Isolation and Metabolic Characteristics of Rat and Chicken Enterocytes

By MALCOLM WATFORD,* PATRICIA LUND and HANS A. KREBS

*Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.*

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1. The recent recognition of the metabolic, as opposed to absorptive, functions of the small intestine prompted efforts to improve the preparation of metabolically competent columnar absorptive cells ('enterocytes') and to study their metabolic properties. 2. With this preparation, linear rates of O_2 consumption are obtained for 40 min at 37°C that are more than 50% higher than rates reported by other authors. 3. Among added substrates, glucose, glutamine and glutamate are the preferred fuels of respiration. The main nitrogenous products of glutamine metabolism are NH_3 , alanine and glutamate. Glutamine carbon was not detectable in citrulline or proline, in contrast with the findings of Windmueller & Spaeth [(1974) *J. Biol. Chem.* **249**, 5070–5079] in the vascularly perfused small intestine. 4. The rates of O_2 uptake in the presence of glutamine or glutamate are sufficient to account for the formation of the carbon skeleton of alanine from the amino acid substrate, i.e. the ratio of O_2 used/alanine formed is greater than 1.5. 5. Added ADP and ATP are rapidly degraded to AMP and IMP to a large extent by release of hydrolytic enzymes from the enterocytes into the medium. 6. Chicken enterocytes isolated by the same method are more stable; linear rates of O_2 uptake are maintained for 60–70 min.

The viscera of the portal bed are known to play a major role in the removal of glutamine from the circulation (Addae & Lotspeich, 1968; Ishikawa *et al.*, 1972; Marliss *et al.*, 1971; Ishikawa, 1975). The site of this glutamine utilization was identified by Windmueller & Spaeth (1974, 1975) to be the small intestine, where glutamine makes a large contribution to the respiratory fuel of the intestinal mucosa, as first observed by Finch & Hird (1960) and by Neptune (1965). According to Windmueller & Spaeth (1974, 1975) NH_3 , alanine, citrulline and proline are the main nitrogenous products derived from glutamine. Formation of citrulline from glutamine is of special interest because it bears on the question of whether, or to what extent, arginine is an essential amino acid (Rose, 1938).

Early work on the metabolism of the isolated intestinal mucosa was carried out on scrapings (Warburg *et al.*, 1924; Dickens & Weil-Malherbe, 1941). In the mid-1960s methods for the isolation of intact absorptive columnar epithelial cells from the mucosa of the small intestine ('enterocytes') were elaborated by Harrer *et al.* (1965), Huang (1965), Perris (1966), Stern & Jensen (1966), Sjöstrand (1968), Webster & Harrison (1969), Iemhoff *et al.* (1970), Evans *et al.* (1971), Reiser & Christiansen (1971), Padron *et al.* (1972), Bihler & Cybulsky (1973) and Hülsmann *et al.* (1974). Most of these methods involved incubation of the small intestine with

enzymes and/or chelating agents, which make the intercellular junctions ineffective.

In the present paper a method for the isolation of metabolically competent enterocytes is described based on those of Perris (1966), Stern & Jensen (1966) and Reiser & Christiansen (1971). The cell suspensions obtained represent a well-defined population of columnar absorptive cells, but do not include all epithelial cells of the mucosa. Goblet cells represent less than 10% and crypt cells are absent. As measured by the rate of O_2 uptake, the metabolic capacity of the rat enterocyte suspension is substantially (50%) higher than any previous preparation. The main object of the work was a study of glutamine metabolism, and other fuels of metabolism, especially also of the role of glutamine as a precursor of citrulline.

Materials and Methods

Rats

Wistar rats (about 200g) were obtained from Charles River U.K. Ltd., Manston Road, Margate, Kent, U.K., and were fed on Oxoid (Oxoid Ltd., London S.E.1, U.K.) pasteurized breeding diet for rats and mice. For some experiments animals were starved for 48 h before use.

All experiments reported in this paper were carried out on cells from fed rats. The cell yield from the intestine of 48 h-starved rats was less than half that from fed rats, but rates of the metabolic processes examined were not significantly different.

* Present address: Laboratoire de Néphrologie, Hôtel Dieu de Montréal, 3840 Rue St. Urbain, Montréal, Québec, Canada.

Chickens

Male Rhode Island Red $\times$ Light Sussex chickens (5–7 weeks old) were obtained from Orchard Farm, Great Missenden, U.K., and were fed on 'Chick starter crumbs, ACS BOCM SILCOCK' supplied by B.B.O. Farmers, Eynsham, Oxon, U.K.

Reagents

Glutaminase (grade V) was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Other purified enzymes and coenzymes were products of Boehringer Corp. (London) Ltd., London W.5, U.K. Bovine serum albumin (fraction V; Miles Laboratories, Slough, Bucks., U.K.) was dialysed as described by Krebs *et al.* (1974).

