ascorbic acid will be unaffected by hydroquinone. Thus the higher hydroquinone level in tissues in ascorbic acid-treated subjects could be explained. Previous reports[12,13] on the lowering of tyrosinase activity by the action of ascorbic acid support these results.

Amelanosis due to tryptophan may be attributed to its selective toxicity[10] to melanotic cells while that due to ascorbic acid is attributed to its reducing action. On the other hand, the induced melanosis due to psoralen (plus sunlight or UV) has not been clearly explained, but the overall effect of administration of this pigmentogenic agent is to lower the hydroquinone level. This suggests that activation of melanogenesis requires an overall lowering of the tissue inhibitor concentration, and points out that the interference of the agents causing acceleration or retardation of melanogenic process. The suggestions put forward by Chakraborty et al.[9] as well as by Nordlund and Lerner[16] support this idea. It was suggested by Chakraborty et al.[9] that psoralen could interfere with inhibitors of tyrosinase to augment melanogenesis, while Nordlund and Lerner[16] suggest that psoralen (plus UV) may damage a postulated inhibitory agent that is responsible for killing pigment cells, producing vitiligo. Recent isolation of an inhibitor by Vijayan et al.[17] from vitiliginous skin is in conformity with the experimental results and the suggestions presented here.

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Noninvolvement of a rat intestinal folate binding protein in physiological folate absorption

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Summary. Folic acid and its derivatives are observed to be instantaneously bound to rat intestinal mucosal cell curface binding protein. Various parameters were measured such as the optimal pH for binding (6.5), the saturation kinetics observed for folate binding and the avid affinity with which polyglutamyl folates are preferentially bound to the binder. A comparison of the observed results with the conditions reported as favorable for intestinal folate absorption in the rat precludes the involvement of this folate-binding protein in the physiological absorption of folates.

Specific folate binding proteins (FABP) have been reported in serum, milk, leukocytes and a variety of tissues such as liver, kidney and intestinal mucosa[1-6]. FABP have been purified from cows' milk[7], human milk[8], umbilical cord serum[9], human choroid plexus[10] and intestinal mucosa[6]. The physiological functions of FABP have not been well established though it is implicated in folate absorption and transport[11,12]. Here we report observations on a folate binder in the intestinal mucosal cell surface and assess its role as a carrier in the physiological absorption of the vitamin.

Materials and methods. Folic acid, tetrahydrofolic acid, 5-formyltetrahydrofolic acid and 5-methyltetrahydrofolic acid were all obtained from Sigma Chemical Co., USA. (2[14]-C) folic acid with specific activity of 54.3 mCi/mmole was obtained from Amersham, U.K. Dihydrofolic acid was synthesized as described earlier[13]. Yeast folates which consist of almost 80–90% polyglutamyl forms, as characterized in our laboratory earlier[14], served as a source of polyglutamyl folates.

Male albino rats of the Wistar strain fed a complete laboratory stock diet and weighing 150–170 g were used throughout the study. 24-h fasted rats were killed, and the upper two-thirds of the small intestine removed and washed with ice-cold 0.9% saline. The intestine was slit open and the mucosal cells were gently scraped off with a fine scalpel and suspended in Ringer solution.

Mucosal cells equivalent to 40 mg protein were suspended in 5 ml Krebs-Ringer phosphate medium, pH 6.5, containing 0.4 μCi (2-^{14}C) folic acid and incubated at 37 °C with constant shaking. Aliquots of 0.1 ml each were removed at various time intervals during incubation, and filtered under suction through 0.22 m μ pore size millipore filters. The filters with trapped cells were washed thoroughly with 30 ml of 154 mM cold saline, dried and their radioactivity determined. The experiment was repeated at various pH values and at various other ^{14}C-folate concentrations.

In studies measuring competition by other folate compounds for the folate binding sites, various concentrations of the particular folate derivative under study were incubated with cells for 5 min at 37 °C prior to the addition of radioactive folic acid. Incubation with shaking was continued for another 5 min prior to trapping and washing the cells on the millipore filters. The relative amounts of various folate derivatives required for a 50% displacement of ^{14}C-folic acid binding were recorded. All radioactivity measurements were made in a Beckman Liquid Scintillation Counter LS 100 using Bray's solution.

Results. As can be seen in figure 1 the binding of ^{14}C-folic acid

to the intestinal mucosal cells is very rapid and is essentially complete within a minute of the addition of mucosal cells to labelled folic acid. The pH of the folate binding to the epithelial cells seems to be very specific with an optimum around 6.5–7 (fig. 2). The binding of ^{14}C-folate to the mucosal cells showed saturation kinetics, the maximum amount bound being in the presence of 1.2 µM folate/mg cell protein (fig. 3).

