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Kinetics of Uptake of L-Leucine and Glycylsarcosine into Normal and Protein Malnourished Young Rat Jejunum

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Summary

The impact of malnutrition on peptide and amino acid absorption has been studied in the immediate postweaning period. At this time peptide uptake is quantitatively more important than amino acid uptake and the vulnerability of the infant to malnutrition is great. Everted rings of rat jejunum were used to investigate the uptake of the peptide glycylsarcosine (Gly-Sar) and the amino acid L-leucine. The animals had been weaned on to isocaloric diets containing 18% or 4% protein. The rats deprived of protein at this age showed a marked growth disturbance with considerable reduction in gut length in addition to poor weight gain. Mediated influx of Gly-Sar and leucine per centimeter of jejunum was reduced in the malnourished animals: V_{max}, 77 ± 7.1 (SEM) and 65 ± 3.6 compared with 85 ± 10.6 and 77 ± 4.4 nmol·min⁻¹·cm⁻¹·, respectively. But, when expressed in relation to body weight, the maximal transport capacity showed a marked increase with malnutrition, values being 126 and 111 nmol⁻¹·cm⁻¹·100 g⁻¹ body weight compared with 39 and 35 nmol⁻¹·cm⁻¹·100 g⁻¹ body weight for Gly-Sar and leucine respectively.

Abbreviation

Gly-Sar, glycylsarcosine

The onset of malnutrition in the immediate postweaning period in infancy is a common problem in the non-industrialised world. One of the crucial factors determining the malnourished infants potential for catch-up growth is the capacity to absorb

protein. The kinetics of peptide and amino acid uptake in postweaning malnutrition or even in young animals has not been studied extensively. Rubino and Guandalini (14) showed that peptide absorption may be quantitatively more important in very young animals than in adults whereas Himukai et al. (5) showed that intact peptide absorption was greater in the suckling and weanling guinea pig than in adults although there was no difference in amino acid absorption. Their studies were, however, confined to a limited range of concentrations and were not corrected for non-mediated transport.

The impact of protein restriction with or without calorie deprivation on amino acid and peptide transport has been investigated in adult man (1) and experimental animals (4, 6, 7, 8, 12, 15, 16, 18). The results have been conflicting, but in general, peptide transport was shown to be less affected than amino acid transport (7, 8, 9).

The present paper reports the results of an investigation into the kinetics of influx of an amino acid and a dipeptide into jejunum from young weanling rats subjected to protein malnutrition, a situation in which the structural and functional effects on the gut are considerable. Gly-Sar was chosen as the dipeptide as it is very resistant to hydrolysis and the complicating effects of uptake of amino acid released by hydrolysis at the brush-border are small. Any extracellular hydrolysis of Gly-Sar by enzymes released into the incubation medium was suppressed by working at pH 5.0 (11, 19). L-Leucine was the amino acid used. It was preferred to glycine because it had a higher affinity for uptake.

MATERIALS AND METHODS

Animals. Weanling (20-d-old), male, Wistar rats (Specific Pathogen Free), weighing approximately 50 g, were housed three rats per cage. Food, in powder form, was presented in small pots and both food and water were allowed *ad libitum*. Animals were maintained on the diets for 4 wk and weighed weekly. The experiments were carried out on two groups of animals. Animals in Group A, were used for the measurement of inulin space and the uptake of Gly-Sar whereas those in group B were used for the measurement of the uptake of leucine.

Diet. The compositions of the normal and low protein diets are shown in Table 1.

Chemicals. [1-^{14}C]Gly-Sar was kindly supplied by Dr S Wilkinson of the Wellcome Research Laboratories, Beckenham, Kent, U.K. Labeled L-[1-^{14}C]leucine was obtained from the Radio Chemical Centre, Amersham, Bucks. Unlabeled Gly-Sar and L-leucine was obtained from the Sigma Chemical Co., St. Louis, Missouri, U.S.A. All other reagents were analytical or scintillation grade.