Preparation of isolated epithelial cells from the rat small intestine

The method of Reiser & Christiansen (1971) was modified with regard to the saline media. Three salines were used: (1) the medium of Krebs & Henseleit (1932) from which CaCl_2 was omitted, (2) the same to which 0.25% (w/v) dialysed serum albumin and 5 mM-EDTA were added, (3) the medium of Krebs & Henseleit (1932) with 2.5% (w/v) dialysed serum albumin. After many preliminary tests the final procedure adopted was as follows. Rats were killed by a blow on the head, thus avoiding the use of anaesthetics, which seemed to increase the release of mucus, and a length of small intestine of about 90 cm, beginning 5 cm below the pylorus and ending about 10 cm before the junction with the colon, was removed promptly. A syringe containing about 40 ml of ice-cold saline (1) saturated with O_2/CO_2 (19:1) was attached to the proximal end and the lumen rinsed with the saline. Rinsing fluid remaining in the lumen was removed by squeezing the intestine gently along its length. Then one end was ligated and the lumen was filled with about 18 ml of saline (2). This amount of fluid was sufficient to distend the intestine slightly. The other end was ligated and the intestine was incubated at 37°C for 15 min in a 250 ml conical flask containing 100 ml of saline (1) with shaking (60–70 oscillations/min). During the incubation the flask was continuously gassed with the O_2/CO_2 mixture. The intestine was then opened, drained and washed with ice-cold saline (3). This removed loose cells from the tips of the villi and some mucus. It was refilled with saline (3) and patted with finger tips for 1 min on an ice-block covered by a polythene sheet, as described by Reiser & Christiansen (1971). By this treatment cells were released into the lumen. The lumen was drained into polystyrene tubes. Contact with glass was avoided at this and subsequent stages because isolated cells are liable to adhere to glass, and to clump. The cells were centri-

fuged at 500g for 3 min and washed once with approx. 4 vol. of saline (3). Finally the packed cells were resuspended in 4 vol. of saline (3) by drawing them up several times into a 10 ml wide-mouthed polypropylene pipette. This suspension contained approx. 10–20 mg dry wt. of tissue per 2 ml.

Incubations other than those involving manometry were carried out in 25 ml polypropylene conical flasks containing 2 ml of the cell suspension plus 2 ml of saline (3) supplemented with substrates. The gas space was filled with O_2/CO_2 (19:1). The flasks were shaken at 37°C in a Dubnoff-type shaker at 60–70 oscillations per min and incubations were stopped by the addition of 0.14 ml of 60% (v/v) HClO_4 . Metabolite analyses were carried out on the neutralized supernatant.

Preparation of chicken enterocytes

Well-fed chickens weighing about 500 g were anaesthetized by intraperitoneal injection of Nembutal in aqueous solution (60 mg/kg). The whole small intestine except the duodenum was removed and treated as described for the rat. The yield of cells was relatively high (up to 5 g wet wt.). Larger birds gave relatively low yields.

O_2 consumption

O_2 consumption was measured in the special manometer cups described by Krebs *et al.* (1974) containing the equivalent of about 20 mg dry wt. of cells in 4 ml of saline (3). The gas phase was O_2/CO_2 (19:1). To minimize damage to the enterocytes the vessels were shaken rather slowly at 37°C at 72–78 oscillations/min with amplitude 2.5–3.0 cm, when the cell concentration could be doubled without diffusion of O_2 becoming rate-limiting. At lower O_2 concentration (air/ CO_2 , 19:1) the rates of O_2 uptake were lower and the proportion of glucose appearing as lactate was increased. Diffusion of O_2 became rate-limiting at low O_2 concentration or at shaking speeds below 50 oscillations/min under the experimental conditions.

Determination of metabolites

Glucose, lactate, pyruvate, alanine, glutamate, aspartate, acetoacetate, 3-hydroxybutyrate and NH_3 were determined as described previously (Cornell *et al.*, 1974). Glutamine was determined with glutaminase and glutamate dehydrogenase by the method of Lund (1974). The ATP assay of Lamprecht & Trautschold (1974) was modified by using 2.5 mM-glucose instead of the recommended 40 mM because glucose-NADP⁺ reductase present in some glucose 6-phosphate dehydrogenase preparations causes a 'creep' at the high glucose concentration. ADP and AMP were determined as described by Adam (1963).

Enzyme assays

The activities of 'malic' enzyme, phosphoenolpyruvate carboxykinase and oxaloacetate decarboxylase were assayed at 37°C in freshly homogenized enterocyte suspensions. 'Malic' enzyme (NADP⁺-requiring malate dehydrogenase) was assayed as described by Stickland (1959), phosphoenolpyruvate carboxykinase in the direction of oxaloacetate formation as described by Holton & Nordlie (1965), and oxaloacetate decarboxylase as described for 'supernatant decarboxylase' by Dean & Bartley (1973).

Results

Handling of isolated enterocytes

O₂ consumption. The manometric measurement of the rate of O₂ consumption was used as a criterion of metabolic function because it gives information on changes with time. In exploratory tests the yields of enterocytes and their rates of O₂ uptake were erratic. Various factors, including those described below, were considered. Eventually cell preparations were obtained that gave linear rates of O₂ consumption for 30–40 min at 38°C, but we were unable to prevent the rate falling to about 70% at 50 min.

