Folate Binding by the Brush Border Membrane Proteins of Small Intestinal Epithelial Cells†

Garth I. Leslie and Peter B. Rowe*

ABSTRACT: Isolated small intestinal cells from the rat failed to accumulate folic acid while retaining the capacity to transport leucine. These cells, however, were capable of binding folic acid with a binding constant (K_b) of 3.98×10^{-5}. Examination of the binding phenomenon revealed that there was a broad specificity in binding in that the pteroic acid moiety appeared to be the structural determinant for binding and that this could be modified by either the successive addition of glutamic acid residues or by the nature and position of a variety of one-carbon substitutions. Isolation of the brush border or absorptive region of these cells demonstrated that these membranes contained a protein with a high affinity for folic acid. Sephadex G-150 chromatography illustrated and sodium dodecyl sulfate–polyacrylamide gel electrophoresis confirmed that folic acid is bound by a protein with a molecular weight greater than 100,000, but that, during the solubilization process with 0.2% dodecyl sulfate, polypeptides, retaining the capacity to bind folic acid, ranging in molecular weight from 16,000 to 24,000 are formed. The nature of the extraction procedures employed in both isolating the brush border membranes and in solubilizing the proteins would indicate that the folate-binding protein is firmly embedded in the membrane matrix.

Theories of membrane-active transport are almost universally based on the existence of specific metabolite-carrier proteins which are considered to constitute an integral part of the membrane. The structural organization of these proteins within the lipoprotein matrix is as yet obscure. The currently favored models of membrane structure, however, propose that the proteins are not limited to the exterior, but that at least part of the hydrophobic regions of these molecules extend into the predominantly lipid membrane core. It has in fact been proposed, and recent work by Bretscher (1971) would support the concept, that certain protein molecules extend from one side of the membrane to the other. A molecular orientation such as this would of course be ideal for a molecule with a metabolite-carrier or metabolite-binding function.

Despite the isolation from bacteria of a number of membrane proteins which almost certainly serve a significant role in the active transport of different metabolites (Pardee, 1968), as yet few mammalian membrane proteins have been isolated with this potential property. The membrane proteins of mammalian cells are presently undergoing exhaustive study and a large body of information has been accumulated, particularly with reference to the mammalian red blood cell and the mitochondrion (Kaplan and Criddle, 1971). The major problems confronting researchers in this field are (i) the difficulties in obtaining sufficient quantities of largely homogeneous membrane material from the heterogeneous cell population of a mammalian organ. This is amplified by the problems of isolation of each of the different types of membrane within the eukaryotic cell, (ii) the technological problems surrounding the development of suitable methods for the isolation of proteins from these membranes, the rigorous methods currently in use, possibly destroying or modifying any functional role served by the native protein, and (iii) the relative lack of availability of specific markers for these proteins which will bind firmly enough to withstand subsequent purification procedures.

In man, folic acid is absorbed from the gastrointestinal tract primarily in the proximal small intestine, but in the rat is would appear that the distal small intestine is also involved (Burgen and Goldberg, 1962). A metabolic disorder in man (Lanzkowsky, 1970), resulting from a genetic defect in the absorption of folic acid, would suggest the existence of a specific protein (or proteins) involved in the absorption mechanism for folic acid and its derivatives. It was considered that such a protein might well constitute part of the membranes of the brush border or absorptive region of the small intestinal cell. The attempted isolation of such a protein was rendered feasible by the availability of specifically labeled [3′,5′,9-^{3}H$_3$]folic acid of high specific activity and by the development by Kimmich (1970) of an isolated small intestinal cell preparation suitable for the study of the mechanisms of absorption and transport of different metabolites.

Materials and Methods

Materials. Female Wistar rats weighing between 150 and 200 g were used for all intestinal cell preparations; [3′,5′,9-^{3}H$_3$]folic acid (specific activity 28 Ci/mmole), [2-^{14}C]folic acid (specific activity 55.3 mCi/mmole), and uniformly labeled L-[^{14}C]leucine (specific activity 150 mCi/mmole) were obtained from the Radiochemical Centre, Amersham. Hyaluronidase (type I), bovine serum albumin (fraction V), folic acid, dihydrofolic acid[1] (H_2Fol), tetrahydrofolic acid (H_4Fol), and *p*-aminobenzoylglutamic acid were purchased from the Sigma Chemical Co. Standard protein markers and dansylated amino acids were Schwarz-Mann products. Pteroic acid, 5-HCO-H_4Fol, and 10-methyl-4-aminopteroylglutamic

† From the Department of Child Health, University of Sydney, N.S.W., Australia. *Received October 8, 1971.*

* To whom correspondence should be addressed at the Institute of Child Health, Royal Alexandra Hospital for Children, Camperdown, N.S.W. 2050, Australia.

[1] Abbreviations used are: H_2Fol, 7,8-dihydrofolic acid; H_4Fol, 5,6,7,8-tetrahydrofolic acid; Dilantin, sodium 5,5-diphenylhydantoin; ABzGlu, *p*-aminobenzoylglutamic acid.

acid (Methotrexate) were gifts from Cyanamid-DHA (Dr. Alan Hellestrand). Pure Dilantin was a gift from Parke-Davis and Co. (Dr. Brian Lucas). Folic acid diglutamate and triglutamate derivatives were synthesized in our laboratory by Dr. Martin Silink using the method described by Krumdieck and Baugh (1969); 10-HCO-H_4Fol was synthesized by a method previously described (Rowe, 1968); 5,10-CH_2-H_4Fol and 5-CH_3H_4Fol were synthesized by established methods (Osborne *et al.*, 1960; Keresztesy and Donaldson, 1961). Other chemicals were of reagent grade obtained from regular commercial sources.