Experimental procedure. Rats were killed by cervical fracture and the whole small intestine was removed. After stripping off the mesentery the length was measured by laying the gut horizontally without stretching and without eversion alongside a ruler on a sheet of filter paper. Jejunum was taken to be the segment of intestine between 15–30-cm distal to the pylorus. A segment about 2 cm long was cut from this section, rinsed, cut open, blotted, and weighed on tared aluminum foil. The segment was then placed in an oven at 105°C for 24 h, cooled, re-weighed and the dry weight measured. A second small segment of jejunum was placed in formol-saline for subsequent microscopic examination.

The experimental procedure and measurement of 2-min uptake of peptide into rings of everted jejunum were as previously described (10, 11, 17, 19). Rings were incubated in 0.5 ml of substrate for 2 min at 37°C under O_2, with shaking at 100 stroke/min. After removal from the incubation medium, the rings were rinsed in NaCl solution (154 mmol/l) at 4°C, blotted, and eluted for 5 min in 1 ml of sulphosalicylic acid (60 g/l) in a boiling water bath.

After centrifugation, 0.5 ml of supernatant was added to 15 ml of scintillation fluid (Packard Scintillator 299) Packard Instruments Limited, Caversham, Berks., U.K. and radioactivity was measured in a liquid scintillation spectrometer (Packard Tricarb Model 3380). Uptake was expressed as $nmol \cdot min^{-1} \cdot cm^{-1}$ or as $nmol \cdot min^{-1} \cdot cm^{-1} \cdot 100 \ g^{-1}$ body weight after correction for substrate in the inulin space, 5.4% ($n = 9$) and 6.5% ($n = 9$) at 2 min for controls and low-protein-fed animals respectively. All estimates of uptake were the mean value of measurement of uptake into at least 12 rings from at least 12 animals.

Estimation of non-mediated uptake. The method of estimating non-mediated uptake was as described by Matthews et al. (11). In this method the substrate was treated as a competitive inhibitor of its own mediated transport and the results were plotted in a form described by Preston et al. (13). After extrapolation to an infinitely high concentration of "inhibitor" the uptake remaining gave the non-mediated uptake. Subtraction of the non-mediated uptake from total uptake gave mediated uptake.

Calculation of kinetic constants. Kinetic constants were calculated by weighted non-linear regression analysis of the data, after correction for non-mediated uptake, by the method of Duggleby (2).

Statistical analysis. The significance of the difference of means was determined by the *t* test and the significance of the difference between uptake curves by the method of Fisher (3).

RESULTS

Morphologic data. The control rats grew steadily throughout the period of observation reaching a mean weight of 220 ± 5.0 g SEM in Group A and 222 ± 4.6 g in group B at wk 7 (Table 2). The rats on the low protein diet on the other hand grew very little, only reaching a mean weight of 61 ± 1.6 g in Group A and 59 ± 1.5 g in Group B at wk 7. The animals on the low protein diet remained active, had no diarrhoea, and none died as a result of the restricted diet.

The low protein diet produced a significant reduction ($P < 0.001$) in the total length of the small intestine, in both Groups A and B (Table 3). There was also a significant reduction ($P < 0.001$) in the thickness of the jejunum in these animals, shown by the increase in the length of intestine per g of jejunum. The significant decrease ($P < 0.001$) in weight/cm of the jejunum was not the result of an alteration in water content as the difference in dry weight, as a percentage of wet weight, between the controls and the low-protein-fed rats was not significant.

Histologic examination of sections of the jejunum stained with haematoxylin and eosin indicated a reduction in thickness of all components of the gut wall. There was a marked reduction in villous height (partial villous atrophy) a finding in human malnutrition.

