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DEVELOPMENT OF AMINO ACID TRANSPORT BY THE SMALL INTESTINE OF THE CHICK EMBRYO

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SUMMARY

1. Amino acid transport by the small intestine of the chick was measured using the *in vitro* tissue-accumulation method. The developmental patterns for L-valine, glycine and L-lysine differ in a number of respects. Active transport of L-lysine was detected 4 days before hatching, that for L-valine 2 days before hatching, whereas active transport of glycine was first noted 1 day after hatching. The increase in active transport near hatching, seen with each amino acid tested, was most rapid with L-valine.

2. The anaerobic glycolytic capacity of the small intestine decreased during the first week after hatching. The capacity for active transport under anaerobic conditions also decreased during this period. Immediately after hatching the rate of anaerobic transport of L-valine was 80 % of the aerobic rate and 10 days later 5 %. Although the rate of efflux and K_m were essentially constant at those stages, the v_{max} for L-valine uptake immediately after hatching was twice as great as at the later stage.

3. The active transport of L-valine was shown to be Na^+-dependent at the developmental stages examined. ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was detected in subcellular fractions of the chick small intestine as early as 4 days before hatching. Prior to hatching its specific activity increased more rapidly in the mitochondrial than in the brush-border and microsomal membrane fractions.

INTRODUCTION

Detailed studies on the development of active amino acid transport in organs with an absorptive function include those with the mammalian yolk sac[1,2], intestine[2,3] and kidney[4]. The time of appearance of active metabolite transport during embryogenesis depends upon a number of factors, including the particular transport system examined and the species employed.

There has been increasing evidence to link the transport of certain metabolites, especially sugars and amino acids, with the activity of a membrane-bound ($Na^+ + K^+$)-ATPase[7,8]. Although the development of sugar transport by the chick yolk sac and small intestine has been investigated[5,6], the development of active amino acid transport has received less attention and this is the subject of the present study. ($Na^+ + K^+$)-ATPase was assayed in various subcellular fractions of the small intestine of the chick

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to ascertain whether a relationship exists between the appearance of active amino acid transport and the activity of the enzyme.

Significant differences in developmental patterns for transport of various amino acids were observed, suggesting that the amino acid transport systems of the small intestine of the chick develop independently.

MATERIALS AND METHODS

Chick embryos, White Plymouth Rock, were purchased from the Cobb Breeding Corporation, Concord, Mass., at various embryonic stages and incubated at 39° with 60% relative humidity until used. Hatching occurs 21 days after fertilization and the developmental age is given by the number of days after fertilization, followed in parentheses by the number of days before (—) or after (+) hatching. Chicks were given water *ad libitum* at hatching, and starter mash 2–3 days later. Chicks older than day 23(+2) were fasted 12 to 24 h before sacrifice.

Embryos and hatched chicks were decapitated, the small intestine was excised from the distal end of the duodenal loop to the ileo-caecal junction, and rinsed briefly in 0.15 M NaCl at room temperature. The small intestine from chicks older than day 24(+3) was everted over a glass rod and cut into rings approx. 5 mm long[9]. Before day 23(+2) the small intestine was too fragile for eversion, therefore 5 to 10 mm segments were cut lengthwise prior to incubation. The segments were pooled and placed in erlenmeyer flasks containing 2.0 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 5.0 mM glucose and previously gassed with 95% O_2:5% CO_2 or 95% N_2:5% CO_2 (ref. 10). The total net weight of the segments never exceeded 100 mg per flask. The ^{14}C-labeled amino acid (final concentration, 1.0 mM; specific activity, 0.1 mC/mmole) was added and the flasks were gassed for 30 sec, closed immediately with rubber stoppers and incubated at 37° for 30 min. In experiments designed to test the effects of Na^+ deprivation on active transport, Tris–HCl (pH 7.4) was substituted for NaCl in the incubation medium. Segments were preincubated for 10 min with metabolic inhibitors before the addition of labeled amino acid. At the end of the incubation period, the tissue was removed, rinsed in 0.15 M NaCl at room temperature, blotted on filter paper, weighed and placed in test tubes containing 2.0 ml of distilled water. The tubes were placed in a water bath at 100° for 10 min, allowed to cool and centrifuged at 1000 × *g* for 3 min[11]. Portions (0.2 ml) of the supernatant fluids and the final incubation media were counted in 10 ml of BRAY's scintillation fluid[12] in a Packard Tri-Carb liquid scintillation spectrometer.