Epithelial Cell Isolation (modification of the method described by Kimmich, 1970). The entire length of the small intestine was removed from six rats and washed through with 20 ml of ice-cold 150 mM sodium chloride. The intestine was cut into 1-in. sections and slit lengthwise to form small sheets. These were placed in 100 ml of a standard incubation medium consisting of 120 mM sodium chloride, 3.0 mM dipotassium hydrogen phosphate, 1.0 mM magnesium chloride, 1.0 mM calcium chloride, 1.0 mg/ml of bovine serum albumin, and 20 mM Tris-Cl buffer (pH 7.4). To this was added hyaluronidase (1.0 mg/ml) and incubation was carried out for 30 min at 37° in a New Brunswick Scientific Co. Gyrotory water bath at 100 cycles/min.

The epithelial cells still adherent to the underlying mucosa were removed by gentle agitation with the tip of a fine plastic pipet. Examination of the residual intestinal sheets under a dissecting microscope revealed that the mature cells had been stripped from the villi, while the immature crypt cells were still largely intact. The cell suspension was filtered through fine cheesecloth to remove any fragments of intact mucosa.

The filtrate was centrifuged at 4° at 200*g* for 5 min, the supernatant fluid discarded, and the cell pellet was gently resuspended in 10 ml of ice-cold incubation medium and recentrifuged at 200*g* for 5 min. This process was repeated twice in order to ensure complete removal of the hyaluronidase. The final cell pellet was resuspended in 10–20 ml of incubation medium. The intestine of six rats yielded epithelial cells equivalent to approximately 200 mg of cellular protein. The entire operation occupied about 1.25 hr and the quality of the preparation was assessed by examination under phase-contrast microscopy, by staining with Trypan Blue and by measurement of the glycolytic activity, amino acid transport capacity, and invertase activity. Cell viability was estimated by determining the fraction of the cell population capable excluding 0.2% Trypan Blue (Girardi *et al.*, 1956).

Lactic Acid Production. Cellular glycolytic activity was measured by estimating the production of lactic acid. Isolated cells equivalent to approximately 80 mg of cellular protein were suspended in 10 ml of the incubation medium, supplemented with 5.0 mM glucose, in a 50-ml capacity plastic bottle. Incubation with shaking was carried out at 37°. Duplicate 1.0-ml aliquots were removed at various time intervals and added to an equal volume of 25% trichloroacetic acid. After centrifugation, the lactic acid content of the supernatant fluid was determined by the method of Barker and Summerson (1941).

L-*Leucine Accumulations.* Cells equivalent to 40 mg of protein were suspended in 5.0 ml of the incubation medium, containing 5.0 mM L-leucine and 0.2 μCi of L-[^{14}C]leucine. Duplicate 0.1-ml samples were removed at various time intervals during incubation, with shaking at 37°. These samples were filtered with suction on 25-mm 0.8-μ pore-size Millipore filters and washed with 10 ml of ice-cold 150 mM sodium chloride. The filters were oven-dried and the radioactive content was estimated in a Packard TriCarb liquid scintillation spectrometer with an efficiency of 75%. For this purpose the filters were placed in glass vials containing 10 ml of Instagel (Packard Instrument Co.), in which the filters became transparent

Folic Acid Transport and Binding Studies. These were carried out in a manner identical with the amino acid transport studies, except of course, that folic acid with either ^{3}H or ^{14}C radioactive label was used in the incubation medium over a wide range of concentrations. The counting efficiency in Instagel for samples containing the tritium label was of the order of 45%.

In studies involving the use of potential inhibitors of cell binding of folic acid, the particular derivative being tested was incubated with the cells for 5 min at 37° prior to the addition of the radioactive folic acid. Incubation with shaking was continued for a further 5 min prior to filtering and washing. In the case of the studies with *N*-ethylmaleimide, however, the period of incubation in the presence of this compound was extended to 30 min.

Brush Border Isolation. The brush borders of the suspended intestinal cells were isolated by a modification of the method of Porteus (1969). All the steps in the procedure were carried out at 0–4°. The cells isolated from six rats, equivalent to 200 mg of protein, were suspended in 20 ml of incubation medium and centrifuged at 10,000*g* for 10 min. The cell pellet, gently resuspended in a small volume of medium, was brought to a total volume of 120 ml with 2.5 mM Na_2EDTA (pH 7.0). The suspension was then homogenized in a Sorvall Omni-Mixer at a speed setting of 4 for 45 sec. The homogenate was centrifuged at 1000*g* for 10 min, the supernatant fluid was discarded, and the pellet was suspended in 60 ml of 5.0 mM Na_2EDTA (pH 7.0) using a Sorvall OmniMixer as before. The last two steps were repeated at least twice until the precipitate, under phase-contrast microscopy, contained only free nuclei and brush borders. This precipitate, suspended as before in 60 ml of 5.0 mM Na_2EDTA, was mixed with an equal volume of a solution containing 100 mM potassium orthophosphate, 50 mM potassium citrate, and 154 mM potassium chloride, adjusted to pH 7.0. This mixture was then allowed to stand for 30 min in a 500-ml capacity plastic cylinder. This procedure resulted in the formation of a flocculent precipitate arising from disruption of the nuclei. The turbid supernatant was decanted through fine cheesecloth which retained any of the nuclear sediment. The filtrate was centrifuged at 1000*g* for 10 min and the brush border pellet was again resuspended in 60 ml of 5.0 mM Na_2EDTA (pH 7.0) and carried through the nuclear precipitation procedure again. The final brush border pellet was suspended in 10 ml of 2.5 mM Na_2EDTA (pH 7.0) and precipitated by centrifugation at 1000*g* for 10 min. This pellet was suspended in 1.0–2.0 ml of water. From six rats, yielding approximately 200 mg of cellular protein, an average of 1.5 mg of brush border protein was obtained.

