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DEVELOPMENTAL ASPECTS OF CYSTINE TRANSPORT IN RAT INTESTINAL SEGMENTS

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SUMMARY

1. An increase in the uptake of [^{35}S]cystine was observed in gut segments from 7- and 25-day-old rats after incubation for 30 and 60 min with 0.1 mM cystine compared with uptake by segments from newborn and adult gut.

2. A steady state in the level of ^{35}S was reached after 30-min and 60-min incubation with gut segments from 7-day-old sucklings and adults, respectively; and the intracellular form of ^{35}S was recovered as cysteine in all stages of development.

3. Cystine transport by developing gut is less susceptible to both anoxia and sodium deprivation than the mature gut.

4. The apparent K_m of transport for both developing and adult gut was 0.167 mM cystine, suggesting that the mechanism of active transport is the same. However the v_{max} of uptake of cystine was approx. 4 times more rapid in the 7-day-old neonate than in both the 15-day old and the adult.

5. The rate of cyst(e)ine efflux from the gut was more rapid in the 7-day old than in the adult.

6. The pH range for optimum translocation of cystine into gut segments was from 6.3 to 6.5.

INTRODUCTION

The possibility that transport of cystine and cysteine by mammalian tissues is an important parameter in cystinuria and cystinosis has led to studies *in vitro* of accumulation of these sulfur amino acids by everted gut segments and sacs, and human and rat kidney slices. Two reports, one by THIER *et al.*[1] and the other by ROSENBERG *et al.*[2], showed impaired transport of cystine and other dibasic amino acids in human intestinal mucosal biopsies studied *in vitro*; whereas ROSENBERG, CRAWHALL AND SEGAL[3] showed that active intestinal transport of cysteine in cystinuric patients was normal. The latter report demonstrated that separate mechanisms are involved in transport of cystine and cysteine by the human gut. Despite the fact that there appears to be separate mechanisms, however, the transported extracellular cystine is recovered as intracellular cysteine in studies *in vitro* with gut segments and everted gut sacs, as well as with other tissues[3–5].

Studies of the structural and physiological development of the small intestine of the rat have shown morphological changes and post-natal absorption of macromolecules up to 20 days after birth[6–9], but little work has been reported on changes in amino acid transport in intestine of neonatal rats. The purpose of the present investigation, therefore, has been to delineate the developmental aspects of cystine transport by the gut to determine whether or not rates and mechanisms of cystine transport in the young differ from those in the adult.

MATERIALS AND METHODS

Animals

Sprague–Dawley adult and neonatal rats were purchased from Huntingdon Farms, West Conshohocken, Pa. Pups were weaned when 21 days old and thereafter maintained on a Purina rat chow diet and water *ad libitum*.

Chemicals

Cystine, cysteine·HCl and *N*-ethylmaleimide were purchased from Calbiochem, Los Angeles, Calif. L-[^{35}S]Cystine, with a specific activity of 34 mC/mmole, was obtained from Schwarz Bio-Research, Orangeburg, N.Y. The purity of each lot of radioactive cystine was checked by high-voltage electrophoresis, as previously described[3]. [*carboxy*-^{14}C]Inulin, with specific activity of approx. 1.32 mC/g inulin, was obtained from New England Nuclear, Boston, Mass.

Experimental procedure

Animals were killed by decapitation. Immediately after an adult was killed, a 125-mm section of the jejunum, 140 mm below the ligament of Treitz, was removed and segments were prepared after eversion on a glass rod[10]. In experiments with neonates, the jejunal sections were removed starting approx. 50–70 mm below the ligament of Treitz, and Clay-Adams intramedic tubing, approx. 125 mm long and wide enough for easy insertion into the gut section, replaced the stirring rod. The remainder of the procedure was the same.