Requirement for albumin. The protective effect of albumin was discovered by Perris (1966). Increasing the albumin concentration of the isolation medium from 0.25 to 2.5% (w/v) halved the cell yield. Low yields were also obtained when albumin was omitted. On the other hand the optimum concentration for the incubation medium was 2.5% (w/v), but albumin did not prevent the decline in O₂ consumption observed after 40 min. Perris (1966) found 1% (w/v) albumin in the incubation medium superior to 2% (w/v), perhaps because undialysed albumin was used, which contains impurities including considerable amounts of acetate and ethanol (see Krebs *et al.*, 1974).

Effect of calcium. Calcium is known to promote aggregation of enterocytes (Kimmich, 1970). The presence (2.5 mM) or absence of calcium in the incubation medium had no effect on either the initial rate of O₂ uptake or on the decline after 40 min.

Alternative manometer vessels. Attempts to prevent the deterioration of the cells during incubation by coating the manometer cups with silicone, or paraffin wax or methyl methacrylate, were unsuccessful. Similarly, manometer flasks made of Perspex or polypropylene made no difference.

Alternative procedures. Isolation of enterocytes at room temperature and/or incubation at 25°C or 30°C did not prevent the decline of O₂ consumption after 40 min, nor was it prevented by the addition of blood serum, of trypsin or pepsin inhibitors or by the presence of 10 mM-glucose at all preparative stages. With 1 mM-EDTA the yields of cells were erratic and

sometimes less than 20% of those obtained with 5 mM-EDTA.

Composition and appearance of cell population

The cells isolated by the method described were predominantly (more than 80%) enterocytes of columnar shape; the brush border was clearly visible under phase-contrast microscopy. The cells were well dispersed immediately after isolation but sometimes tended to clump on standing and during incubation.

Clumping can be prevented by the addition of 2 mM-DL-dithiothreitol (which had no effect on the O₂ consumption). But even in the presence of the thiol the enterocytes did not pack well on centrifugation, as shown by the high wet weight/dry weight ratio. The mean wet weight/dry weight ratio of the pellet was 12.2 in untreated suspension and 9.5 in the presence of dithiothreitol. Bronk & Leese (1973) and Hanson (1975) found ratios of 6.25 and 3.65 respectively for rat mucosal scrapings. Because of these variations, metabolic rates were expressed by reference to dry weight.

Care must be taken to use rats free from infection with protozoal parasites, such as *Hexamita* or *Giardia*, in the intestine. Infections can be readily controlled by including in the drinking water Emtryl (1,2-dimethyl-5-nitroimidazole), a product of May and Baker, Dagenham, Essex, U.K.; it is also marketed as dimetridazole.

Effect of added substrates on the rate of O₂ consumption

Glucose, glutamine and glutamate were the only substances that caused major increases in the rate of O₂ consumption (Table 1). The effects of glucose and glutamine were partially additive. The absolute rates and the increases on addition of the substrates were initially similar in rat and chicken enterocytes, but the O₂ uptake of chicken enterocytes, in contrast with those of the rat, did not decrease significantly within an incubation period of 60 min (Table 2). This applied to the endogenous respiration as well as the increased rate on addition of glucose and glutamine. In the presence of glucose, the rates for the rat were about 50% higher than those reported by Perris (1966), the highest so far recorded in the literature. The higher rates may be connected with our use of a bicarbonate-buffered saline, which in other tissues has been found to be superior to phosphate-buffered salines. Addition of oleate (1 mM), butyrate (5 mM), propionate (2 mM) or acetate (5 mM) did not significantly increase the rate above the endogenous, nor did oleate or butyrate form ketone bodies. On the other hand, acetoacetate gave some increase (Table 1), with about 20% of the acetoacetate removed appearing as 3-hydroxybutyrate and the remainder (about 1.5 µmol/min per g dry wt.) presumably being

completely oxidized. Although glucose is oxidized via pyruvate, added pyruvate or lactate plus pyruvate gave only small increases over the endogenous rate (Table 1). Equivocal results were obtained with alanine (5 mM), asparagine (5 mM), aspartate (5 mM), 3-hydroxybutyrate (5 mM), succinate (5 mM) or citrate (5 mM). Any fatty acids present in the dialysed albumin did not serve as a major fuel, because replacing this albumin by albumin defatted as described by Chen (1967) did not decrease the endogenous respiration.

The addition of oxidizable substrates did not prevent the fall in the rate of O_2 consumption of rat enterocytes after 30–40 min. Except for the higher rates of O_2 consumption, the findings are in general agreement with those recorded in the literature, though Neptune (1965) found a ready oxidation of acetate, in contrast with the present findings.

Products formed from glucose, glutamate and glutamine by rat enterocytes

On addition of glucose alone about 70% of the glucose removed was accounted for by lactate and small amounts of pyruvate. There were also small increments of alanine and glutamate over the endogenous amount (Table 3). The free NH_3 formed is

probably mainly derived from adenine nucleotides (see Table 4). Uncertainties as to the source of the amino acids formed do not allow accurate calculations on whether, or to what extent, glucose suppresses endogenous respiration. The respiratory quotient of 0.85 for scrapings of rat ileum (Dickens & Simer, 1930) would suggest that fatty acids are the main endogenous substrate.