General treatment of transport results. Values for total influx of [^{14}C]Gly-Sar and [^{14}C]leucine into jejunum from control and

Table 1. *Composition of standard and low protein diets**

	Control diet (%)	Low protein diet (%)
Maize starch	51.6	65.7
Casein	18.1	4.0
D-Glucose	10.9	10.9
Cellulose	9.1	9.1
Corn oil	4.5	4.5
Salts	4.5	4.5
L-Methionine	0.18	0.18
Vitamins	1.1	1.1

* The diets were fortified with the following vitamins: Vitamin A, Ergocalciferol, Vitamin E, Thiamine, Riboflavin, Vitamin B_6, Calcium pantothenate, Vitamin B_{12}, choline chloride, and Vitamin K; and with the following salts: KH_2PO_4 $CaCO_3$ NaCl, $MgSO_4$, $FeSO_47H_2O$, $MnSO_44H_2O$, KI, $CuSO_4$, $ZnCO_3$, and $CoCl_2$.

Table 2. *Change in body weight in rats on standard and low protein diets*

Group	Diet	Number	3	4	5	6	7
			Wk postpartum [mean weight (g) ± SEM]				
A*	Standard	15	52 ± 0.91	91 ± 2	136 ± 3	184 ± 4	220 ± 5
	Low protein	17	51 ± 0.8	51 ± 0.8	53 ± 1.2	57 ± 1.6	61 ± 1.6
B†	Standard	12	51 ± 7.5	91 ± 2.3	134 ± 2.3	178 ± 3	223 ± 4.6
	Low protein	12	52 ± 0.9	53 ± 1.1	54 ± 1.2	56 ± 1.3	59 ± 1.5

* Group A animals used in measurement of inulin space and uptake of glycylsarcosine.
† Group B animals used in uptake of leucine.

Table 3. *Changes in total length, weight, and water content of intestine from rats fed on standard and low protein diet (values are mean ± SEM)*

Group	Diet	Number	Total length small intestine (cm)	Length intestine per g wet wt (cm)	Weight of intestine per cm (g)	Dry weight % of wet weight
A*	Standard	12	104 ± 2	22.01 ± 0.74	0.0420 ± 0.0013	21.7 ± 0.5
	Low protein	14	77 ± 1‡	33.46 ± 1.21‡	0.0304 ± 0.0010‡	21.2 ± 0.3
B†	Standard	12	110 ± 2	22.86 ± 0.62	0.0440 ± 0.0012	21.70 ± 0.62
	Low protein	12	77 ± 1‡	38.84 ± 1.18‡	0.0257 ± 0.00075‡	21.70 ± 0.33

* Group A rats used in uptake of glycylsarcosine.
† Group B rats used in uptake of leucine.
‡ $P < 0.001$.

low-protein-fed rats were plotted by the method of Hofstee (V against V/S) where V is the rate of influx and S is the concentration of substrate. In all cases the Hofstee plots were obviously biphasic. A typical example is shown in Figure 1 for the Hofstee plot of influx of Gly-Sar into jejunum from the low-protein-fed rats. After correction for non-mediated uptake all the Hofstee plots became linear and it was possible to derive values for apparent K_t and V_{max} for mediated influx by the method of Duggleby (2) (Table 4).

Although this method gives standard errors of the parameters, these are only an approximate guide to the precision of the parameters and cannot be relied on to determine whether or not differences in parameter values are statistically significant (2). Individual t tests at each concentration showed that at some concentrations, but not all, uptake was significantly lower in the low-protein-fed animals. To test if the aggregate of these probabilities was significant, a method described by Fisher (3) was used. In this method the aggregate of the independent probabilities is tested for significance using χ^2 tables. It was found that in the case of Gly-Sar the probability of the aggregate of the 12 tests occurring by chance was less than 0.001 and in the case of leucine less than 0.01. Finally, observation of the curves (Fig. 2) revealed that for Gly-Sar and leucine uptake, from 11 out of the 12 concentrations studied in the protein malnourished rats, uptake was lower than in the control rats. By a simple sign test this observation is highly significant ($P < 0.005$).

The results show that in the protein malnourished animals, although mean body weight is approximately 73% less, gut length is only 30% and maximal transport capacity per cm only 10% less than in the control animals (Table 3 and 4); therefore, transport is preserved relative to body weight. Sugiyama *et al.* (18) suggested that body weight may be a better basis for expressing uptake in animals on different dietary regimes that result in gross differences in body weight. It can be seen from Table 4 that, expressed on the basis of body weight, there is a 3-fold increase in both peptide and amino acid uptake in the protein malnourished animals compared with the controls.