Immediately before beginning the study, the gut sections were cut into segments 1–5 mm in length. Adult tissue pools consisted of three segments, one from each of three animals. In studies with neonatal tissues, the pool consisted of 4–6 segments from each of 4–6 pups. The total wet weights of pooled tissues from adults ranged from 15 to 25 mg and from neonates from 60 to 20 mg per incubation bottle. Segments were incubated in 25-ml Nalgene bottles containing 2 ml Krebs–Ringer bicarbonate buffer (pH 7.3) and either 0.1 mM [^{35}S]cystine containing 0.7 μC/ml or [*carboxy*-^{14}C]-inulin with 0.25 μC/ml incubation medium. The bottles were gassed for 30 sec with O_2:CO_2 (95:5), capped immediately with rubber stoppers and incubated for 30 min in a Dubnoff shaker at 37° unless stated otherwise.

At the end of the incubation period, tissues were removed, rinsed in saline, blotted, weighed and treated in one of two ways: either immersed in distilled water for equilibration of the free amino acid content of the tissue with water by a method previously described[11], or homogenized for 45 sec in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 20 mM *N*-ethylmaleimide followed by precipitation of tissue

proteins with 1 ml of 10 % trichloroacetic acid. Both treatments required centrifugation for 6 min at 3000 × *g*. A 0.2-ml sample of the tissue pool supernatant and a 0.2-ml sample of the corresponding incubation medium were each counted in a liquid scintillation spectrometer.

A 0.05-ml sample of the supernatant from tissue homogenation in *N*-ethylmaleimide and trichloroacetic acid deproteinization was spotted on Whatman 3MM paper and run for 2 h in a Gilson high-voltage electrophorator (4000 V, 250 mA) in 6.8 % formic acid in water, as previously described[3]. At the end of the run, the paper was air dried, the paper strips were marked according to the cystine and cysteine–*N*-ethylmaleimide standards simultaneously chromatographed. The marked areas were cut into small sections, placed in counting vials, and counted in a liquid scintillation spectrometer. The percentage of labeled cysteine–*N*-ethylmaleimide was based on the percentage of radioactivity in cysteine–*N*-ethylmaleimide compared with the total radioactivity recovered in cystine and cysteine–*N*-ethylmaleimide after correction for cystine trapped in the extracellular fluid tissue space[12].

Total tissue water represented the difference between the wet tissue weight and the tissue weight after drying under vacuum on a platinum weighing pan at 100° for 24 h. This value was expressed as percent wet tissue weight.

Extracellular space was based on the volume of distribution of [*carboxy*-^{14}C]-inulin. The percentage of extracellular space was calculated from the total radioactivity recovered from the aqueous tissue supernatant (counts/min per ml) divided by the wet tissue weight. Calculations were similar to those reported previously[13]. The values for intracellular fluid space represent the difference between total tissue water and extracellular fluid space.

$[^{35}S]_{in}/[^{35}S]_{out}$ is the ratio of the concentration of ^{35}S per ml of intracellular fluid to that per ml of medium, calculated as previously described[1].

In studies of the effects of different substrate concentrations on cystine transport, $[^{35}S]_{in}/[^{35}S]_{out}$, corrected for the diffusion component of transport, were divided by the initial specific activities of cystine to determine the μmoles cystine transported per ml of intracellular fluid. These data were converted to μmoles cystine transported per min and were plotted according to Lineweaver–Burk to determine the apparent K_m and v_{max} of the complex system. Studies on the effects of anaerobiosis were carried out in Krebs–Ringer bicarbonate buffer (pH 7.3) gassed with N_2–CO_2 (95:5).

In efflux studies, gut segments were incubated for 30 min at 37° in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.3) containing 0.1 mM [^{35}S]cystine. The tissues were removed, washed rapidly in saline, lightly blotted and transferred immediately to 3 ml Krebs–Ringer bicarbonate buffer. The new bottle was gassed for 30 sec with O_2:CO_2 (95:5) and incubated in a Dubnoff metabolic shaker at 37°. At 4-min intervals, a 0.2-ml aliquot of the incubation medium was withdrawn and placed in a scintillation counting vial. The bottle was re-gassed for 30 sec and returned to the shaker until the next time period, when the procedure was repeated.