The addition of glutamine alone caused formation of glutamate, alanine, aspartate and NH_3 . NH_3 formation was approximately equivalent to glutamine removal. Glutamate accounted for about 40% of the glutamine removed. Of the fraction of glutamine degraded beyond the stage of glutamate, about 50% was accounted for as alanine (i.e. 30% of the total glutamine removed) and 20% as aspartate (i.e. 12% of the total glutamine removed). NH_3 , alanine and glutamate have already been shown by Matthews & Wiseman (1953), Neame & Wiseman (1957, 1958), Aikawa *et al.* (1973) and Windmueller & Spaeth (1974) to be major end products of intestinal glutamate and glutamine metabolism *in vivo*. The one to one stoichiometry between glutamine removed and NH_3 formed indicates that the hydrolysis to glutamate is the first step of glutamine degradation and that glutamate dehydrogenase is not significantly involved in the formation of α -oxoglutarate. Transamination of glutamine with pyruvate via glutamine- α -oxo acid aminotransferase cannot be a major reaction because glutamine removal and alanine formation are not much affected when ample pyruvate is available (i.e. when glucose is also present). The rate of O_2 consumption in the presence of glutamine (or glutamate) is sufficient for the formation of the carbon skeleton of alanine from the amino acid substrate in that formation of pyruvate from α -oxoglutarate requires $1.5 \mu\text{mol}$ of O_2 and the ratio of the increment of O_2 used/alanine formed is greater than 1.5 in the experiments.

The addition of glutamine plus glucose caused an increase in the yield of alanine and a decrease in glutamate and aspartate. The rates of glutamine and glucose removal were the same as when either substance was added alone, as found by Hanson & Parsons (1977) in the isolated perfused small intestine, but the amount of glutamine broken down beyond the stage of glutamate was increased to 80%

Table 1. *Effect of added substrates on the rate of O_2 consumption of rat enterocytes*

The rates of O_2 consumption were measured manometrically as described in the Materials and Methods section. Rates were calculated from the 15–30 min period of incubation. The data are means $\pm$ S.E.M., with the numbers of observations in parentheses.

Substrate	Rate of O_2 uptake ($\mu\text{mol}/\text{min}$ per g dry wt.)
None	9.64 ± 0.24 (29)
Glucose (10 mM)	19.7 ± 0.33 (42)
Glutamine (5 mM)	15.5 ± 0.43 (11)
Glutamine (5 mM)+ glucose (10 mM)	21.3 ± 0.46 (15)
Glutamate (5 mM)	15.2 ± 0.89 (4)
Lactate (10 mM)+ pyruvate (1 mM)	13.0 ± 1.4 (3)
Pyruvate (10 mM)	11.8 ± 0.93 (6)
Acetoacetate (5 mM)	12.5 ± 0.49 (3)

Table 2. *Time course of O_2 consumption of chicken enterocytes*

Chicken enterocytes were prepared as described in the Materials and Methods section. The O_2 consumption was followed manometrically.

Substrate added	Rate of O_2 uptake ($\mu\text{mol}/\text{min}$ per g dry wt.)		
	20–30 min	40–50 min	60–70 min
None	11.0 ± 1.3 (3)	11.8 ± 0.9 (3)	11.5 (2)
Glucose (5 mM)	19.1 ± 1.1 (6)	19.3 ± 1.9 (6)	17.0 ± 2.0 (3)
Glutamine (5 mM)	15.0 (2)	14.8 (2)	12.9 (2)
Glutamine (5 mM)+glucose (5 mM)	21.8 (2)	21.6 (2)	19.3 (2)

Table 3. *Metabolic changes on addition of glucose, glutamine and glutamate in enterocyte suspensions*

Cells were incubated for 20 min at 37°C. The initial concentration of glucose was 10 mM and that of amino acid, 5 mM. The data are means $\pm$ S.E.M. for metabolite removal (—) or production (+) expressed as $\mu\text{mol/min per g dry wt.}$, with the numbers of observations in parentheses.