In order to assess the binding specificity of the sites, a range of folic acid derivatives were tested for their ability to compete for the binding sites. The table indicates the relative concentration of various folate compounds required for a 50% displacement of ^{14}C-folate bound to the FABP. Taking the concentration of unlabelled folic acid required for a similar displacement (50%) of labelled folate as 1, dihydrofolic acid and polyglutamyl folates had an affinity almost equal to that of folic acid in competing for the binding sites, their relative concentration ratios being 1.04 and 1.19 respectively. Tetrahydrofolate, 5-methyl- and 5 formyltetrahydrofolates were less efficiently bound, their affinities for the FABP being 2.38, 5.95 and 11.42 times lower, respectively, than folic acid.

Discussion. The role of intestinal FABP in the physiological absorption of folate compounds is not unequivocally established. We have reported earlier[15], confirming the original suggestions of Blair et al.[16], that folates are taken up in the intestine by a process of passive diffusion as the neutral species in an acidic microenvironment (preferably around pH 4.0) which is generated and regulated in the unstirred glycocalyx layer by an intestinal brush border Mg^{2+}-ATPase. However, the optimal pH of the folate binding is pH 6.5 (fig. 2), a pH at which physiological folate absorption would be greatly reduced[15]. Moreover, polyglutamyl folates, which are the main dietary forms of the vitamin, preferentially bind to the receptor (table). These forms are unsuitable for intestinal absorption per se as they have to be hydrolyzed to simple monoglutamyl forms by the action of intestinal folyl conjugase[17], an enzyme of pancreatic origin in the rat[18], with a pH optimum of 4.2. The above evidence would preclude a role for the folate binding protein under study as a carrier involved in folate absorption.

We have also reported[19] a concentration-dependent passive uptake of folates in the intestine, whereas folate binding to the intestinal FABP showed saturation kinetics (fig. 3). Further, we observed earlier[19] that no correlation existed between the kinetics of the appearance of absorbed folate in the blood stream and the binding of folate simultaneously in vivo to the mucosal cell receptors. Whereas ingested folate builds up maximally in the blood stream 30 to 45 min following ingestion, after which the concentration in the blood declines, the folate activity bound to the mucosal cell receptors is consistently high even 5 h after folate ingestion. No precursor-product relationship seemed to exist between folates bound to the mucosal surface and folates appearing in the blood stream after intestinal folate absorption[19]. These results taken together suggest that the intestinal FABP is not involved in the physiological absorption of dietary folates.

Relative binding competition by various folate derivatives for folic acid bound to mucosal cells

Competing folate compound	Concentration (M) required to reduce ^{14}C-folate bound by 50%	Relative concentration required for 50% reduction in ^{14}C-folate*
Folic acid	2.1×10^{-4}	1.00
Dihydrofolic acid	2.2×10^{-4}	1.04
Tetrahydrofolic acid	5.0×10^{-4}	2.38
5-formyltetrahydrofolic acid	24.0×10^{-4}	11.42
5-methyltetrahydrofolic acid	12.5×10^{-4}	5.95
Polyglutamyl folate	2.5×10^{-4}	1.19

Intestinal mucosal cells equivalent to 40 mg protein were incubated in 5 ml Ringer's phosphate buffer pH 6.5 with various concentrations of different folate derivatives for 5 min after which 0.4 µCi ^{14}C-folic acid (2 µM) was added, and the incubation continued for 5 more min after which the binding of ^{14}C folic acid was determined. * The values were obtained by dividing the concentration of the folate derivative that produced 50% inhibition by the concentration of unlabelled folic acid that produced the same degree of inhibition.

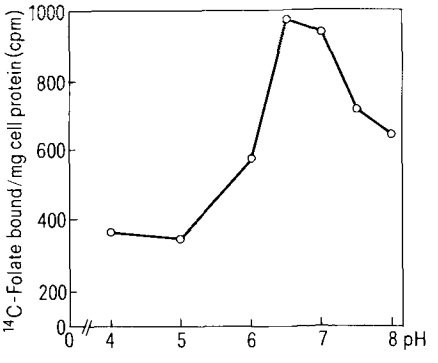

Figure 2. ^{14}C-Folate binding to intestinal mucosal cells as a function of pH.

