Amino Acid Balance and Imbalance. IX. Effect of Amino Acid Imbalance on Blood Amino Acid Pattern.* (27566)

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The rate of growth of an experimental animal may be depressed if an amino acid mixture that lacks one or more indispensable amino acid(s) is added to its diet. This effect, which is attributed to a dietary imbalance of amino acids, is most readily demonstrated in young growing animals fed a low protein diet, and sometimes occurs when the dietary addition consists of less than 1% of only one or 2 amino acids that are not limiting for growth(1-4). The depression in growth rate is accompanied by a depression in food intake(5-7), sometimes within a few hours(4). Both growth and food intake are stimulated by increasing the quantity of amino acid (or acids) that is most limiting for growth(4-6,8-10) and, if the imbalance is not too severe, by administration of insulin which stimulates food consumption and thereby increases the intake of the most limiting amino acid(8).

The biochemical basis for the gross effects of amino acid imbalances is not known. The nutritional observations tend to implicate reactions that lead to wasting or inefficient utilization of the most limiting amino acids. Rats fed an imbalanced diet retain less nitrogen (9,10), excrete more of the most limiting amino acid in the urine(3,11), may have elevated blood urea concentrations(8) and may require more of the most limiting amino acid in the diet to maintain a growth rate equal to that of the control group(5).

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On the other hand, a direct effect of the imbalanced mixture in depressing appetite could result in depressed nitrogen retention (10) and a need for a greater amount of the limiting amino acid. Mellinkoff(12) has suggested that the blood amino acid pattern may directly or indirectly influence the desire for food. However, there have been few studies of plasma amino acid patterns after feeding diets that depress appetite. Since amino acid imbalances cause a marked drop in food intake, the blood amino acid patterns of rats fed on imbalanced diets have been examined, and the results of an initial study are reported below.

Methods. Male rats of the Holtzman strain, weighing between 120-150 g were fed as described below and were supplied with water *ad libitum*.

Diets. The basal diet used was essentially that described earlier(10). This contained fibrin, 6; salt mixture, 5(13); corn oil, 5; vitamin supplement, 0.25(13); choline chloride, 0.15 and "dextrin" (moist corn starch heated at 115° for 2 hours, then dried and ground) as the carbohydrate to make up 100%. Fat soluble vit. A, D and E were included in the corn oil to provide the following concentrations per 100 g of the diet: A, 400 I.U.; D, 40 I.U.; and α-tocopherol, 10 mg. The protein-free diet was of the same composition except that fibrin was replaced by "dextrin". Imbalances were created by adding a mixture of 0.4% DL-methionine and 0.6% DL-phenylalanine(5), or a mixture of indispensable amino acids lacking histidine (5, see footnote to Fig. 1) to the basal diet. The additions of amino acids were compensated for by adjusting the percentage of dextrin.

Protein depletion. Rats that weighed between 120-130 g were fed the protein-free diet for 4 days. Those that lost approximately 15-20 g were separated into groups

consisting of 10 rats each. They were starved for 12 hours and employed for studying, i) rate of food intake of various diets, and ii) plasma amino acid pattern.

Single daily feeding. Male rats weighing about 150 g were trained to accept a single daily feeding according to the procedure outlined previously(10). During the training period the rats were fed the basal diet for only one hour each day. Only rats that gained the same amount of weight and which were consuming the same amount of food were used for studying blood amino acid pattern.

Measurements of rate of food intake. Three groups of 10 protein-depleted rats each, after being starved for 12 hours were fed, respectively, weighed amounts of: (i) the basal diet; (ii) the imbalanced diet containing methionine and phenylalanine; (iii) the imbalanced diet containing the amino acid mixture lacking histidine. Food intake was recorded every half hour for the first 3 hours and subsequently as shown in the results. Corrections were made for food spilled during the feeding trials.

Analysis of blood samples for amino acid pattern was carried out using rats that consumed equal amounts of the different diets during the feeding period. Protein-depleted rats that consumed 5-6 g of the basal or the imbalanced diet containing methionine plus phenylalanine during the feeding period of 2 hours (Fig. 1) were used for study of blood amino acid pattern. Groups were killed by ether anesthesia 1, 3 and 5 hours after the feeding period. For preparation of samples for analysis of amino acids, blood samples from a minimum of 10-12 rats were pooled. Blood was withdrawn by heart puncture and plasma separated from the heparinized blood by centrifugation. The samples of plasma were deproteinized and analyzed for free amino acids using the method of Moore and Stein(14,15) with an automatic amino acid analyzer (Beckmann, Spinco model). The fasting level of amino acids was determined in plasma from protein-depleted rats fasted for a period of 24 hours.

Since the protein-depleted rats consumed only about 4 g of the imbalanced diet containing an amino acid mixture lacking histidine in the limited feeding period (Fig. 1), rats trained to accept a single daily feeding were used in studies of this diet. They were fed the imbalanced diet for 2 days consecutively before experiment because their food intake fell only on the second day. On the day of an experiment, control rats were pair-fed the basal diet. Groups were killed at intervals of 1, 3½ and 6½ hours after the 1-hour feeding period and blood was collected by heart puncture for determination of amino acid concentrations(14,15). Rats trained on the basal diet were starved for 24 hours and samples of blood were analyzed to obtain "fasting" values for this experiment.