An alternative method of isolating the brush borders (Forstner *et al.*, 1968) was also tried, but resulted in relatively lower yields. There was, additionally, a not insignificant contamination by nuclei and nuclear debris.

Invertase Assays. Assays for invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) was performed by a modification of the method of Fisher and Kohtes (1951). In the modification the glucose released from the sucrose substrate was measured by the *o*-tolidine method of Marks (1959).

Dodecyl sulfate–polyacrylamide gel electrophoresis was

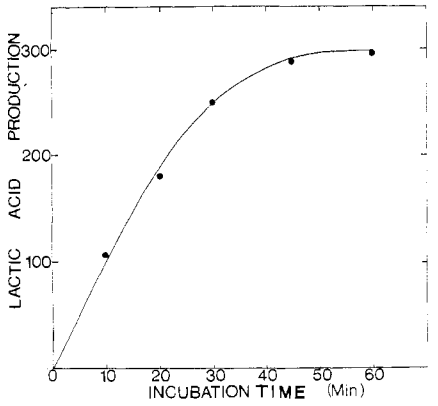

FIGURE 1: Lactic acid production by isolated small intestinal epithelial cells. The results are expressed as nmoles of lactic acid produced per mg of cellular protein. The graph illustrates a representative experiment in which duplicate 1.0-ml samples are removed from the cell incubation mixture at various time intervals.

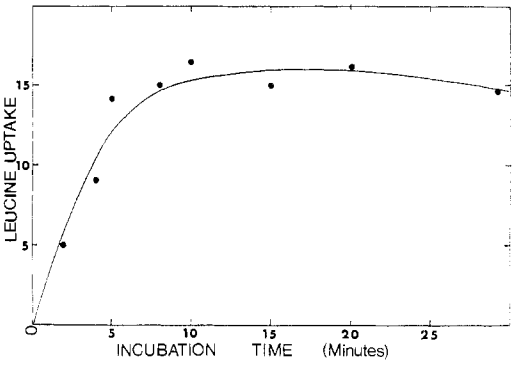

FIGURE 2: Leucine accumulation by isolated small intestinal epithelial cells. Results are expressed as nmoles of leucine incorporated by the cells per mg of cellular protein. The graph illustrates the results of a single experiment with duplicate samples removed at each time point.

used for the determination of the molecular weight distribution of the membrane proteins. A variety of techniques was employed based on those described by Shapiro *et al.* (1967), Weber and Osborn (1969), Ornstein (1964), and Davis (1964). In an attempt to evaluate higher molecular protein bands, 5% acrylamide gels containing 0.1% dodecyl sulfate (6.0 × 0.5 cm) were employed. These gels contained a 30:1 ratio of acrylamide to methylenebisacrylamide. Electrophoresis was undertaken at a range of pH values from pH 8.3 in a 40 mM Tris-glycine buffer, which proved to be the most effective, to pH 4.5 in a 0.9 M sodium acetate buffer. Electrode buffers contained 0.1% dodecyl sulfate. In order to obtain better definition of the lower molecular weight components, use was made of a 12.5% acrylamide gel containing 0.1% dodecyl sulfate and a 20:1 ratio of acrylamide to methylenebisacrylamide. The buffer used was 0.1 M Tris-phosphate at pH 6.8. This method was an adapation of that described by Swank and Munkres (1971), except that 8.0 M urea was not included in the gel. Samples were prepared by standing in ice for 3 hr in the presence of 0.2% dodecyl sulfate and 20 mM β-mercaptoethanol. Marker proteins were subjected to identical treatment.

With both types of gel, electrophoretic runs were carried out at 2.5–3.0 mA/gel for time periods determined by the migration of a Bromophenol Blue dye marker. The average time for a run in a 5% gel at pH 8.3 was 30 min compared to 3 hr for a 12.5% gel at pH 6.8.

At the completion of the run the gels were removed and either (i) fractionated by slicing into 2.5-mm sections and dissolved in Instagel for counting in a scintillation spectrometer or (ii) washed and fixed in 20% sulfosalicylic acid and stained for protein by a modification of the method described by Kaplan and Criddle (1971). In the modified method the gels were stained with 1.0% Amido Black in 40% ethanol and 7.0% acetic acid and subsequently destained in a 40% ethanol and 7.0% acetic acid mixture. The stained gels were scanned at 625 mμ in a Gilford 2410 linear transporter coupled to a Gilford 2400 spectrophotometer, (iii) stained for carbohydrate by the periodic acid–Schiff reaction (Clarke, 1964), or (iv) stained for lipid with 0.5% Rhodamine B in 50% ethanol.

N-Terminal Analysis of Proteins. This procedure was carried out by the dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) method described by Gray (1968). Approximately 500 μg of protein was dissolved in 0.5 ml of 8.0 M urea buffered with 0.5 M sodium bicarbonate. To this was added 0.5 ml of dansyl chloride (20 mg/ml in acetone) and the reaction was allowed to proceed overnight at 25°. Protein which precipitated during labeling was collected by centrifugation at 1000*g* for 20 min and washed once with water. The soluble protein remaining in the supernatant fluid was desalted by passage through a 10.5 × 1.5 cm Sephadex G-25 column. Each protein sample therefore yielded two fractions after dansylation. These fractions were lyophilized, suspended in 1.0 ml of 6.7 N HCl, and hydrolyzed under vacuum for 18 hr at 105°. The hydrolysate was lyophilized and dissolved in the appropriate solvents for thin-layer chromatography in two dimensions on silica gel G thin-layer plates (Deyl and Rosmus, 1965). Three different solvent systems were used for the identification of dansylated N-terminal residues. These were (i) benzene–pyridine–acetic acid (16:4:1, v/v), (ii) chloroform–ethanol–acetic acid (38:4:3, v/v), and (iii) 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v). System i was always employed as the first solvent with system ii used in the second dimension. System iii was used in the second dimension for the identification of dansylated cystine residues.