DISCUSSION

The effects of dietary restriction on the kinetics of uptake of peptides and amino acids in the young animal have not been reported previously. Our results show that the effects of protein malnutrition on the young rat are different from those on the adult rat both morphologically and physiologically. Protein dep-
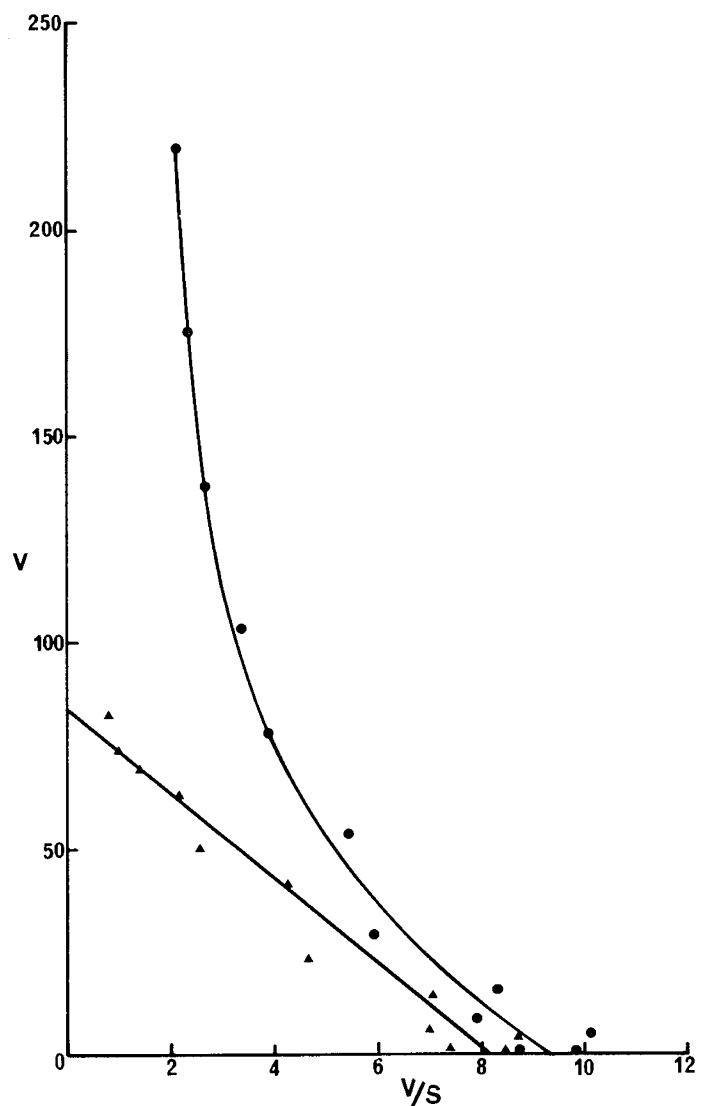

Fig. 1. Hofstee plots of uptake from [^{14}C]Gly-Sar before (●——●) and after correction for the non-mediated component of uptake (▲——▲) by jejunum from low protein fed rats.

Table 4. *Kinetic constants (±SE) and non-mediated transport for glycylsarcosine (Gly-Sar) and leucine uptake in rats fed standard and low protein diet*