Product formed Substrate added ...	Metabolite removal or production					
	None	Glucose	Glutamine	Glucose+ glutamine	Glutamate	Glucose+ glutamate
Glucose	—	-12.0 ± 0.6 (4)	—	-11.3 ± 0.3 (5)	—	-10.3 ± 0.8 (3)
Glutamine	—	—	-11.0 ± 1.0 (5)	-12.1 ± 0.5 (5)	—	—
Glutamate	+0.45 (2)	$+0.70 \pm 0.2$ (4)	$+4.3 \pm 0.4$ (5)	$+2.5 \pm 0.3$ (5)	-2.2 ± 0.2 (3)	-2.3 ± 0.01 (3)
Alanine	+0.05 (2)	$+0.77 \pm 0.03$ (4)	$+3.2 \pm 0.4$ (5)	$+6.1 \pm 0.75$ (5)	$+1.1 \pm 0.2$ (3)	$+3.0 \pm 0.2$ (3)
Aspartate	—	+0.15 (2)	+1.25 (2)	+0.50 (2)	+0.36 (2)	+0.05 (2)
Lactate	$+1.3 \pm 0.4$ (3)	$+16.4 \pm 1.1$ (4)	$+1.90 \pm 0.32$ (5)	$+18.6 \pm 0.9$ (5)	$+1.6 \pm 0.2$ (3)	$+14.1 \pm 1.8$ (3)
NH ₃	+0.35 (2)	+0.60 (2)	$+10.3 \pm 0.3$ (5)	$+8.6 \pm 1.1$ (5)	$+1.2 \pm 0.4$ (3)	$+0.70 \pm 0.1$ (3)
Pyruvate	$+0.13 \pm 0.03$ (3)	$+0.92 \pm 0.11$ (7)	$+0.20 \pm 0.01$ (4)	$+0.71 \pm 0.08$ (8)	$+0.34 \pm 0.11$ (3)	$+0.92 \pm 0.16$ (3)
O ₂	-9.64 ± 0.24 (29)	-19.7 ± 0.33 (42)	-15.5 ± 0.43 (11)	-21.3 ± 0.46 (15)	-15.2 ± 0.89 (4)	-20.3 ± 1.26 (4)

of the total glutamine removed. Some 64% of the amino nitrogen could be accounted for as alanine and 5% as aspartate. Alanine therefore accounted for approx. 50% of the glutamine nitrogen removed. The production of NH₃ was not significantly affected by the addition of glucose.

Citrulline was not detectable as a product of glutamine metabolism under the present experimental conditions on the basis of (i) amino acid analysis with a JEOL amino acid analyser, (ii) t.l.c. and (iii) chemical analysis by the method of Archibald (1944) as modified by Jones *et al.* (1961). Because of non-separation of alanine and citrulline in test (i) the bulk of alanine was removed from the samples with alanine dehydrogenase as described by Williamson (1974) before amino acid analysis. The small peaks remaining were concluded to be residual alanine, as the calculated concentration was directly related to the initial alanine concentration of the sample. Moreover, the peaks were not increased when enterocytes were incubated under conditions most likely to promote citrulline synthesis (10 mM-glucose; 2.5 mM-ornithine; 2.5 mM-NH₄Cl; 1 mM-ADP). For test (ii) samples were first concentrated by freeze-drying after elution with aq. 2M-NH₃ from Amberlite IR-120 columns. No spot corresponding to citrulline was detectable on spraying with Ehrlich's reagent.

Automatic amino acid analyses to identify other products of glutamine metabolism gave no indication that proline was increased by the presence of glutamine.

Enzymes involved in the formation of alanine from glutamine

In the absence of flux through glutamate dehydrogenase, the rapid degradation of glutamate must be initiated by aminotransferase reactions of the type described by Krebs & Bellamy (1960) and Borst & Slater (1960). For a net production of alanine from glutamine in the enterocyte, α -oxoglutarate must form pyruvate. This involves enzymes additional to the tricarboxylic acid cycle. There are three possibilities: from malate via 'malic' enzyme (EC 1.1.1.40), from oxaloacetate via oxaloacetate decarboxylase, or from oxaloacetate via phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40). All three enzymes were present in sufficient activities to account for the formation of alanine from glutamate. Oxaloacetate decarboxylase (see Dean & Bartley, 1973) had the highest activity [$6.1 \mu\text{mol/min per g dry wt.}$ at 37°C (five observations)]. The activities of 'malic' enzyme and phosphoenolpyruvate carboxykinase were 4.0 and $2.3 \mu\text{mol/min per g dry wt.}$ respectively (means of two observations; see also data of Anderson, 1970; Tyrell & Anderson, 1971). Starvation or a high-carbohydrate or high-protein diet made no difference to the activities of the three enzymes, in contrast with parallel assays on rat liver, where the high-carbohydrate diet increased the activity of 'malic' enzyme by 13-fold.

In the intact enterocytes 1 mM-mercaptopycolinate, an inhibitor of phosphoenolpyruvate carboxykinase (DiTullio *et al.*, 1974), had no effect on alanine for-

mation, as also reported by Hanson & Parsons (1977). Presumably, therefore, either 'malic' enzyme or oxaloacetate decarboxylase could be responsible for the formation of pyruvate.