Protein determinations were carried out according to the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Results

Cell Viability and Metabolic Function. Approximately 90–95% of the isolated cells excluded Trypan Blue immediately after their preparation. At 4 hr some 60% of cells still excluded the dye.

Lactic acid production remained linear for at least 30 min after isolation, but there was an appreciable decrease after this time (Figure 1). The linearity of production was not maintained for as long a period as described by Kimmich (1970) with small intestinal cells from the chicken, but the rate of production was remarkably similar to his reported values over the initial 30-min time period.

Leucine accumulation is shown in Figure 2. The rapid accumulation attaining a maximum at approximately 7 min is in close agreement with data from Reiser and Christiansen (1971), and supports Kimmich's observation on the amino acid transport capacity for this cell system.

Folic Acid Transport. The isolated cells from the rat appeared to fulfil the parameters of performance with respect to metabolic activity, viability, and transport as demonstrated by Kimmich (1970). In view of the demonstrable accumulation by the cells of simple molecules such as leucine, it was of interest to determine whether a far more complex molecule, folic acid, could also be transported. These cells, however, under these conditions, did not apparently accumulate folic acid over a 60-min time interval.

In these experiments the cells were suspended in the standard incubation medium, containing 2.5 μCi of [^{3}H]folic acid/ml with carrier folic acid supplementation where necessary in order to achieve a range of concentrations from the nanomolar to the millimolar level. At neither 4, 25, nor 37° could any increase in the amount of folic acid associated with the cells as a function of time, be demonstrated. At any one concentration of folic acid, the maximum binding to the cells was achieved as soon as rapid mixing occurred, and thus did not vary significantly with time. At a concentration of folic acid of 1.85×10^{-5} M in the incubation medium, the cells bound 16.0 pmoles/mg of protein.

Binding of Folic Acid. The binding of folic acid by the cells was a saturable process and a Lineweaver–Burk plot revealed a binding constant (K_b) of 3.98×10^{-5} M. This binding of folic acid could be related to specific binding sites which anatomically one would expect to be related to the membranes of the luminal brush border region of the cell. Additionally or alternatively the binding may be largely nonspecific, perhaps related to lateral cell walls which are now freely exposed to the incubation medium.

In order to assess the absolute specificity of the binding sites for folic acid, a wide range of folic acid derivatives was tested for their ability to compete for these binding sites. Table I demonstrates that, compared to folic acid itself, folic acid diglutamate and the reduced derivatives H_2Fol and H_4Fol were equally effective in competing with the isotope for the binding site. The triglutamate derivative of folic acid was somewhat less efficient.

Of the various reduced one-carbon carrier derivatives of folic acid, 5,10-CH_2H_4Fol was an effective binding inhibitor compared to 5-HCO-H_4Fol and 10-HCOH_4Fol. Pteroic acid, which constitutes the major component of the folic acid molecule, competed equally effectively for its binding site. *p*-Aminobenzoylglutamic acid, however, produced no effect whatsoever on folic acid binding. Similarly Methotrexate, a structural analog of folic acid, renowned for its tight stoichiometric binding to the enzyme folate reductase, was without effect in relatively high concentrations. 5-Me_3THF, in the light of the significant effect produced by 5-HCO-THF, interestingly was also ineffective.

Dilantin, which has been postulated to interfere with the absorptive mechanism for folic acid (Bernstein *et al.*, 1970), did not effect its cell binding, while *N*-ethylmaleimide actually produced a significant increase in binding. This latter effect was pursued further (*vide infra*).

In order to characterize a possible specific binding site on the brush borders, cell suspensions were incubated with [^{3}H]folic acid for 5 min at 37°. The brush borders were isolated and despite the rigorous purification procedure a significant amount of folic acid remained associated with this cell membrane fraction. There was, in fact, an average fivefold increase in the specific activity in terms of folic acid bound per milligram of protein for the brush border fraction compared to the intact cell. Invertase specific activity was increased tenfold in the brush borders compared to the intact cells.

The folic acid tagged brush borders were solubilized in 0.2% sodium dodecyl sulfate for 2 hr at 4°. An aliquot containing 7.8 mg of protein was chromatographed on a Sephadex G-150 column (230 × 15 mm), suspended in 25.0 mM Tris-chloride buffer (pH 7.5) containing 0.1% dodecyl sulfate. Chromatography was carried out at room temperature of 25° with a pressure head of 46 cm. Figure 3 was a typical elution pattern in which the protein profile revealed two major peaks, one moving immediately behind the void volume indicating a minimum molecular weight of at least 1000,000, and the other in the region between albumin and cytochrome *c* markers, this latter peak corresponding to a molecular weight of the order of 25,000. The radioactivity profile revealed three major peaks. The first corresponded to the leading protein peak, the second was related to the second major protein peak, and the third was in the position of free folic acid. The appearance of free folic acid was not unexpected in the light of the nature of the solubilization procedure in dodecyl sulfate which results in marked changes in the conformation and charge of protein molecules (Reynolds and Tanford, 1970). The presence of 0.2% dodecyl sulfate was critical for the release of proteins which could not be effected by simple homogenization or by nonionic detergents in functional concentrations.

