MgSO_4$ and 11 mM glucose. Pteroylglutamic acid was present initially on the mucosal side at a concentration of $1 \cdot 10^{-6}$ M, and at zero concentration on the serosal side. Fig. 1 shows that sacs taken between 7 and 70 cm from the pylorus transported pteroylglutamic acid at a similar rate, but sacs taken from the more distal part of the small intestine were less effective in this respect.

Metabolic fate of pteroylglutamic acid on transport

To investigate the possibility of metabolism on transport in the rat, everted sacs were incubated for 1 h at 37° with [^{3}H]pteroylglutamic acid on the mucosal side at a concentration of $1 \cdot 10^{-7}$ M or $1 \cdot 10^{-6}$ M and zero concentration on the serosal side, and the serosal solution was analysed for radioactive components at the end of the incubation. Thin-layer chromatography using either *n*-propanol–1 % aqueous ammonia (200:100, by vol.) or 0.1 M phosphate buffer (pH 7.0) containing 1 % mercaptoethanol as the solvent system was employed for analysis of the serosal solution. Using the *n*-propanol–ammonia system, one band of radioactivity was

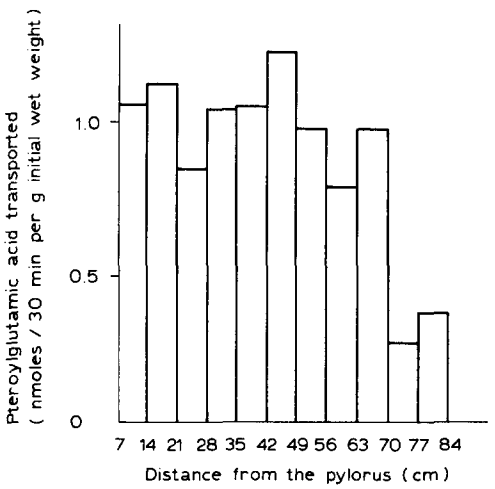

Fig. 1. Site of pteroylglutamic acid transport in the small intestine of the rat. Sacs were incubated in 0.11 M sodium phosphate buffer (pH 6.2) containing 35.7 mM NaCl, 5.5 mM KCl, 1.8 mM $MgSO_4$ and 11 mM glucose. Pteroylglutamic acid was present on the mucosal side of the sac only. The initial concentration was $1 \cdot 10^{-6}$ M and incubation was at 37° for 30 min. Each column represents the mean of 3 values. 6 animals were used. The transport of pteroylglutamic acid is given as total net transport across the mucosal surface (tissue uptake + transport into the serosal solution).

observed. The radioactive material cochromatographed with unlabelled pteroylglutamic acid and had an R_F value of 0.1. Using a similar system BLAIR AND SAUNDERS[20] obtained good separations of pteroylglutamic acid, 5-formyltetrahydropteroylglutamic acid and 5-methyltetrahydropteroylglutamic acid, with R_F values of 0.15, 0.36 and 0.55, respectively. When the phosphate buffer solvent system was employed in our experiments, a major band of radioactive material with an R_F value of 0.4 was observed. This material cochromatographed with pteroylglutamic acid. A minor radioactive component with an R_F of 0.8 was also observed but was not identified. In this sytsem an R_F value of approx. 0.8 was observed for both 5-formyltetrahydropteroylglutamic acid and 5-methyltetrahydropteroylglutamic acid markers. These compounds gave a clear separation from radioactive and unlabelled pteroylglutamic acid. The behaviour of polyglutamate derivatives of pteroylglutamic acid in these systems was not studied, but other workers using the phosphate buffer system with paper chromatography have achieved good separations of pteroylglutamic acid from the diglutamate and triglutamate derivatives of pteroylglutamic acid[21,22].