Adenine nucleotides in isolated enterocytes

The decline in O_2 uptake in rat, but not in chicken, enterocytes suggested that losses of adenine nucleotides may lead to the deterioration. At the start of the incubation the total adenine nucleotide content of rat and chicken cell suspensions was similar (Table 4). On incubation the total fell to about half the initial value within 20 min in rat enterocytes, but changed very little during the subsequent 40 min. In chicken enterocytes the fall after 20 min was only 13% and after 60 min only 21%. The fall in rat enterocytes thus does not parallel the decrease in the rate of O_2 consumption. Bronk & Leese (1973) noted similar losses of adenine nucleotides in scrapings, rings and everted sacs of rat intestine. ADP added at zero time or at 30 min produced a temporary increase in O_2 uptake, as also found by Bronk & Leese (1973), whereas ATP or adenosine had no effect. Investigation of the fate of the added ADP showed (Table 5) that it is removed very rapidly by the cells; after 2 min about two-thirds had been converted into AMP and after 40 min about 90% of the adenine nucleotides

present had been interconverted or deaminated. The same changes occurred at a somewhat lower rate in the cell-free medium obtained by centrifuging the enterocyte suspension after 20 min incubation. Thus the enzymes responsible for the decomposition of the adenine nucleotides are released into the medium at an early stage of incubation. Paper chromatography showed that IMP was the major end product, and added IMP was quantitatively recovered after 40 min incubation. Added ATP also rapidly disappeared, giving initially ADP, AMP and eventually IMP and NH_3 .

Discussion

Isolated enterocytes as experimental material

The high rate of O_2 consumption by cells prepared by the present procedure ($19.7 \mu\text{mol/min per g dry wt.}$ with glucose as substrate), at least 50% higher than the highest value recorded in the literature for rat enterocytes (Perris, 1966), is probably due to the use of dialysed albumin and of a medium containing physiological concentrations of bicarbonate and CO_2 . A loss of metabolic capacity on prolonged incubation was also noted by Stern (1966) and by Perris (1966). Bronk & Leese (1973) suggest that the loss may be caused by diffusion of adenine nucleotides and enzymes from intestinal preparations.

Table 4. *Adenine nucleotides in incubated isolated rat and chicken enterocytes*
Enterocytes were incubated with 10 mM-glucose. The data are means $\pm$ S.E.M. ($\mu\text{mol/g dry wt.}$).

Time (min) ...	Rat			Chicken		
	0	20	60	0	20	60
Number of observations ...	24	9	8	7	3	5
ATP	4.3 ± 0.5	3.1 ± 0.5	1.5 ± 0.4	5.7 ± 0.7	4.6 ± 0.5	3.8 ± 0.4
ADP	7.9 ± 0.5	3.7 ± 0.4	3.3 ± 0.6	3.5 ± 0.5	3.9 ± 1.1	3.5 ± 0.5
AMP	4.2 ± 0.5	1.8 ± 0.5	2.9 ± 0.7	2.8 ± 0.4	1.9 ± 1.2	2.0 ± 0.7
Total	16.4 ± 0.9	8.7 ± 1.1	7.8 ± 1.1	12.1 ± 0.9	10.5 ± 1.9	9.5 ± 1.3

Table 5. *Fate of ADP added to enterocyte suspension or incubation medium*

The enterocyte suspension (19.4 mg dry wt. per flask) was incubated with 10 mM-glucose for 20 min. ADP (final concentration 1 mM) was then incubated with (a) the cell suspension or (b) the cell-free medium obtained by centrifugation. The data are $\mu\text{mol/ml}$ of suspension or medium from a representative experiment.

	Time after addition of ADP (min)	ATP	ADP	AMP	Total	NH_3
	0	0.01	1.06	0.03	1.1	0.03
Suspension (a)	2	0.07	0.27	0.67	1.01	0.17
	10	0.01	0.08	0.61	0.69	0.41
	40	0.01	0.06	0.04	0.10	0.90
Medium (b)	2	0.13	0.61	0.33	1.07	0.12
	10	0.09	0.31	0.54	0.94	0.13
	40	0.04	0.04	0.67	0.75	0.60

Isolated enterocytes do not replace vascular perfusion of the intestine, if only because there are other tissue components in the intestinal wall that have important metabolic functions. In addition, in the case of enterocytes, exposure of the entire cell surface to the same environment is an unphysiological situation.

The question of citrulline synthesis in the intestinal wall

A net release of citrulline into the portal vein *in vivo* has been reported in man by Felig & Wahren (1971), in sheep by Wolff *et al.* (1972) and in dog by Weber *et al.* (1976). However, the evidence that glutamine is the citrulline precursor is inconclusive. In the experiments of Weber *et al.* (1976) release of citrulline and ornithine was balanced by uptake of arginine. Wolff *et al.* (1972) used fed animals, so that absorption of amino acids from the lumen may have contributed to the concentrations found in the portal vein. Even in starved animals hydrolysis of endogenous luminal protein can complicate arteriportal-venous-difference measurements (see Elwyn, 1970). The most convincing evidence that glutamine can be converted into ornithine remains the incorporation of ^{14}C from [^{14}C]glutamine into citrulline reported by Windmueller & Spaeth (1974) in the isolated vascularly perfused small intestine. The failure to detect citrulline synthesis in the present experiments may be taken to indicate either that any synthesis occurs in cell elements not present in the suspension, for example in the crypt cells, or that the rate is exceedingly low.