Confirmation of the radioactivity associated with the brush borders and with protein eluted from the brush borders as being in fact due to folic acid was achieved with paper chromatography on Whatman No. 1 paper with 50 mM phosphate buffer (pH 6.0). The [^{3}H]folic acid used in these experiments is labeled specifically in the 3′,5′ positions of the benzoic acid moiety and in the 9 position of the pteridine moiety. The tritium in these positions is not freely exchangeable (Zakrzewski *et al.*, 1970). Under the conditions of the experi-

TABLE I: Inhibition of Binding of [^{3}H]Folic Acid by Folic Acid, Its Analogs, and Derivatives.

Addition	Concn (M)	[^{3}H]Folic Acid Bound[a] Control	Test	% Inhibn
Folic acid	1.1×10^{-4}	14.3	5.1	64.4
H_2Fol	1.2×10^{-4}	15.9	6.7	58.2
H_4Fol	7.3×10^{-4}	18.8	7.5	60.3
Pteroic acid	1.6×10^{-4}	11.2	4.5	60.0
Folic acid diglutamate	1.2×10^{-4}	12.1	4.2	65.3
Folic acid triglutamate	2.2×10^{-4}	10.0	5.7	43.0
5-HCO-H_4Fol	4.2×10^{-5}	16.2	12.5	22.7
10-HCO-H_4Fol	1.7×10^{-5}	21.5	17.6	19.5
5,10-CH_2H_4Fol	1.7×10^{-4}	15.1	8.35	44.7
5-CH_3H_4Fol	5.8×10^{-5}	19.8	19.0	
Methotrexate	8.0×10^{-4}	16.1	16.6	
p-ABzGlu	1.0×10^{-4}	12.71	13.07	
Dilantin	8.7×10^{-5}	15.0	15.5	
N-Ethylmaleimide	2.0×10^{-4}	15.3	22.8	+50[b]

[a] Measured as moles ($\times 10^{14}$) of [^{3}H]folic acid bound per mg of cellular protein. The concentration of [^{3}H]folic acid in the incubation medium was 8.9×10^{-8} M. [b] *N*-Ethylmaleimide, in fact, stimulates the binding of [^{3}H]folic acid by 50%.

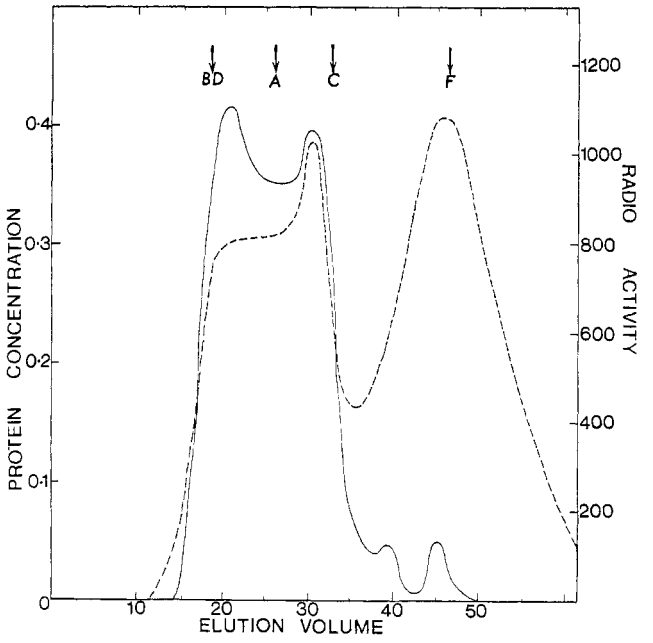

FIGURE 3: Elution profile of 7.8 mg of protein, obtained from brush borders solubilized in 0.2% dodecyl sulfate, chromatographed on a 230 × 15 mm Sephadex G-150 column with 25 mM Tris-Cl buffer (pH 7.5) containing 0.1% dodecyl sulfate. Protein concentration (continuous line) is expressed as mg/ml, radioactivity (broken line) as dpm/ml, and elution volume in mls. The markers (arrows) are Blue Detran (BD), average mol wt 2,000,000; bovine albumin (A), mol wt 68,000; cytochrome *c* (C), mol wt 12,400, and folic acid (F), mol wt 441. The two major leading protein peaks were pooled to constitute what is referred to in the text as pool I.

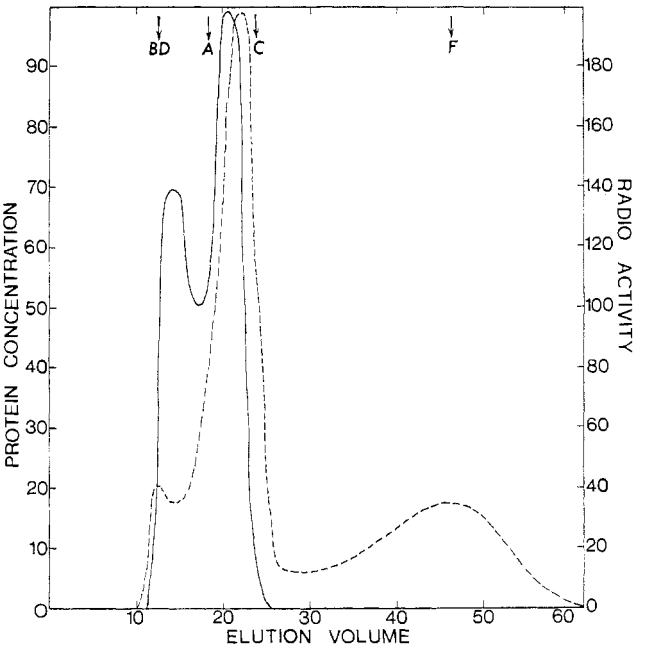

FIGURE 4: Elution profile of 1.9 of pool I protein chromatographed on a 210 × 15 mm Sephadex G-150 column with 25 mM Tris-C buffer (pH 7.5) containing 0.1% dodecyl sulfate. Protein concentration (continuous line) is expressed as μg/ml, radioactivity (broken line) as dpm/ml, and elution volume is in mls. The molecular weight markers were the same as those shown on Figure 5. The leading protein peak constituted pool II and the major second peak constituted pool III.