In studies on the effects of pH, no one buffer was appropriate[14]. At pH above 7.4, the sodium bicarbonate of Krebs–Ringer buffer was replaced by 0.1 M Tris buffer of the appropriate pH. At pH 5.0 and 5.5, 0.1 M sodium acetate replaced the bicarbonate. At pH 6.0, 6.5 and 7.0, Krebs–Ringer phosphate buffers were used.

RESULTS

Total tissue water and extracellular and intracellular fluid spaces

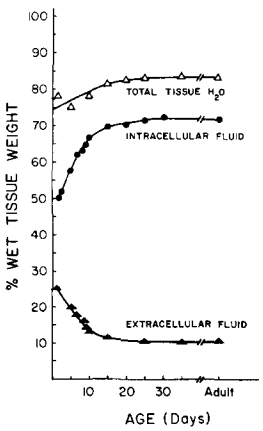
Changes during development in the total tissue water, intracellular and extracellular fluid spaces of jejunal segments are plotted in Fig. 1. It shows the increase in total tissue water and intracellular space with the concomitant decrease in extracellular space from the first to the tenth day of development with no significant changes from the 10th day to adulthood. Although in the early neonatal stages, a larger extracellular fluid space could be a reflection of membrane disruption, since up to the 10th day after birth the tissue is fragile and does not have the rigidity of the adult gut, electron micrographs at 60300 magnification show the membranes to be intact.

The effects of varying times of incubation on extracellular fluid space also were investigated. The extracellular space in gut segments from 7- and 8-day-old neonates increased from 12 to 17 % after 15–45-min incubation, respectively. Segments from adult gut, however, showed no significant changes in extracellular fluid space over a 60-min incubation period.

In considering the effects of pH on active transport, changes in extracellular fluid space were also determined. The data showed that at pH from 8 to 5 there was no significant difference in the extracellular fluid space of adult gut incubated 30 min; however, the gut from 7-day olds showed a gradual increase in extracellular fluid space from 18 to 23 % when incubated in buffers ranging from pH 7 to pH 5, respectively.

Pattern of cystine transport by the intestinal segments during development

The results of studies of cystine transport in developing gut are plotted in Fig. 2,



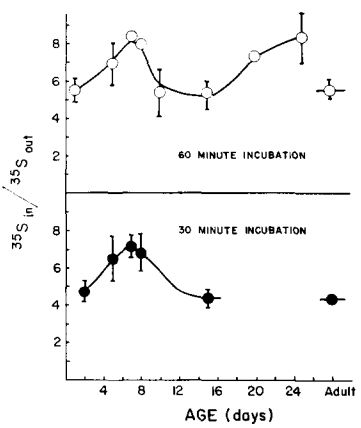


Fig. 1. Changes in tissue fluid spaces of rat gut segments during development after incubation in Krebs–Ringer bicarbonate buffer (pH 7.3) for 60 min with inulin (see MATERIALS AND METHODS). Total tissue water is expressed as percent of wet tissue weight. The extracellular fluid values are based on determinations of inulin spaces and are plotted as percent of wet tissue weights. Intracellular fluid spaces represent the difference between percent total tissue water and percent extracellular fluid space. Each point represents the average of 6 determinations.

Fig. 2. Uptake of 0.1 mM [^{35}S]cystine by gut segments from developing rats. For procedure, see MATERIALS AND METHODS. Complete data appear in Table I.

TABLE I

CYSTINE TRANSPORT BY INTESTINAL SEGMENTS

$[^{35}S]_{in}/[^{35}S]_{out}$ is the ratio of the concentration of ^{35}S per ml of intracellular fluid to that per ml of medium.