Substrate	Diet	K_t (mmol/l)	V_{max} (nmol·min^{-1}·cm^{-1})	V_{max} (nmol·min^{-1}·cm^{-1}·100 g body wt^{-1})	Non-mediated transport (nmol·min^{-1}·cm^{-1}· mmol^{-1})
Gly-Sar	Standard	8.5 ± 1.31	86 ± 10.61	39 ± 4.8	2.18
Gly-Sar	Low protein	9.5 ± 1.01	77 ± 7.1	126 ± 11.6	1.34
Leucine	Standard	5.5 ± 0.48	77 ± 4.4	35 ± 1.9	0.358
Leucine	Low protein	6.7 ± 0.56	65 ± 3.6	111 ± 6.0	0.0978
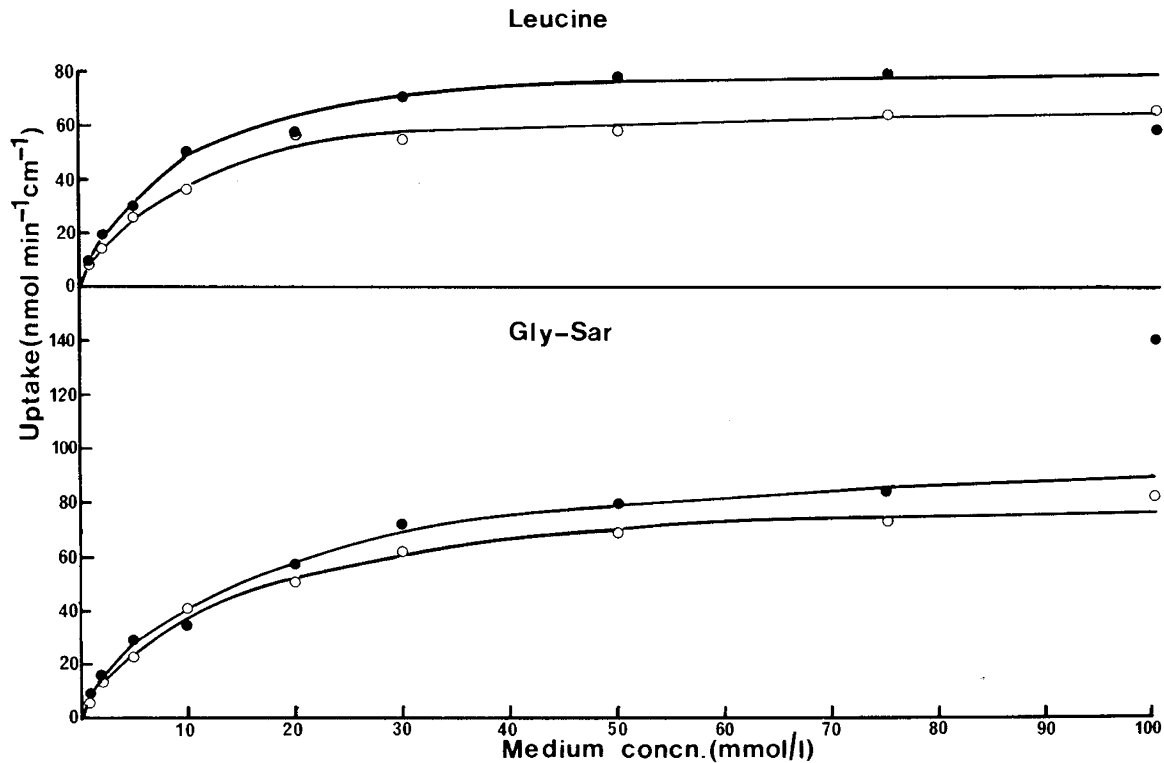
Fig. 2. Uptake of [^{14}C]Gly-Sar and [^{14}C]leucine, after correction for the non-mediated component, into rings of everted jejunum from control (●——●) and low protein fed (O——O) rats over the concentration range 1–100 mmol/l.

rivation in the immediate postweaning period caused a reduction in maximal mediated transport capacity for both Gly-Sar and leucine when uptake was expressed on the basis of unit length. There was no preservation of peptide uptake relative to amino acid uptake, neither was there an initial increase in absorption as found by Lis *et al.* (7, 8) in short-term protein deprivation in adult rats. Furthermore, the reduction observed in this study was not as great as that seen in long-term protein malnutrition in the adult rat (7, 8). The malnourished young animals had a much greater reduction in gut length compared with the controls than malnourished adult animals in which the reduction in mean intestinal length was only of the order of 5% (7).