ment, folic acid would be expected to break down to form [3′,5′-$^3H_2$]*p*-aminobenzoylglutamic acid and pteridine-6-carboxylic acid. The oxidation to form the latter compound releases the C_9 tritium as tritiated water, *i.e.*, the pteridine derivative is unlabeled. In the chromatographic system utilized, folic acid had an R_F of 0.1 and *p*-aminobenzoylglutamic acid moved with an R_F of 0.9. Of the initial isotope used, more than 99.9% of the radioactivity migrated with folic acid. This was the case also with the isotope associated with the brush borders and its protein fractions and with the isotope eluted from the chromatography column in the position of free folic acid.

Similar experiments on brush borders isolated from cells incubated with uniformyl labeled [^{14}C]folic acid, yielded virtually identical results, although necessarily the radioactivity yields were much lower.

The combined protein peaks (pool I) from the Sephadex column were concentrated by ultrafiltration on a PM 10 membrane (exclusion mol wt 10,000) in an Amicon 8MC ultramicrofiltration apparatus, and rechromatographed on Sephadex G-150 under the same conditions (Figure 4). The protein elution pattern showed two peaks similar to those in Figure 3, but the bulk of the protein was now associated with the second lower molecular weight peak around 25,000. The radioactivity profile showed a major peak in the molecular weight range of 16,000 with minor peaks corresponding to the high molecular weight peak and to free folic acid. The leading higher molecular weight protein peak (pool II) and the trailing lower molecular weight peak (pool III) were concentrated as before.

Sephadex chromatography of the brush border membrane proteins from cells that had been exposed to a potent inhibitor of folic acid binding, lent strong support to the specificity of binding by the eluted proteins. The protein elution profile was identical with the controls and significantly the radioactivity associated with the protein peaks accurately reflected the decreased binding shown by the intact cells.

Cell binding of folic acid was increased 50% by treatment of the cells with *N*-ethylmaleimide. Isolation of the brush borders from these cells, however, showed that there was no increase in binding to this cell fraction or to the proteins eluted from Sephadex chromatography of the brush border proteins. This would indicate then that the increase in binding to the cells was a nonspecific phenomenon resulting from interreaction of the *N*-ethylmaleimide with cell membrane proteins, and that the membrane proteins with nonspecifically bound folic acid were removed in the course of the isolation of the brush borders.

Dodecyl sulfate–polyacrylamide gel electrophoresis was performed on the solubilized brush borders, the pooled protein (pool I) eluted from the Sephadex chromatography of the brush borders (Figure 3) and the concentrated pools of the leading protein peak (pool II) and the major protein peak (pool III) resulting from chromatography of pool I (Figure 4).

Figure 5 demonstrates the protein and radioactivity profile obtained on electrophoresis of solubilized brush borders on a 5.0% acrylamide–dodecyl sulfate gel at pH 8.3. Some 50% of the protein was below 45,000 molecular weight which correlated well with the Sephadex chromatography elution profile (Figure 3). Folic acid was distributed as the free acid, as a major peak in region of proteins of mol wt 24,000 and as minor peaks associated with three higher molecular weight proteins including one which barely penetrated the gel. No positive stains were obtained for either lipid or carbohydrate.

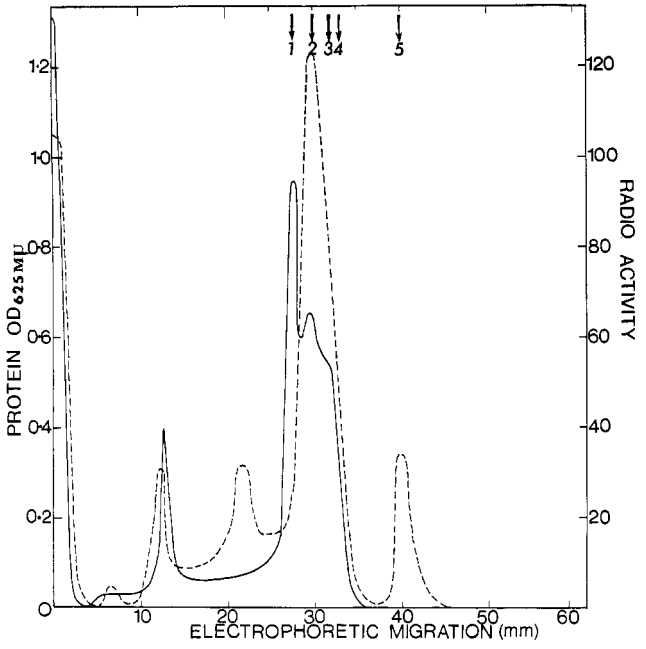

FIGURE 5: Protein and radioactivity profile of dodecyl sulfate–polyacrylamide gel electrophoresis of solubilized brush border protein on a 5% polyacrylamide gel with a 30:1 polyacrylamide: methylenebisacrylamide ratio, containing 0.1% dodecyl sulfate run at pH 8.3 in a 40 mM Tris-glycine buffer. Protein concentration (continuous line) was determined by the absorbance at 625 mμ of the Amido Black stain. Radioactivity (broken line) is expressed as dpm/2.5-mm section of gel. The markers (arrows) were (1) ovalbumin, mol wt 45,000; (2) chymotrypsinogen (beef pancreas), mol wt 25,000; (3) myoglobin (sperm whale), mol wt 17,800; (4) cytochrome *c* (horse heart), mol wt 12,400; and (5) folic acid, mol wt 441.