Incubation time (min)	*Animal age (days)*	*Number of experiments*	*Cystine recovered as intracellular cysteine* (%)*	*Animals per experiment*	$[^{35}S]_{in}/[^{35}S]_{out} \pm S.E.$
30	Adult	15	100	3	4.39 ± 0.19
60	(35 to 40)	6	100	3	5.61 ± 0.35
60	1	4	100	6	5.49 ± 0.60
30	2	3		6	4.72 ± 0.46
30	5	3		6	6.53 ± 1.16**
60	5	5	100	6	6.92 ± 1.07
30	7	5		6	7.11 ± 0.50***
60	7	1	100	6	8.35
30	8	3		6	6.79 ± 0.99***
60	8	1		6	7.90
60	10	4	100	6	5 28 ± 1.24
30	15	5		6	4.39 ± 0.45
60	15	5	100	6	5.19 ± 0.70
60	20	3	100	6	7.18 ± 0.26***
60	25	4	100	6	8.24 ± 1.36***

* Corrected for cystine trapped in extracellular tissue space[12].

** $P < 0.02$ compared to adult tissue incubated the same time, as determined by Student's t distribution by the Small sample method which applies the Bessel correction.

*** $P < 0.01$.

and the data are recorded in Table I. The plot shows a diphasic increase in cystine transport; the first rise appearing with gut segments from 7-day-old rats and the second phase when the animals are 25 days old. An examination of Table I points out two important facts: (1) that there is a wide range of variability in cystine transport by developing gut, and (2) that the increase in transport by gut segments from 7- and 25-day-old animals is highly significant.

Intracellular cystine–cysteine ratios

The data presented in Table I show that at all stages of development 100% of the intracellular ^{35}S is recovered as cysteine–*N*-ethylmaleimide after reaction of the tissue pool with *N*-ethylmaleimide and electrophoretic separation of cysteine–*N*-ethylmaleimide and cystine. Since [^{35}S]cystine uptake results in intracellular [^{35}S]-cysteine accumulation, and hence a difference between the intracellular and extracellular molecular species, distribution ratios for [^{35}S]cystine represent a radioactivity gradient only. One molecule of cystine transported is represented by two molecules of intracellular cysteine. The assumption has been made that the reduction of cystine is rapid and not rate limiting to the transport process.

Kinetics of cystine uptake

More thorough studies were undertaken to determine whether there were differences in both the steady-state ^{35}S and rate of cystine uptake in 7-day, 15-day and adult tissues. The results appear in Fig. 3. A steady state in ^{35}S transport into gut

segments from 7- and 15-day-old animals was reached after 45-min incubation. However, in the adult, accumulation of ^{35}S continued to increase beyond this period of time.

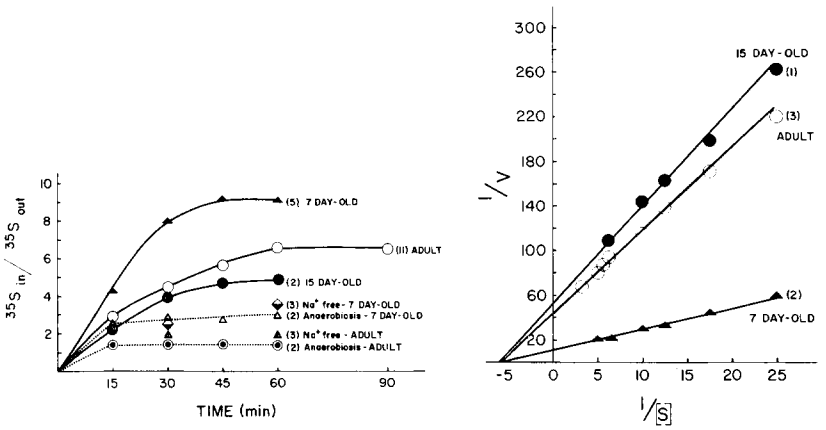


Fig. 3. Uptake of 0.1 mM [^{35}S]cystine by gut segments from 7- and 15-day sucklings and adults after varying periods of incubation in Krebs–Ringer bicarbonate buffer (pH 7.3) at 37° aerobically, together with studies of [^{35}S]cystine uptake by 7-day-old and adult rats under anaerobiosis and sodium deprivation. The number of experiments are in parentheses.