Variation with pteroylglutamic acid concentration

Jejunal sacs were incubated with various concentrations of pteroylglutamic acid on the mucosal side of the sacs and zero concentration on the serosal side. The incubating solution was the phosphate-buffered solution at pH 6.2. It can be seen from Fig. 2a that a saturable process is effective at pteroylglutamic acid concentrations over the range $1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M. Fig. 2b shows the Lineweaver–Burk plot for this range of concentrations. A similar relationship was observed when the sacs were incubated in Krebs bicarbonate buffer. A K_t of $0.7 \cdot 10^{-6}$ M and a maximum velocity of 2.3 nmoles per g wet weight per 30 min were calculated.

Transport with no concentration gradient

When pteroylglutamic acid was present on both sides of the sac in the same concentration ($1 \cdot 10^{-6}$ M), and the incubating solution was the modified phosphate buffer (see MATERIALS AND METHODS), the concentration in the serosal solution after 1 h was greater than that in the mucosal solution. Ratios of 1.31 ± 0.04 (6 sacs) were obtained for the serosal to mucosal concentrations. Measurements of the electrical potential differences across the sac walls in the same 6 experiments gave values of 3.4 ± 0.7 mV after 1 h. Calculations of the ratios of serosal to mucosal concentrations of a dianion at equilibrium using this value for the potential difference were made using the Nernst equation. A ratio of 1.29 was calculated, giving good agreement with that obtained by measurement. Thus there was no transport of pteroylglutamic acid against an electrochemical gradient. When Krebs bicarbonate saline (pH 6.0) with 11 mM glucose was the incubating medium, increased serosal to mucosal concentrations were observed at $1 \cdot 10^{-7}$ M concentration of pteroylglutamic acid but not at $1 \cdot 10^{-6}$ M concentration (see Table I). Although increased serosal to mucosal concentrations were obtained at $1 \cdot 10^{-7}$ M concentration in this medium, there was no net transport of pteroylglutamic acid from the mucosal to the serosal solution. The increased ratios were due to a more rapid uptake of pteroylglutamic acid into the tissues from the mucosal side of the sac than from

the serosal side. The tissues were able to concentrate pteroylglutamic acid with respect to both the mucosal and the serosal solutions (see Table I).

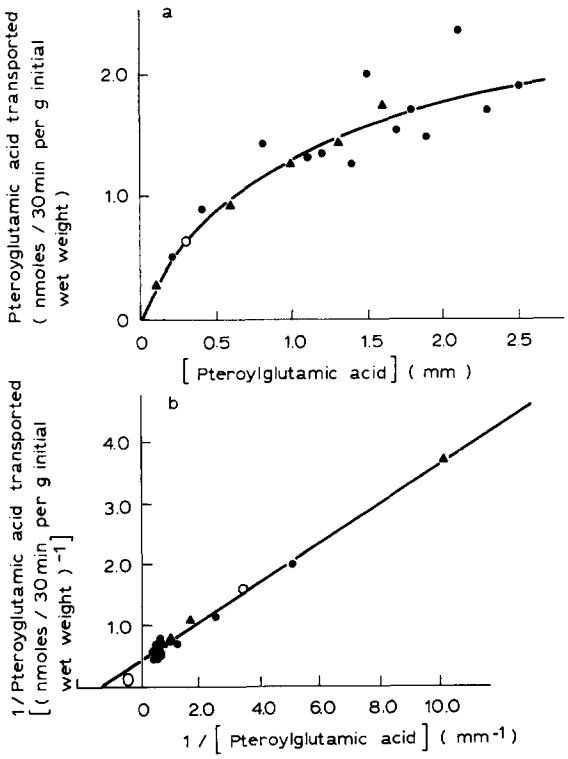

Fig. 2. Variation of rate of pteroylglutamic acid transport with concentration. Jejunal sacs were incubated for 30 min at 37° in 0.11 M sodium phosphate buffer (pH 6.2) containing 35.7 mM NaCl, 5.5 mM KCl, 1.8 mM $MgSO_4$ and 11 mM glucose. a, variation of rate of pteroylglutamic acid transport with increasing pteroylglutamic acid concentration; b, Lineweaver–Burk plot for pteroylglutamic acid transport. ●, single value; ○, mean of 2 values; ▲, mean of 3 values. Transport of pteroylglutamic acid is given as total net transport across the mucosal surface (tissue uptake + transport into the serosal solution).