The hypothesis that synthesis of ornithine from glutamate can occur in mammals is attractive from the nutritional point of view. In the adult, arginine is not an essential amino acid. It must therefore be postulated that it can be synthesized. Smith *et al.* (1967) found synthesis from proline, but not from glutamate, in several rat tissues, especially in kidney and liver. The gut was not assayed. According to Herzfeld & Raper (1976) proline oxidase is absent from the adult. However, the small intestine possesses the enzymic machinery to form citrulline and proline from glutamate (see also Windmueller & Spaeth, 1974). In spite of this, we consider the question of citrulline synthesis in the gut to be still unanswered.

Alanine formation in the small intestine

It has been known for some time that glutamate and glutamine entering the mucosa give rise to alanine (Matthews & Wiseman, 1953; Neame & Wiseman, 1957, 1958; Aikawa *et al.*, 1973; Windmueller & Spaeth, 1974). The present data on the rates of O_2 uptake and the effect of glucose on the yields of alanine show that the carbon skeleton of alanine can be provided either by oxidative degradation of glutamate to pyruvate or by pyruvate

supplied through glycolysis. Similar conclusions were reached by Hanson & Parsons (1977). This bears on the physiological significance of the unusually high aerobic glycolysis in the intestinal mucosa, which has been known since the work of Warburg *et al.* (1924) and Dickens & Weil-Malherbe (1941). The high rate of glycolysis could not be satisfactorily explained on physiological grounds. It would now appear that it provides pyruvate needed for transamination. This in turn is important because the penetration of glutamate from the portal blood into liver cells would be too slow to clear the blood of excess glutamate, and because glutamate may be toxic to the brain if its concentration in the blood plasma rises (Lemkey-Johnston & Reynolds, 1974; Pizzi *et al.*, 1977). Alanine on the other hand is readily metabolized by the liver. Thus the mucosal conversion of glutamate nitrogen into alanine nitrogen may be looked on as a detoxication mechanism which protects the peripheral circulation from an overload of glutamate.

Site of glutamine degradation

There is a striking difference between the rates of glutamine disposal in the wall of the small intestine and in the liver. Both tissues possess a high activity of the phosphate-dependent glutaminase (EC 3.5.1.2). Intestinal mucosa utilizes plasma-derived glutamine *in vivo* (Windmueller & Spaeth, 1974), whereas there is evidence that liver does not (Lund & Watford, 1976). However, the enzymes differ in their kinetic constants. The K_m for glutamine of the intestinal enzyme is 4 mM (Pinkus & Windmueller, 1976) and 28 mM for the liver enzyme (Huang & Knox, 1976). This explains why the intestinal enzyme is much more active under normal physiological conditions. Measurements by Windmueller & Spaeth (1978) in the rat *in vivo* in the post-absorptive state show that arterial glutamine (at 0.51 mM) is removed by the small intestine at a rate of $0.191 \mu\text{mol/min per g wet wt.}$ At the same time alanine appears in the portal blood at a rate of $0.138 \mu\text{mol/min per g wet wt.}$

Note Added in Proof (Received 29 December 1978)

A paper published after the present paper was submitted (Ross *et al.*, 1978) reported the synthesis of ornithine from glutamate in homogenates of mucosa from rat small intestine. The rate in the fully supplemented system was $31.8 \mu\text{mol/90 min per g fresh wt. of tissue}$, a rate that is low enough to explain our failure to detect the formation of citrulline from glutamine under our experimental conditions.