Fig. 4. Lineweaver–Burk plot of data derived from calculations described in text. $1/[S]$ expressed as 1/mM cystine, $1/v$ expressed as min/mM cystine. In an experiment, each determination represented the average of triplicate values at each substrate concentration with tissue pools from 4 animals. The number of experiments are in parentheses.

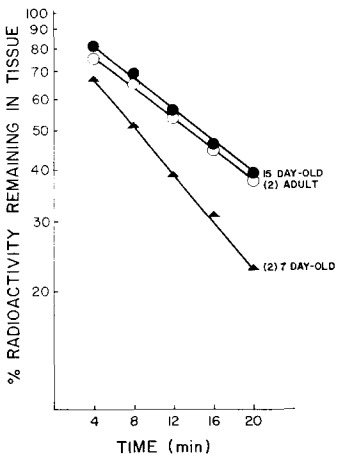
Since the velocity of cystine transport in 7-day-old sucklings is different from adults, the possibility that there are different mechanisms of cellular ^{35}S accumulation was considered. Various concentrations of cystine were incubated for 30 min with gut segments from 7-, 15-day olds and adults. Fig. 4 is a Lineweaver–Burk plot of values calculated as described in MATERIALS AND METHODS of this report. An apparent K_m value of 0.167 mM cystine for uptake of cystine in gut segments from developing and adult rats with v_{max} of 0.091 mM cystine/min, 0.019 mM/min and 0.023 mM/min for 7-day old, 15-day old and adults, respectively, shows that the maximum rate of transport in the 7-day neonate is about 4 times more rapid than in the 15-day old and adult. Although these numerical values are derived from studies of crude and complex systems, the presence of different mechanisms of transport seems highly improbable.

Comparison of efflux rates

In view of the reported morphological differences between the jejunum of the developing and adult rat[6–10] and the possibility that differences in rate and the pathway of efflux might exist were considered. The results of such an investigation are plotted in Fig. 5. The studies reveal that there is a more rapid efflux of cyst(e)ine from jejunal segments from 7-day-old animals than from either 15-day neonates or adults. Considering the segment to be a two compartment system, the fact that the steady-state ^{35}S levels are higher in the 7-day-old tissue compared to the adult (Fig. 3) in the face of higher efflux rates in the 7-day old indicates the influx rate in the latter is increased to a greater extent than the efflux rate.

Effects of anaerobiosis, sodium deprivation and buffers of different pH on [^{35}S]cystine transport

The effects of sodium deprivation and anaerobiosis are recorded in Fig. 3. The data show that when adult gut segments are incubated either under anaerobiosis or in sodium-free medium, $[^{35}S]_{in}/[^{35}S]_{out}$ approximated 1, which indicated the lack of a concentration gradient and active transport. However, gut segments from 7-day neonates transported cystine actively at approx. 45 % of their normal rate during the first 15 min of incubation. The effect of pH on accumulation of ^{35}S is presented in Fig. 6. The graph shows that cystine transport is optimum from pH 6.3 to 6.5 and does not differ in 7-day-old or adult tissue.



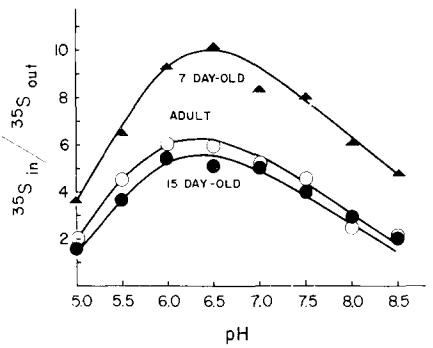


Fig. 5. Semilogarithmic plot of ^{35}S efflux from intestinal segments. The procedure is described under MATERIALS AND METHODS. Each point represents the mean of 4 samples. The number of experiments are in parentheses.