TABLE I

PTEROYLGLUTAMIC ACID TRANSPORT WITH NO INITIAL CONCENTRATION GRADIENT BETWEEN THE MUCOSAL AND SEROSAL SOLUTIONS

Sacs were incubated in Krebs bicarbonate saline (pH 6.0) containing 11 mM glucose for 1 h at 37°. Serosal and mucosal concentrations in nmoles/ml are compared with tissue concentrations in nmoles/g final wet weight. Values are given as the means with the standard error of the mean and the number of experiments in parentheses.

Initial concn. × 10^7 (*M*)	*Serosal concn.* / *Mucosal concn.*	*Tissue concn.* / *Mucosal concn.*	*Tissue concn.* / *Serosal concn.*
10	0.87 ± 0.05 (6)	0.89 ± 0.11 (6)	0.97 ± 0.11 (6)
1	1.35 ± 0.15 (6)	2.43 ± 0.28 (6)	1.81 ± 0.10 (6)

Effect of glucose

Pteroylglutamic acid transport was appreciable in the absence of added glucose whether Krebs bicarbonate saline or the phosphate buffer was the incubating medium. Fig. 3a shows the effect of various concentrations of glucose on pteroylglutamic acid

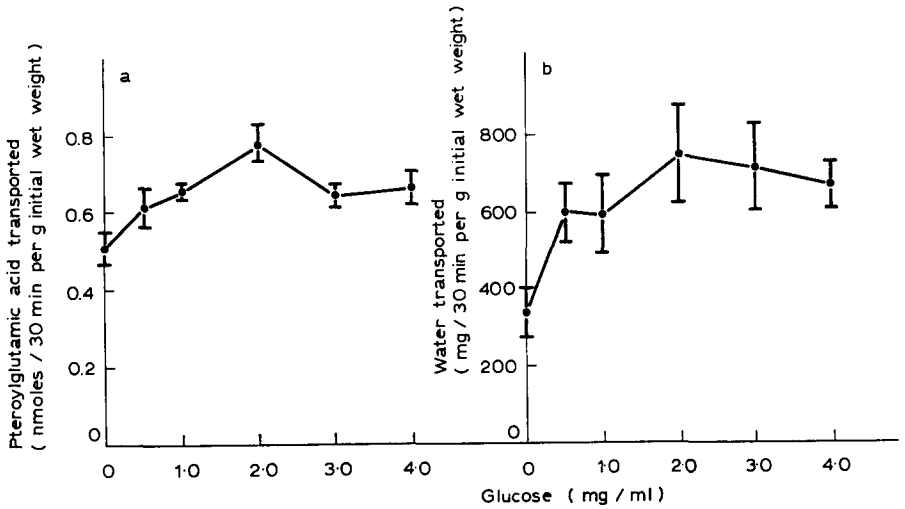

Fig. 3. Effect of glucose on (a) rate of pteroylglutamic acid transport and (b) rate of water transport. Sacs were incubated for 30 min at 37° in Krebs bicarbonate saline. Values given are the means of 6 sacs with the standard error of the mean. Pteroylglutamic acid was present initially on the mucosal side of the sac only, the concentration being $1 \cdot 10^{-6}$ M. Transport is given as total net transport across the mucosal surface (tissue uptake + transport into the serosal solution).