Total tissue water represents the difference in wet tissue weight after incubation and the tissue weight after drying at 105° for at least 12 h. The extracellular fluid space was estimated[13] by incubation of intestinal segments in medium containing [^{14}C]inulin (12.5 mC/g). Intracellular fluid represents the difference between total tissue water and the extracellular fluid. From the values of the wet tissue weight, extra- and intracellular fluids, in addition to the counts/min of amino acid in the tissue pool and medium at the end of incubation, the distribution ratio was calculated. The distribution ratio is defined as the counts/min per ml of intracellular fluid divided by counts/min per ml of extracellular fluid*.

* We wish to thank Dr. Leah Lowenstein for instruction in this technique which was developed in her laboratory.

Intestinal segments were incubated for 30 min and homogenized in 1.0 M $HClO_4$ at 0° in a Potter–Elvehjem homogenizer. The acid-soluble supernatant fluid was neutralized with KOH, and the $KClO_4$ removed by centrifugation. The solution was applied to 1 mM chromatography paper and developed in a descending system with *n*-butanol–glacial acetic acid–water (12:3:5, v/v/v) for 17 h. Amino acids were localized by dipping the paper through 0.2 % ninhydrin in acetone, and heating at 105° for 3 min[14]. The radioactive zones were localized by cutting the paper into strips 2 cm × 1 cm; these were placed in 5 ml of liquid scintillation cocktail (4 g 2,5-diphenyloxazole + 50 mg 1,4-bis-(5-phenyloxazolyl-2)-benzene/l of toluene) and counted in the scintillation counter.

The efflux of [^{14}C]valine from preloaded intestinal segments was measured by first incubating segments at 37° for 30 min with L-[^{14}C]valine as described above. The segments were removed, rinsed briefly in saline at 0°, lightly blotted and transferred to 3.0 ml of oxygenated medium without L-valine. The flasks were gassed for 30 sec and incubated at 37°. At various intervals, 0.2 ml portions of the medium were removed and counted with 10 ml of BRAY's solution. After each sampling, the flasks were re-gassed for 30 sec and the shaking continued. After 30 min the tissue was treated as described above. The results are plotted as the percentage of [^{14}C]valine remaining in the tissue segments *vs.* incubation time.

Earlier experiments indicated that the rate of uptake of L-[^{14}C]valine was linear for the first 10 min at 37°; therefore, kinetic determinations were carried out after a 5 min incubation. Intestinal segments were incubated in bicarbonate buffer with L-[^{14}C]valine (0.5–10.0 mM, specific activity 0.01–0.2 mC/mmole). The results are presented in a Lineweaver–Burk plot.

For determination of anaerobic glycolysis, intestinal segments were incubated in Krebs–Ringer bicarbonate buffer previously gassed with 95 % N_2:5 % CO_2 and containing 25.0 mM glucose. The evolution of CO_2 was measured in a Gilson constant pressure respirometer at 37° and reported as $Q_{CO_2}^{N_2}$ (μl CO_2/mg dry wt. per h); it was calculated for the first 60 min period.

The development of ($Na^+ + K^+$)-ATPase was studied in the small intestine of the chick. Small intestines from chicks younger than day 23 (+2) or mucosal scrapings from older chicks were homogenized with 40–50 ml of 5.0 mM EDTA (pH 7.4) for 30 sec at 0° with a Virtis "45" homogenizer operated at a reduced blade speed. The method of fractionation of the crude homogenate was similar to that of QUIGLEY AND GOTTERER[15]. The brush-border pellet isolated from the crude homogenate at 800 × *g* for 10 min was resuspended in 2.5 mM EDTA (pH 7.4). The mitochondrial-membrane pellet (10000 × *g* for 15 min) and the microsomal-membrane pellet (105000 × *g* for 60 min) were resuspended as above. The protein content of the suspensions ranged from 1–6 mg/ml. Examination of the fractions by phase-contrast microscopy showed that the mitochondrial and microsomal fractions were free from contamination by brush borders. ($Na^+ + K^+$)-ATPase was assayed in the membrane fractions according to the procedure of QUIGLEY AND GOTTERER[15] by subtracting the ouabain-insensitive ATPase activity from the total ATPase activity. Specific activity is expressed as μmoles P_i/mg protein per h; total activity as μmoles P_i/h per small intestine. Inorganic phosphate was determined by the method of LELOIR AND CARDINI[16], protein according to LOWRY *et al.*[17] using bovine serum albumin as standard.