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References

- Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–577, Academic Press, London and New York
- Addae, S. K. & Lotspeich, W. D. (1968). *Am. J. Physiol.* **215**, 278–281
- Aikawa, T., Matsutaka, H., Yamamoto, H., Okuda, T., Ishikawa, E., Kawano, T. & Matsumura, E. (1973) *J. Biochem. (Tokyo)* **74**, 1093–1095
- Anderson, J. W. (1970) *Biochim. Biophys. Acta* **208**, 165–167
- Archibald, R. M. (1944) *J. Biol. Chem.* **156**, 121–142
- Bihler, I. & Cybulsky, R. (1973) *Biochim. Biophys. Acta* **298**, 429–436
- Borst, P. & Slater, E. C. (1960) *Biochim. Biophys. Acta* **41**, 170–171
- Bronk, J. R. & Leese, H. J. (1973) *J. Physiol. (London)* **235**, 183–196
- Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181
- Cornell, N. W., Lund, P. & Krebs, H. A. (1974) *Biochem. J.* **142**, 327–337
- Dean, B. & Bartley, W. (1973) *Biochem. J.* **135**, 667–672
- Dickens, F. & Simer, F. (1930) *Biochem. J.* **24**, 1301–1326
- Dickens, F. & Weil-Malherbe, H. (1941) *Biochem. J.* **35**, 7–15
- DiTullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) *Biochem. J.* **138**, 387–394
- Elwyn, D. H. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), vol. 4, pp. 523–557, Academic Press, London and New York
- Evans, E. M., Wigglesworth, J. M., Burdett, K. & Pover, W. F. R. (1971) *J. Cell Biol.* **51**, 452–464
- Felig, P. & Wahren, J. (1971) *J. Clin. Invest.* **50**, 2703–2714
- Finch, L. R. & Hird, F. J. R. (1960) *Biochim. Biophys. Acta* **43**, 268–277
- Hanson, P. J. (1975) D.Phil. Thesis, University of Oxford
- Hanson, P. J. & Parsons, D. S. (1977) *Biochem. J.* **166**, 509–519
- Harrer, D. S., Stern, B. K. & Reilly, R. W. (1965) *Nature (London)* **203**, 319–320
- Herzfeld, A. & Raper, S. M. (1976) *Biochim. Biophys. Acta* **428**, 600–610
- Holton, D. S. & Nordlie, R. C. (1965) *Biochemistry* **4**, 723–731
- Huang, K. C. (1965) *Life Sci.* **4**, 1201–1206
- Huang, Y. Z. & Knox, W. E. (1976) *Enzyme* **21**, 408–426
- Hülsmann, W. C., Van den Berg, J. W. O. & de Jonge, H. R. (1974) *Methods Enzymol.* **32B**, 665–673
- Iemhoff, W. G. T., Van den Berg, J. W. O., de Pijper, A. M. & Hülsmann, W. C. (1970) *Biochim. Biophys. Acta* **215**, 229–241
- Ishikawa, E. (1975) *Adv. Enzyme Regul.* **14**, 117–136
- Ishikawa, E., Aikawa, T. & Matsutaka, H. (1972) *J. Biochem. (Tokyo)* **71**, 1097–1099
- Jones, M. E., Anderson, A. D., Anderson, C. & Hodes, S. (1961) *Arch. Biochem. Biophys.* **95**, 499–507
- Kimmich, G. A. (1970) *Biochemistry* **9**, 3659–3668
- Krebs, H. A. & Bellamy, D. (1960) *Biochem. J.* **75**, 523–529
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) *Alfred Benzon Symp.* **6**, 726–750
- Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, pp. 2101–2110, Academic Press, London and New York
- Lemkey-Johnston, N. & Reynolds, W. A. (1974) *J. Neuropathol. Exp. Neurol.* **33**, 74–97
- Lund, P. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, pp. 1719–1722, Academic Press, London and New York
- Lund, P. & Watford, M. (1976) in *The Urea Cycle* (Grisolia, S., Baguena, R. & Mayor, F., eds.), pp. 479–488, John Wiley and Sons, New York
- Marliss, E. B., Aoki, T. T., Posefsky, T., Most, A. S. & Cahill, G. F. (1971) *J. Clin. Invest.* **50**, 814–817
- Matthews, D. M. & Wiseman, G. (1953) *J. Physiol. (London)* **120**, 55P
- Neame, K. D. & Wiseman, G. (1957) *J. Physiol. (London)* **135**, 442–450
- Neame, K. D. & Wiseman, G. (1958) *J. Physiol. (London)* **140**, 148–155
- Neptune, E. M. (1965) *Am. J. Physiol.* **209**, 329–332
- Padron, J., Gallagher, J. T. & Kent, P. W. (1972) *Br. J. Exp. Pathol.* **54**, 347–351
- Perris, A. D. (1966) *Can. J. Biochem.* **44**, 687–693
- Pinkus, L. M. & Windmueller, H. G. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1574, abstr. no. 1118
- Pizzi, W. J., Barnhart, J. E. & Fanslow, D. J. (1977) *Science* **196**, 452–454
- Reiser, S. & Christiansen, P. A. (1971) *Biochim. Biophys. Acta* **225**, 123–139
- Rose, W. C. (1938) *Physiol. Rev.* **18**, 109–136
- Ross, G., Dunn, D. & Jones, M. E. (1978) *Biochem. Biophys. Res. Commun.* **85**, 140–147
- Sjöstrand, F. S. (1968) *J. Ultrastruct. Res.* **22**, 424–442
- Smith, A. D., Benziman, M. & Strecker, H. J. (1967) *Biochem. J.* **104**, 557–564
- Stern, B. K. (1966) *Gastroenterology* **51**, 855–864
- Stern, B. K. & Jensen, W. E. (1966) *Nature (London)* **209**, 789–790
- Stickland, R. G. (1959) *Biochem. J.* **73**, 646–654
- Tyrell, J. B. & Anderson, J. W. (1971) *Endocrinology* **89**, 1178–1185
- Warburg, O., Posener, K. & Negelein, E. (1924) *Biochem. Z.* **152**, 309–344
- Weber, F. L., Maddrey, W. C. & Walser, M. (1976) *Am. J. Physiol.* **232**, E263–E269
- Webster, H. L. & Harrison, D. D. (1969) *Exp. Cell Res.* **56**, 245–260
- Williamson, D. H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, pp. 1679–1685, Academic Press, London and New York
- Windmueller, H. G. & Spaeth, A. E. (1974) *J. Biol. Chem.* **249**, 5070–5079
- Windmueller, H. G. & Spaeth, A. E. (1975) *Arch. Biochem. Biophys.* **171**, 662–672
- Windmueller, H. G. & Spaeth, A. E. (1978) *J. Biol. Chem.* **253**, 69–76
- Wolff, J. E., Bergman, E. N. & Williams, H. H. (1972) *Am. J. Physiol.* **223**, 438–446