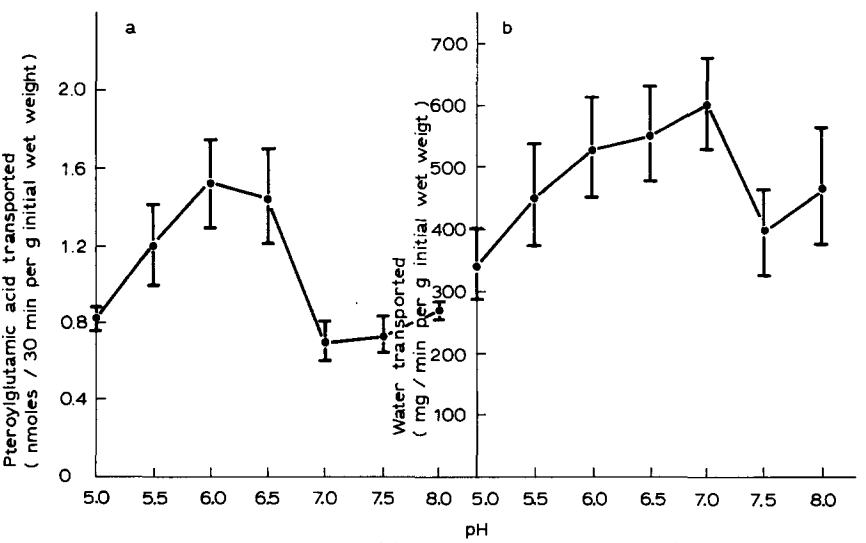

Fig. 4. The effect of pH variation of (a) rate of pteroylglutamic acid transport and (b) rate of water transport. Sacs were incubated for 30 min at 37° in Krebs bicarbonate saline containing 11 mM glucose. The pH of the solutions were adjusted with dilute HCl or dilute NaOH. Values given are the means of 6 sacs with the standard error of the mean. Pteroylglutamic acid was present initially only on the mucosal side of the sac, the concentration being $1 \cdot 10^{-6}$ M. Transport is given as total net transport across the mucosal surface (tissue uptake + transport into the serosal solution).

transport, with Krebs bicarbonate as the incubating medium. Pteroylglutamic acid was initially present at $1 \cdot 10^{-6}$ M concentration on the mucosal side and zero concentration on the serosal side. It can be seen that pteroylglutamic acid transport under these conditions is markedly stimulated by glucose. Water transport in the same experiments was also stimulated in a similar manner (Fig. 3b). These results suggest that pteroylglutamic acid may be transported to some extent by solvent drag with the water flow. When sacs were incubated in the modified phosphate buffer (see MATERIALS AND METHODS), the stimulation of both water and pteroylglutamic acid transport by glucose was far less pronounced than when Krebs bicarbonate saline was the incubating medium.

Effect of pH variation

As mentioned above, when solutions are placed in the lumen of the small intestine, the pH values of the solutions alter, and the direction of the pH change depends on the region of the small intestine under investigation. In our experiments, when everted sacs were made from the jejunum and incubated in Krebs bicarbonate saline, the pH values of both the mucosal and serosal solutions tended to alter to give values in the range 6.5–7.0. This phenomenon was considered to be of importance, since the rate of diffusion of weak electrolytes (in this case pteroylglutamic acid) is known to be affected by changes in the pH of the suspending medium[23,24]. Fig. 4a shows the variation of pteroylglutamic acid transport in the jejunal sacs with the initial pH of the bathing solutions. An optimum pH of 6.0 was observed. Pteroylglutamic acid was initially present on the mucosal side of the sac only, at a concentration of $1 \cdot 10^{-6}$ M. Fig. 4b shows the variation of water transport with pH in the same experiments; a pH optimum of 7.0 was found for this process.

DISCUSSION

Several workers[4–6,9] have demonstrated that pteroylglutamic acid is most efficiently absorbed in the proximal part of the small intestine, but COHEN *et al.*[7] found no variation in the absorptive capacity of different parts of the small intestine. In our experiments pteroylglutamic acid was absorbed over the major portion of the small intestine at similar rates when it was initially present at a concentration of $1 \cdot 10^{-6}$ M (Fig. 1). The reduced rate of transport in the distal part of the small intestine as compared with the more proximal regions could be due to a reduced surface area available for diffusion in the ileum, and the lower rate of water transport in this region[1,25].