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TABLE I

ACCUMULATION OF L-VALINE BY THE CHICK SMALL INTESTINE DURING DEVELOPMENT

Segments were incubated with 1.0 mM L-[^{14}C]valine at 37° for 30 min under aerobic or anaerobic conditions and treated as described in the text. Results are reported as distribution ratio (D.R.) and values are the mean of 4 determinations ± S.E. D.R. is defined as counts/min intracellular fluid divided by counts/min extracellular fluid. Age is given by number of days after fertilization followed by number of days before (−) or after (+) hatching in parentheses.

Incubation conditions	*Day 21 (hatch)*	*Day 22 (+1)*	*Day 23 (+2)*	*Day 30 (+9)*	*Day 31 (+10)*	*Adult*
Aerobic	13.0 (4) ± 0.2	8.1 (2)	5.7 (4) ± 1.2	6.0 (4) ± 0.3	5.5 (4) ± 0.3	3.0 (4) ± 0.2
Anaerobic	10.7 (4) ± 0.3	—	4.1 (4) ± 0.5	1.3 (4) ± 0.2	—	0.6 (4) ± 0.1
Aerobic, 1 mM 2,4-dinitrophenol	7.6 (4) ± 0.3	3.2 (2)	—	—	1.8 (4) ± 0.2	1.0 (4) ± 0.1
Aerobic, 0.15 mM ouabain	5.1 (4) ± 0.7	—	2.5 (4) ± 0.5	4.1 (4) ± 0.3	—	—

Ouabain, EDTA, Tris–HCl and Tris–ATP were obtained from the Sigma Chemical Company, St. Louis, Missouri. L-[^{14}C]lysine (247 mC/mmole), L-[^{14}C]valine (200 mC/mmole), [^{14}C]glycine (116 mC/mmole) and [*carboxyl*-^{14}C]inulin (2.9 mC/g) were obtained from the New England Nuclear Corporation, Boston, Mass.

RESULTS

The total tissue water of intestinal segments was found to be 86 % of the wet weight of the tissue. There was no significant change in this value at the stages examined in the present study. The extracellular fluid space was found to represent approx. 10 % of the wet tissue weight at all stages examined. The inulin space was filled after the first 10 min and rose only slightly after a further 30 min of incubation. In all calculations of the distribution ratio, the total tissue water was taken as 0.86, the extracellular fluid space as 0.10 and the intracellular fluid as 0.76 of the wet tissue weight.

Fig. 1 displays the patterns for amino acid transport. It can be seen that active accumulation of L-valine (*i.e.* distribution ratio >1) does not occur until day 19(—2), with the most rapid increase in distribution ratio occurring between day 20(—1) and day 21 (hatching). When the maximum value of 12.5 was reached, the distribution ratio declined rapidly until 3 weeks after hatching and then remained constant. The pattern for glycine transport appears to differ in a number of respects from that for L-valine. A distribution ratio of 2.0 was not reached until day 21 (hatching); it then increased slowly to a maximum ratio of 4.1 on day 24(+3). The transport of L-lysine appears to be active at the earliest stage examined, and its distribution ratio increased from 2.0 on day 20(—1) to a maximum of 6.0 on day 21, followed by a decline in distribution ratio to a value characteristic for the small intestine of the mature