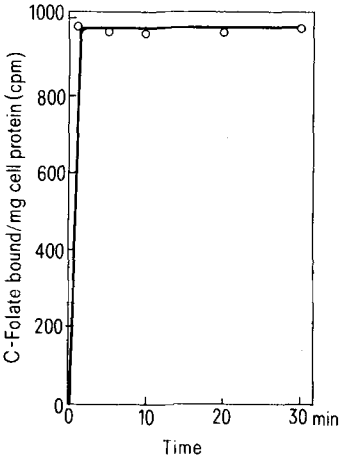

Figure 1. Time course of ^{14}C-folate binding to intestinal mucosal cells.

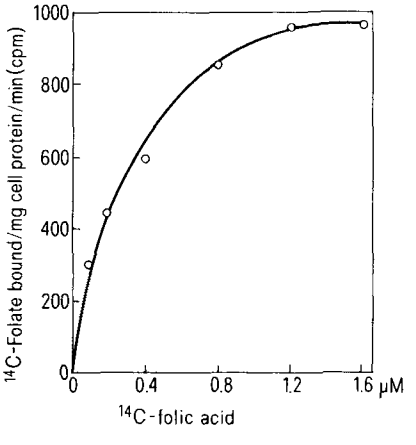

Figure 3. ^{14}C-Folate binding to intestinal mucosal cells as a function of concentration.

We have recently isolated the ^{14}C-folate-tagged brush borders from rat intestinal mucosal cells. After solubilization of these borders with 0.2% sodium dodecyl sulphate and fractionation on Sephadex G 150, a dingle symmetrical protein peak appeared, to which labelled folate was found to be bound[19]. The characteristics of the saturation binding and other properties of this protein indicate that it is a high affinity soluble folate binder similar to the one reported in milk[2], intestine[6], rat kidney[5] and serum[1].

It is suggested that FABP might function in the storage of excess dietary folate as it has an avid affinity for polyglutamyl folates; FABP might possibly control the level of folate absorption by sequestering the polyglutamyl folates from the action of folyl conjugase and releasing them as and when necessary. Alternatively, these bound polyglutamyl folates, having higher cofactor activity than the monoglutamyl counterparts, may have a physiological function regulating intestinal mucosal cell activity.

Monoglutamyl folyl derivatives which as such are more suitable for intestinal absorption have less affinity for the FABP, again suggesting that the FABP activity is not related to the absorption process.

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Formation of histidinoalanine cross-links in heated proteins

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Summary. Formation of the cross-linking amino acid, histidinoalanine, was demonstrated in various proteins when they were heated. This cross-link formation may be partly responsible for the deterioration of food proteins resulting from heat treatment.

It is known that heat treatment of proteins impairs their nutritional values. One of the significant heat-induced changes in proteins is the formation of cross-links between polypeptide chains of proteins[1]. The presence of cross-links reduces the digestibility of the proteins and renders some constituent amino acids biologically unavailable. So far, the formation of isopeptide cross-links[2] and of lysinoalanine cross-links[3] have been demonstrated.

Histidinoalanine or N^{τ}-(2-amino-2-carboxyethyl)-histidine is a bifunctional cross-linking amino acid. It was first found in human dentin collagen[4] and subsequently found in proteins of various human connective tissues[5]. Its content was found to increase with age[5,6]. It has been proposed that histidinoalanine crosslinks might be related to the aging of human connective tissues[5,6].

This paper reports the formation of histidinoalanine in various proteins when they were heated at neutral pH. This cross-link, like those mentioned above, may be responsible for the deterioration of food proteins on heat treatment.

Materials and methods. Bovine serum albumin, ovalbumin and porcine pepsin were obtained from Sigma. Bovine tendon collagen was obtained from the Millipore Corporation. Milk casein was obtained from Merck. Human gamma globulin was obtained from Calbiochem.

The protein sample (10 mg) was dissolved or suspended in 1 ml of potassium phosphate buffer, 0.1 M, pH 6.8 or 7.4, in a sealed tube and heated. After the heat treatment, 1 ml of concentrated HCl was added and acid hydrolysis was performed at 110 °C for 24 h. Cross-linking amino acids were determined as described in the previous paper[6].

Results and discussion. As shown in table 1, histidinoalanine was formed in various proteins when they were heated at

Table 1. Formation of histidinoalanine and lysinoalanine in various proteins on heating. The protein sample (10 mg) was heated at 110 °C for 24 h in 0.1 M potassium phosphate buffer, pH 6.8. These cross-linking amino acids were not detected in unheated controls

Protein	Histidinoalanine formed (nmole/mg)	Lysinoalanine formed (nmole/mg)
Bovine serum albumin	39.0	6.4
Casein	15.3	2.6
Ovalbumin	8.3	2.0
Human gamma globulin	17.7	3.3
Porcine pepsin	7.0	0.1
Bovine tendon collagen	0.7	0.1