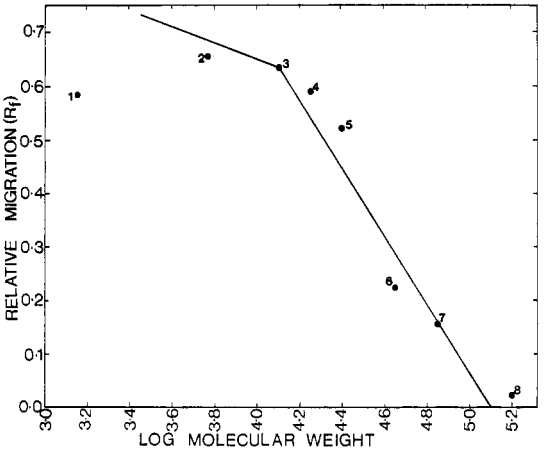

FIGURE 6: Electrophoretic migration of standard protein markers relative to Bromophenol Blue tracking dye (R_F) as a function of the log molecular weight of the markers. The gel was 12.5% polyacrylamide with a 20:1 acrylamide:methylenebisacrylamide ratio containing 0.1% dodecyl sulfate in a 0.1 M Tris-phosphate buffer at pH 6.8. Electrophoresis was undertaken at 3.0 mA/gel. The markers used were (1) bacitracin, mol wt 1450; (2) insulin (beef pancreas), mol wt 5700; (3) cytochrome *c* (horse heart), mol wt 12,400; (4) myoglobin (sperm whale), mol wt 17,800; (5) chymotrypsinogen A (beef pancreas), mol wt 25,000; (6) ovalbumin, mol wt 45,000; (7) albumin (bovine), mol wt 67,000; (8) γ-globulins (human), mol wt 160,000. The plot clearly indicates that there was a good correlation between the R_F and the log molecular weight above molecular weights of 12,400.

All subsequent gel electrophoretic studies were performed on 12.5% polyacrylamide gels with 0.1% dodecyl sulfate at a pH of 6.8 as this technique allowed of the best definition of the molecular weights of the proteins largely involved in this study. That is, the plot of the R_F against the log M_w was approximately linear over the range of some 12,400–160,000 (Figure 6).

Electrophoresis of pool I supported the chromatographic observation that a shift had occurred in the molecular weight distribution of the proteins with a shift into the lower molecular weight range with a major peak in the 180,000–24,000 region associated with tritiated folic acid. Pool II, the higher molecular weight protein pool, obtained by Sephadex chromatography of pool I, was shown on gel electrophoresis to be largely constituted by a range of molecules of molecular weight *below* 57,000 with a large peak in the 20,000 molecular weight region. Radioactivity was associated with the proteins in the 14,000–20,000 molecular weight range and the 80,000–100,000 molecular weight range. This result indicated that the higher molecular weight proteins, to which folic acid was bound, had largely dissociated into a range of lower molecular weight species, but that despite this, a significant amount of folic acid remained bound.

Electrophoretic examination of pool III from Sephadex chromatography confirmed that the bulk of the protein was in the 16,000–30,000 molecular weight range and that radioactivity was associated with proteins in the 16,000–20,000 molecular weight range.

N-Terminal Analyses. In view of the difficulty in identifying the number of protein bands on gel electrophoresis, N-terminal analysis was carried out on the proteins of the brush border and of pool III which eventually contained the bulk of the protein. During dansylation the protein was partly precipitated from both of these samples and hence each sample yielded two fractions for acid hydrolysis, namely soluble dansylated proteins and precipitated dansylated proteins. The results of the analyses are shown in Table II. From the brush border membrane protein, twelve different N-terminal residues were identified. Seven of these were found in pool III protein and it was clear that, in both cases, the protein precipitated during dansylation qualitatively reflected the N-terminal pattern of the whole fraction. This number of N terminals in a minimum number as far as reflecting the number of different polypeptide chains is concerned as certain α-NH_2 groups may be blocked and also a number of polypeptide chains may have the same terminal amino acid. Additionally it is possible that other dansylated N terminals may be present in such small quantities so as to render them undetectable. The major residues obtained from both protein samples were cystine, isoleucine, leucine, and valine.

It would appear then that the bulk of the membrane proteins in the brush borders isolated under these conditions, are constituted by at least seven different polypeptides, largely of the molecular weight range 16,000–24,000 and that folic acid is bound to a polypeptide in the lower region of the molecular weight range.

Discussion

The experiments described have demonstrated that isolated mature cells of the intestinal epithelium fail to accumulate folic acid. A number of factors could explain this. These isolated cells have lost their regular *in vivo* polar orientation and apposition to one another. It is quite possible that this is critical for a transport system which according to Burgen

TABLE II: Distribution of N-Terminal Amino Acids in the Brush Border Proteins and the Low Molecular Weight Proteins (Pool III).

N-Terminal Amino Acid	Brush Border Protein		Pool III Protein	
	Precipitate[a]	Supernatant	Precipitate	Supernatant
Cystine	+[b]	+	+	+
Leucine	+	+	+	+
Isoleucine	+	+	+	+
Valine	+	+	+	+
Glutamic acid	+	+	+	−
Aspartic acid	+	−	+	−
Serine	+	−	+	−
Glutamine	+	+	−	−
Methionine	+	+	−	−
Asparagine	+	−	−	−
Lysine	+	−	−	−
Tyrosine	+	−	−	−

[a] The headings "precipitate" and "supernatant" refer in each case to the appropriate dansylated protein fraction resulting from the dansylation procedure. [b] The designation + or − indicates the presence or absence respectively of the appropriate *N*-dansylamino acid. Quantitation was not attempted, but on the basis of the size and intensity of the fluorescent spots, cystine, leucine, isoleucine, and valine were present in the largest amounts.

and Goldberg (1962) and Smith *et al.* (1970) has a luminal–serosal directionality, *i.e.*, is largely a one-way path. There may be in fact, even *in vivo*, very little if any intracellular accumulation of folic acid in these cells, which possibly act merely as a shuttle, shunting folic acid from the gut lumen into the circulation. The loss of physiological orientation of the cells, hence would lead to complete disruption of the vectorial element of the shuttle.