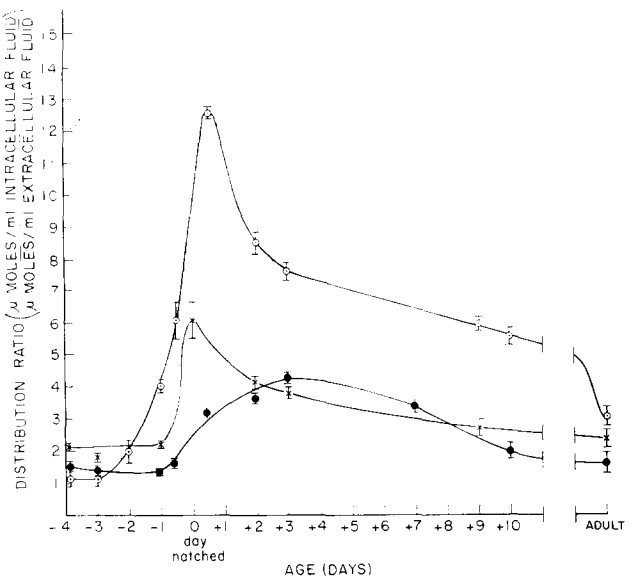

Fig. 1. Accumulation of amino acids by the small intestine of the chick during development. Segments were incubated with 1.0 mM L-[^{14}C]amino acid at 37° for 30 min. Values are the means of 3–4 determinations ± S.E. ○—○, valine; ●—●, glycine; ×—×, lysine.

chicken. Chromatographic analysis revealed that over 95 % of the accumulated amino acid could be recovered as the original compound. This was shown at two stages, day 22(+1) and day 45(+24).

Table I illustrates the effects of various incubation conditions on active transport of L-valine. There was a progressive dependence on aerobic metabolism as shown by the inhibition of L-valine transport under anaerobic conditions immediately after hatching and 10 days later. Inhibition was 20 % on day 21 (hatching) and 95 % on day 31(+10). 2,4-Dinitrophenol (1.0 mM) was also inhibitory in a similar pattern under aerobic conditions. Differences were observed in the rate of anaerobic glycolysis between various intestinal sites; therefore the results are reported separately for the three divisions of the small intestine of the chick. During the first week after hatching there was a 60 % decrease in $Q_{CO_2}^{N_2}$ for the proximal jejunum, *i.e.* 20.7 to 8.3; 36 % for the distal jejunum (14.9 to 9.5) and 27 % for the ileum (12.4 to 9.0). This is illustrated in Fig. 2. In media containing Na^+, ouabain (0.15 mM) inhibited L-valine transport, but did not depress it to the level seen in Na^+-free media. Similar observations were made in glycine and L-lysine transport studies. The inhibition of active valine transport was 90 % or greater at all stages examined when Tris–HCl was substituted for NaCl in a sodium-free medium. This inhibition cannot be explained by

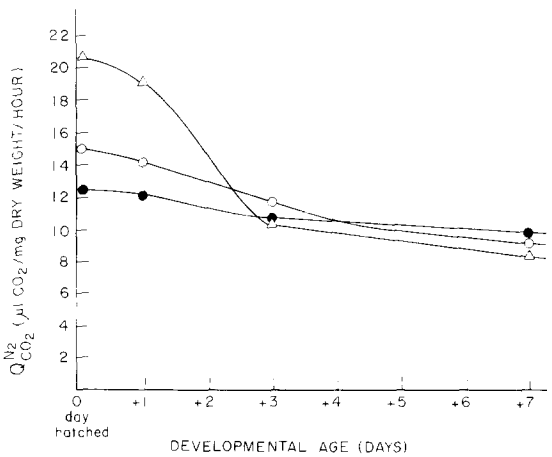

Fig. 2. The rate of anaerobic glycolysis in the small intestine of the chick during the first week after hatching. Values are the averages of 2 determinations. △—△, proximal jejunum; ○—○, distal jejunum; ●—●, ileum.

a change in the extra- or intracellular fluids since the values were unaltered in segments incubated in sodium-free medium as compared to controls incubated in Krebs-Ringer bicarbonate buffer.

Since a marked difference was noted in the active transport of L-valine by intestinal segments at day 22(+10), the kinetics of uptake and efflux of L-valine were examined at those stages. As shown in Fig. 3, the rate of efflux of valine from the day 22(+1) segments was only slightly greater than from day 31(+10) segments. Fig. 4 shows that there was no difference in the apparent K_m but the v_{max} was twice as great for day 22(+1) as compared to day 31(+10) tissue segments.