It has been suggested that in man[12] pteroylglutamic acid is metabolised in the intestinal mucosa and appears on the serosal side of the gut as 5-methyltetrahydropteroylglutamic acid, although other workers[13] have shown that orally administered pteroylglutamic acid enters the portal blood of man unaltered. It has also been shown that in the hamster[8] pteroylglutamic acid is metabolised to 5-methyltetrahydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid. In the rat we found that the folate appearing on the serosal side of everted sacs after transport was probably unaltered pteroylglutamic acid. This finding does not rule out the possibility that pteroylglutamic acid undergoes metabolic change in the mucosa since the folate retained within the tissues was not analysed. The results suggest,

however, that absorbed pteroylglutamic acid may appear initially in the blood of the rat in an unaltered form, although the possibility of conversion to polyglutamate derivatives could not be ruled out.

Over a range of $1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M pteroylglutamic acid concentrations a saturable process was found to be operating for pteroylglutamic acid transport and a K_t of $0.7 \cdot 10^{-6}$ M was obtained. BURGEN AND GOLDBERG[9], using a perfusion technique also demonstrated a saturable process for pteroylglutamic acid transport in the rat and they obtained a K_s value for this process of $4 \cdot 10^{-5}$ M. The saturable process may indicate a facilitated diffusion or an 'active' process or it may represent the uptake of pteroylglutamic acid onto proteins (or a specific protein, such as an enzyme) in the tissues. GHITIS *et al.*[26] and FORD *et al.*[27] have observed that pteroylglutamic acid and some of its derivatives are absorbed onto milk proteins. Pteroylglutamic acid absorbed onto protein in the tissues of the small intestine would be removed from the osmotic equilibrium between the tissue fluid and the mucosal solution, and the entry of pteroylglutamic acid into the tissues by diffusion would therefore be facilitated at these concentrations. The studies on tissue uptake of pteroylglutamic acid at $1 \cdot 10^{-7}$ M concentration showed that the tissues were capable of concentrating pteroylglutamic acid with respect to the bathing solutions (Table III). When pteroylglutamic acid was present on both sides of the gut membrane at the same initial concentration with either Krebs bicarbonate saline or a phosphate-buffered solution as the bathing fluid, it was not transported to the serosal side against the electrochemical gradient existing across the wall of the jejunum. At $1 \cdot 10^{-7}$ M concentration, the uptake of pteroylglutamic acid into the tissues to give a high tissue concentration relative to the concentrations in the bathing solutions was not reflected in an increased transport into the serosal solution. A study of the uptake of pteroylglutamic acid into the wall of the small intestine will be the subject of another communication.

The stimulation of pteroylglutamic acid transport in Krebs bicarbonate saline by glucose may be attributable to the increased water transport in the presence of glucose if pteroylglutamic acid is transported as a result of solvent drag (Figs. 3a, 3b). The parallel manner in which both pteroylglutamic acid and water are transported indicates that pteroylglutamic acid may be transported by solvent drag with the water flow. In the absence of added glucose pteroylglutamic acid transport was appreciable. Under the latter conditions it is possible that pteroylglutamic acid was transported by passive diffusion and as a consequence of glucose-independent water transport.

When pteroylglutamic acid was initially present in the mucosal solution at a concentration of $1 \cdot 10^{-6}$ M, the rate of transport of this compound was shown to vary with the pH of the incubating medium and an optimum pH of 6.0 was observed (Fig. 4a). Water transport exhibited an optimum pH at 7.0 (Fig. 4b). Since the optimum pH for pteroylglutamic acid transport is at a more acid pH than for water transport, the transport of pteroylglutamic acid may not be exclusively dependent on the transport of water. The rate of transport of a weak acid by passive diffusion would also be influenced by pH and would increase with increasing acidity of the incubating medium[23,24]. It is possible, therefore, that pteroylglutamic acid may be transported to some extent by passive diffusion in addition to transport by solvent drag.

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