Fig. 6. Effects of pH on cystine transport. The number in parentheses represented the number of experiments. The value from each experiment was the average of triplicate determinations. The tissue pool per bottle was from 4–6 animals.

DISCUSSION

These studies show both similarities and differences in [^{35}S]cystine transport during development of the small intestine of the rat. The differences are in the velocity of [^{35}S]cystine uptake, in the rate of efflux of [^{35}S]cyst(e)ine, and in the effects of anaerobiosis and sodium deprivation on [^{35}S]cystine transport into gut segments from 7-day suckling and adults. The similarities are in the apparent K_m of transport, the fact that extracellular cystine always is recovered as intracellular cysteine, and that the optimum pH range of 6.3–6.5 for ^{35}S accumulation in gut segments is the same in the developing and adult jejunum. Based on these findings, the pattern of cystine uptake during development seems to be due to changes in the rate of transport rather than the site of transport or differences in the nature of the intracellular labeled material. The higher rate of efflux during a time of higher steady state in the gut of the 7-day-old suggests that the influx rate is increased to a greater extent than the efflux rate.

The presence of a concentration gradient in intestinal segments from the 7-day suckling compared with no active transport into adult gut under anaerobiosis recalls the findings of FAZEKAS, ALEXANDER AND HIMWICH[15], who reported that survival time in newborn rats decreased from 20 min in the 1-day old to about 8 min in the 8-day old. MOTT[16] and WILSON AND LIN[17] corroborated these findings and hypothesized that a more rapid rate of glycolysis resulting in increased lactate production, might in part explain the increased survival time in the newborn as compared with the adult.

It is important to point out that the observed increase in [^{35}S]cystine uptake in gut segments from 7- and 25-day-old rats compared with those from the adult does not parallel the gradual lengthening of microvilli or the development of the absorptive epithelial cell. CLARK[6] reported that approx. 18 days after birth, the absorptive cells no longer contained large vacuoles and numerous tubules but had all the attributes of adult intestine. KOLDOVSKY, SUNSHINE AND KRETCHMER[9] presented stained autoradiographs and data to substantiate his content on that in the intestines of 7-, 11- and 12-day sucklings, the rate of cellular proliferation and division proceeds more slowly than in the weaned animal. If increased cystine uptake were due to the immaturity of the cell one might expect higher rates of uptake at term with a gradual decrease to the normal level by the 18th day after gestation. If uptake were dependent on cellular proliferation, conversely, one would expect very low levels of uptake in the 7-day suckling.

DOELL AND KRETCHMER[18], LECCE[8], HERINGOVA, JIRSOVA AND KOLDOVSKY[19], PELICHOVA *et al.*[20] and ESTERLY[21] have reported postnatal changes and development of enzymatic activities in the small intestine of the rat. DOELL AND KRETCHMER[18] reported that β-galactosidase activity increases from 18 days before gestation until 5 days after term when the activity becomes maximum. After the 5th day, activity decreases until the 18th day when the enzyme activity is comparable with that of the adult. KOLDOVSKY and his co-workers studied the development of intestinal alkaline phosphatase, lipolytic, proteolytic, dipeptidase, and tripeptidase, β-glucuronidase and β-glucosidase activities. In general, they found that the main changes in activities occurred between the 15th and 20th day postnatally. Recently, ESTERLY[21], through histochemical studies of rat gut, showed minimal galactosidase activity in the intestinal mucosa at birth with an increase to high levels by the 4th through the 7th days and a decrease to adult levels after the 14th day. The increased rate of cystine uptake in jejunal segments at one period of time during development, therefore, is not an unusual phenomenon; however, up to the present time a period of increased enzymatic activity after weaning has not been reported for any other enzyme system.

We do not know whether these changes occur with all amino acids or whether these differences in transport have functional counterparts in intestinal absorption. However, there are some studies in the human which indicate that the latter might be true since feeding experiments with different types of cystinurics having low or no transport also had defective cystine absorption[2]. It may be that such increased absorption at a particular stage in development may be related to the body's metabolic demands for amino acids at this particular time.

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