As shown in Table II, there was little change in the specific activity of the

$(Na^+ + K^+)$-ATPase in the brush-border fraction of the small intestine at the stages examined. There was a marked increase in the specific activity of $(Na^+ + K^+)$-ATPase in the mitochondrial membrane fraction from day 17(—4) to day 23(+2). An increase in the units of $(Na^+ + K^+)$-ATPase per small intestine is apparent in all the membrane fractions examined, with the most rapid increase occurring in the mitochondrial membrane fraction.

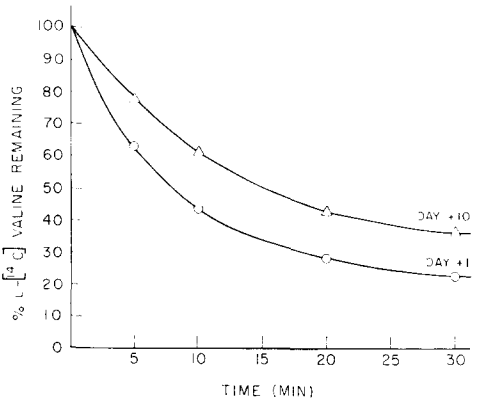

Fig. 3. Efflux of L-[^{14}C]valine from preloaded small intestinal segments of the chick.

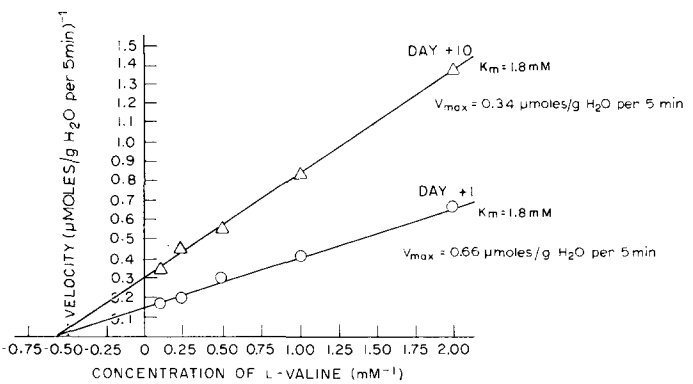

Fig. 4. Effect of L-valine concentration on initial uptake into segments of the chick small intestine. Results are presented in a Lineweaver–Burk plot and values are the means of 3 determinations.

DISCUSSION

The time of appearance of active amino acid transport in the chick embryo varies with each organ. Active transport of α-aminoisobutyric acid, glycine and leucine was reported in the chick heart[18] as early as day 5(—16). The yolk sac of the chick embryo actively accumulates glycine as early as day 7(—14), and gradually loses this ability from day 12(—9) to hatching[6].

It has been shown in the present study that the appearance of active amino acid transport in the small intestine of the chick occurs relatively late in embryonic development. The patterns of two neutral amino acids, L-valine and glycine, appear to differ in the time of appearance of active transport and the maximum distribution

TABLE II

DEVELOPMENT OF INTESTINAL ATPase

Specific activities of ATPase are expressed as μmoles P_i/mg protein per h and are reported as the mean ± S.E. with the number of determinations in parentheses. Units of ATPase are expressed as μmoles P_i/h per small intestine.

Age	*Ouabain-insensitive ATPase*			*$(Na^+ + K^+)$-ATPase*		
	Brush-border	*Mitochondrial*	*Microsomal*	*Brush-border*	*Mitochondrial*	*Microsomal*
A. *Specific activity in subcellular fractions*						
Day 17	3.9 (4) ± 0.5	11.7 (4) ± 1.1	2.6 (4) ± 0.2	1.1 (4) ± 0.1	1.7 (4) ± 0.1	0.8 (4) ± 0.1
Day 20	5.1 (4) ± 0.4	18.4 (4) ± 2.3	4.7 (4) ± 0.3	1.6 (4) ± 0.2	4.0 (4) ± 0.2	1.0 (4) ± 0.1
Day 23	8.6 (4) ± 0.3	28.9 (4) ± 1.9	13.1 (4) ± 0.7	1.4 (4) ± 0.3	7.3 (4) ± 0.1	2.1 (4) ± 0.1
Day 30	9.3 (2)					
B. *Units of ATPase in subcellular fractions*						
Day 17	6.7 (12) ± 0.1	17.3 (12) ± 1.8	5.3 (12) ± 0.3	1.6 (12) ± 0.2	2.5 (12) ± 0.3	1.6 (12) ± 0.1
Day 20	47.5 (12) ± 5.5	38.0 (12) ± 3.5	18.9 (12) ± 1.1	15.4 (12) ± 3.2	8.4 (12) ± 0.3	4.2 (12) ± 0.4
Day 23	135.0 (8) ± 8.6	165.0 (8) ± 21.9	115.4 (8) ± 11.7	22.4 (8) ± 5.4	41.6 (8) ± 4.1	18.5 (8) ± 1.6
Day 30	522.6 (2)			63.0 (2)		