In addition, folic acid is a relatively large molecule from a transport point of view and it is possible that there is a relatively slower flux dictated by its size.

Das and Hoffbrand (1970) also have observed that lymphocytes in culture only accumulated significant amounts of folic acid when stimulated to growth and division by phytohemagglutinin. The intestinal cells used in these studies are mature, nondividing cells and hence one might not expect to see any net folic acid uptake.

Finally, it is possible that, during the isolation procedure, some component of the transport system has been damaged perhaps by proteolytic digestion, which, while not entirely destroying the capacity of the membranes of the brush border to bind folic acid, has inactivated some metabolite-transfer function.

The binding of folic acid to isolated intestinal cells is relatively specific with a binding constant (K_b) of 3.98×10^{-5} which corresponds to the transport constant (K_t) obtained by Burgen and Goldberg (1962) for folic acid in a perfused segment of rat small intestine. The binding site is, however, only relatively specific for folic acid in that fundamentally the pteroic acid moiety appears to be the structural determinant for the binding process. Elongation of the molecule by the successive addition of glutamic acid residues decreases its binding efficiency as does the addition of one-carbon units to the pteroic acid molecule. The addition of a formyl group to the 5 and 10 position decreased the binding efficiency of the molecule, while the addition of a methyl group to these same positions, resulted in a complete failure to bind at the folic acid site. Reduction of the molecule at the 5, 6, 7, or 8 positions did not affect its binding efficiency.

The binding of folic acid to the proteins obtained from Sephadex chromatography would appear to be specific to the extent that the decreased binding to cells in the presence of derivatives such as 5,10-CH_2H_4Fol was reflected in the decreased radioactivity associated with both high and low molecular weight protein peaks.

A number of workers have isolated from bacterial membranes specific carrier proteins or binding proteins for a variety of metabolites (Kaback, 1970). These particular bacterial membrane proteins all have certain common characteristics. They are of molecular weight of the order of 30,000 and have apparently only one specific binding site per molcule with dissociation constants of the order of 10^{-5}–10^{-6} M. No lipid or carbohydrate moieties have been reported to form part of the protein molecule.

In the higher organisms, while few membrane proteins have as yet been described with a potential transport role, some elegant work by Miledi *et al.* (1971) has resulted in the isolation of a cholinergic-receptor protein from the electroplaque membrane of *Electrophorus electricus*. Dunham and Hoffman (1970) have also described the isolation of the ouabain-binding component of human red cell membranes which appear to be identical with the enzyme Na,KATPase.

Most of the detailed studies of mammalian membrane proteins carried out, admittedly to a large extent on red blood cells, emphasize the heterogeneous nature of the protein population (Rosenberg and Guidotti, 1969; Lenard, 1970; Phillips and Morrison, 1970). This would also seem to apply to the membrane proteins of the brush border of the rat small intestine.

The interpretation of these protein patterns is, however, very difficult because of the vigorous extraction procedures currently in use. This is accentuated by the problem of autodigestion of the proteins by a variety of peptidases (Bender *et al.*, 1971; Fairbanks *et al.*, 1971; Steck *et al.*, 1971).

Wallach's group has also examined the relative ease of release of different types of protein from the erythrocyte membrane and has correlated this phenomenon with a model illustrating the relative disposition of different protein components within the membrane.

It is quite possible that some digestion of membrane proteins has occurred during our isolation of the proteins from the brush border membranes of small intestinal cells particularly during the hyaluronidase digestion step where the intact cells would be exposed to some digestive activity by disrupted cells. In support of this, no glycoproteins or lipoproteins were identifiable among these membrane proteins. It is almost certain that the use of divalent cation chelators such as EDTA and citrate has resulted in some loss of membrane stabilization due to the removal of Ca^{2+} or Mg^{2+} ions (Burger *et al.*, 1968). This could well have resulted in the removal of sialoproteins if an extrapolation can be made from the behavior of the externally sited glycoproteins of the bovine erythrocyte membrane.

The inference from this, of course, is that the proteins isolated from the brush border membranes may represent only a small fraction of those present in the intact membrane.

The overall interpretation of the data presented in this work, however, is that folic acid and its analogs and deriva-

tives have a specific affinity for a protein molecule of molecular weight 100,000 in the membrane of the brush border region of the small intestinal cell. Upon exposure to dodecyl sulfate this molecule dissociates into polypeptide subunits of the order of mol wt 16,000–20,000. It is admittedly difficult to interpret the progressive shift of protein molecular weight from a relatively even distribution above and below mol wt 50,000 to a predominance of the species of less than mol wt 50,000 during the course of the isolation of the proteins. Enzymatic digestion, may have played a role, but it is of critical importance to note that the lower molecular weight species constituting pool III did not shift into an even lower molecular weight range. It is interesting moreover that, compared to the N-terminal amino acids demonstrated in the brush borders, no new N terminals had appeared in the pool III lower molecular weight proteins, a predictable event had proteolytic digestion played a major role in the generation of this group of molecules.

Following the argument developed by Steck *et al.* (1971) it would appear that the protein molecule which binds folic acid is firmly anchored in the membrane matrix as it withstood the isolation techniques involved.

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