The observed reduction in maximal mediated transport capacity per cm in the malnourished young rat was not great (on the order of 10%) and when the reduction in gut diameter and villous height is taken into account, this might point to an increase in the number of transport sites per cm in order to preserve uptake of peptide and amino acid. This suggests that in the *in vivo* situation, lack of protein in the diet must have been the rate-limiting factor in growth rather than the number of peptide and amino acid transport sites. On returning to a normal diet the animals would be equipped for an increased uptake of protein digestion products. This point is emphasised if the results are studied in relation to body weight, where there was an increase in maximal mediated transport capacity per unit body weight of both Gly-Sar and leucine in the protein malnourished animals.

The results show that non-mediated transport was markedly lower in the malnourished animals. Whether or not non-mediated transport takes place to a significant extent *in vivo* is a matter of dispute, but in the *in vitro* situation it apparently makes a large contribution to transport and cannot be neglected. The large difference between the value for non-mediated transport of Gly-Sar and leucine may be a reflection of the lipophilic properties of the leucine side chain. A similar difference has been reported in hamster jejunum (15). In view of the finding that significant peptide absorption persists in malnutrition and that there is a probable increase in both peptide and amino acid transport sites, it is important to include amino acids in peptide form in therapeutic diets for developing individuals to allow for maximum absorption of amino nitrogen.

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The Identification and the Excretion Pattern of Isovaleryl Glucuronide in the Urine of Patients with Isovaleric Acidemia

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Summary

We identified isovaleryl glucuronide in the urine of patients with isovaleric acidemia by using gas chromatography-mass spectrometry (GC-MS) and by identifying the products of enzymatic hydrolysis. Conjugation of isovaleryl-CoA with glycine, by the action of glycine-N-acylase, is the main detoxification mechanism in isovaleric acidemia. The identification of isovaleryl glucuronide demonstrates a hitherto unknown, additional detoxification mechanism in patients with isovaleric acidemia. Quantitative analysis of 72 urine specimens from four patients with isovaleric acidemia shows that isovaleryl glucuronide is more likely to be excreted when the amount of urinary 3-hydroxyisovaleric acid excretion is high. This suggests that detoxification via glucuronide conjugation plays an important role when the glycine conjugation system is saturated.

Abbreviations

a.m.u., atomic mass unit
BSA, N,O-bis(trimethylsilyl)acetamide
CI, chemical ionization
EI, electron impact
GC, gas chromatography
MS, mass spectrometry
TMS, trimethylsilyl

Isovaleric acidemia is a recessively inherited disorder of leucine

metabolism, resulting from a deficiency of isovaleryl-CoA dehydrogenase (7, 8). Clinical manifestations include acute attacks of vomiting, acidosis, ataxia, lethargy, and coma (1, 11). The enzyme block leads to the accumulation of isovaleryl-CoA in tissues. During periods of remission, unoxidized isovaleryl-CoA is handled by conjugation with glycine, catalyzed by the action of glycine-N-acylase. Isovalerylglycine thus formed is disposed by urinary excretion (9). When this system is saturated, excess isovaleryl-CoA is hydrolyzed and released as free isovaleric acid causing acute toxic effects. Free isovaleric acid is then omega-1 oxidized or omega oxidized to 3-hydroxyisovaleric (10) or 4-hydroxyisovaleric (13) and methylsuccinic (13) acids, respectively. Small fractions of isovaleryl-CoA are also metabolized to other unusual organic acids including 3-hydroxyisoheptanoic (4) and isovalerylglutamic acid (5).

During the last several years we have identified four patients with isovaleric acidemia by GC and GC-MS. We subsequently analyzed many follow-up samples from these patients. While analyzing these samples, we noticed a well-defined peak which eluted at very high temperatures (methylene unit 22.27 on 10% OV-1) in a number of these samples, in amounts ranging up to 0.50 mg/mg creatinine. In this report, we describe the identification of this compound as isovaleryl glucuronide using GC and GC-MS as well as enzymatic methods.

MATERIALS AND METHODS

Chemical materials. "Tri Sil-BSA Formula P" was purchased from Pierce Chemical (Rockford, IL). High purity *E. coli β-*