ratio measured after hatching. L-Lysine was the only amino acid of those tested to be actively transported at the earliest embryonic stage examined, *i.e.* 4 days before hatching. The amino acids employed in the present study are representative of the major transport mechanisms in the small intestine and have been utilized extensively in developmental studies[1-3]. Although a limited number of amino acids was tested, these observations suggest that the major transport mechanisms develop independently during intestinal maturation. A change in the mucosal surface area of the small intestine during maturation may introduce an additional variable[19]; nevertheless, it is felt that the changes in amino acid transport observed in the present study reflect qualitative changes in the intestinal epithelium.

In the mammalian small intestine certain actively transported sugars have an inhibitory effect on active amino acid transport[20]. It has been proposed that there is a common carrier mechanism for sugars and amino acids in the brush border of the small intestine[21]. Active transport of α-methylglucoside appears earlier in the chick small intestine[6] than the amino acids examined in the present study. The distribution ratio for α-methylglucoside transport was 2.0 on day 17(—4) and rose steadily to 8.0 on day 25(+4), leveling off at 11.0 by day 35(+14). The developmental pattern for sugar transport thus differs markedly from the pattern for amino acids in the chick small intestine and therefore suggests that there are separate membrane carrier mechanisms for sugars and for amino acids.

The mature small intestine of the chicken is completely dependent on its aerobic metabolism for the active transport of amino acids and of sugars[6]. The present study demonstrates that soon after hatching the active transport of amino acids is not completely dependent on aerobic metabolism. Measurements of glycolysis showed that the rate of anaerobic glycolysis of intestinal segments was high at hatching and then declined during the first week after hatching. WILSON AND LIN[22] noted a 25 % decrease in the rate of anaerobic glycolysis in the small intestine of the rabbit during the first week after birth. Glycogen was reported to accumulate in the embryonic chick intestine[23,24] until day 18(—3) after which it was rapidly depleted, perhaps by serving as substrate to support the development and operation of transport processes in the small intestine. Thus the glycolytic pathway provides a major part of the energy needed for active transport after hatching.

After hatching, the ability of the chick small intestine to transport L-valine decreases rapidly. Part of this change may be due to the proliferation of a new population of intestinal epithelial cells displaying different transport properties[25]. The kinetic changes observed in the present study are similar to those reported for cystine transport in the small intestine of the rat after birth[26].

The relationship between the appearance of active amino acid transport and $(Na^+ + K^+)$-ATPase activity remains uncertain. It was shown that $(Na^+ + K^+)$-ATPase activity can be detected as early as day 17(—4), and that it increases rapidly in the mitochondrial membrane fraction prior to hatching. QUIGLEY AND GOTTERER[15] found the major portion of $(Na^+ + K^+)$-ATPase to be originally associated with the mitochondrial subcellular fraction in the mucosa of the small intestine of the rat, but after isolation by sucrose gradient centrifugation the final $(Na^+ + K^+)$-ATPase appeared to be located in the plasma membrane. The reason for the adherence of plasma membranes containing $(Na^+ + K^+)$-ATPase to the mitochondria during fractionation is not known.

Of particular significance in the present study is the observation that the major transport mechanisms for amino acids in the small intestine do not develop synchronously during intestinal maturation. The differences in the time of development of amino acid and sugar transport in the small intestine argue against a common carrier mechanism for amino acids and sugars.

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