Results. The results (Fig. 1) show that rates of food intake of protein-depleted rats ingesting the imbalanced diets are depressed after a few hours. The severity of the depression and the time at which it becomes measurable varies. The very early depressions seen represent the most rapid effects we have observed. With other imbalances the response is slower and the depression is less (Leung and Harper, unpublished observations). If the period of starvation before an experiment is omitted the response may be delayed for as long as 2-3 days(16). With rats that are not protein-depleted a depression in food intake regularly occurs within 24 hours. With rats trained to accept a single 1-2-hour feeding daily a depression occurs the second day. Apparently the effect of an amino acid imbalance on food intake is influenced by several factors. Food intake of the group fed the imbalanced diet containing an amino acid mixture lacking histidine was depressed earlier and more than that of the group fed the imbalanced diet containing methionine and phenylalanine. In previous studies with diets containing different levels of fibrin(5), differences in severity of the effects of these 2 types of imbalance were noted.

The results of analyses of amino acids in the blood of rats ingesting equal amounts of histidine provided by the basal or the diet in which an imbalance was caused by a mixture of amino acids lacking histidine are presented in Table I and Fig. 2. Of the changes observed among the amino acids determined, the

most striking was the marked lowering in concentration of the most limiting amino acid, histidine, in the blood of rats fed the imbalanced diet (Fig. 2). This effect was prominent one hour after the feeding period.

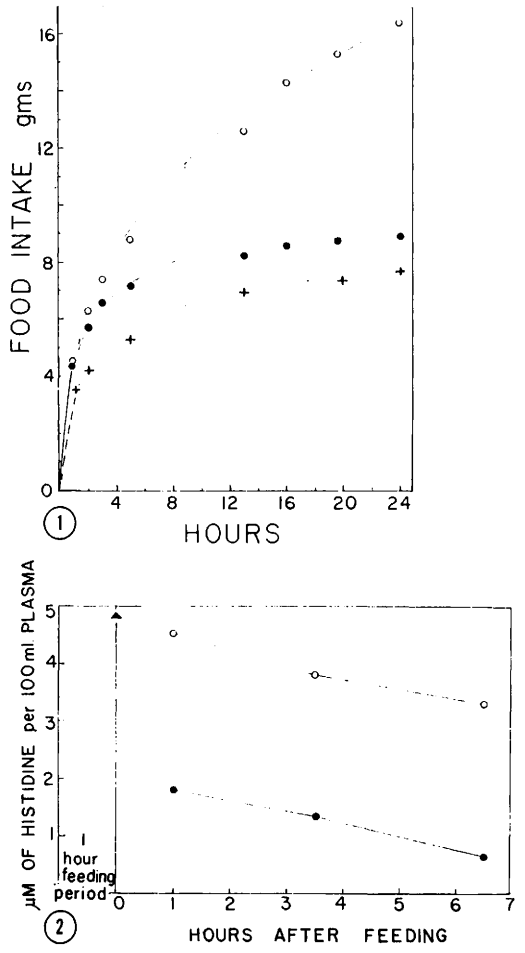

FIG. 1. Food intake of protein-depleted rats starved for 12 hr then fed basal or imbalanced diets. (a) -○-○- Group fed diet containing 6% of fibrin. (b) -●-●- Group fed diet containing 6% of fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine. (c) --×--×-- Group fed diet containing 6% of fibrin + amino acid mixture lacking in histidine. [This contributed in percentage: DL-methionine, 0.4; DL-phenylalanine, 0.6; L-leucine, 0.6; DL-isoleucine, 0.4; DL-valine, 0.4; L-lysine HCl, 0.6; L-arginine HCl, 0.2; DL-threonine, 0.4; and DL-tryptophan, 0.2.]

FIG. 2. Effect of amino acid imbalance on plasma histidine concentration at intervals after feeding a single meal providing 16 mg of histidine. (a) —▲— Fasting level. (Rats trained for single daily feeding fasted for 24 hr.) (b) -○-○- Group fed diet containing 6% of fibrin. (c) -●-●- Group fed diet containing 6% of fibrin + amino acid mixture lacking histidine. See (c), Fig. 1 for amino acid mixture.

After feeding the basal diet for one hour concentrations of several of the indispensable amino acids fell below the fasting level (Table I). Leucine, methionine, histidine, isoleucine, valine and phenylalanine are nearly equally limiting for growth of rats fed this diet(6,17). Leucine and methionine concentrations were depressed 50%, concentrations of the other 4 were depressed less, but all fell below the fasting values. Lysine and threonine which are less limiting for growth fell only slightly. Rats fed the diet containing in addition 3% of the mixture of indispensable amino acids lacking histidine had plasma concentrations of these amino acids that were higher than the fasting levels. Thus, the indispensable amino acid pattern of the blood tended to reflect the dietary amino acid pattern. However, no characteristic pattern was observed for the dispensable amino acids. Concentrations of alanine and asparagine were high in the blood when either diet was fed. The concentration of glycine tended to rise with time. The high concentration of tyrosine and taurine in the blood of rats fed the imbalanced diets indicates the rapid conversion of dietary phenylalanine to tyrosine and methionine to taurine.

Similar but less marked effects were observed when equal quantities of the basal or the imbalanced diet containing methionine and phenylalanine were fed to the protein-depleted rats. In this diet, the balance between the second most limiting group of amino acids (methionine and phenylalanine), and the most limiting group of amino acids (leucine, isoleucine, valine and histidine) is quite delicate(5). Blood concentrations of these 2 groups are shown in Table II. Concentrations of the amino acids added to create the imbalance (methionine and phenylalanine) were high in the blood of rats ingesting the imbalanced diet. However, although both groups ate the same amount of the 4 most limiting amino acids in the diet, concentrations of these fell more from the fasting level in the blood of the imbalanced group than in that of the corresponding control group. "Fasting" values for the 4 most limiting

TABLE I. Effect of Amino Acid Imbalance Due to a Mixture of Indispensable Amino Acids Lacking Histidine on Plasma Amino Acid Concentrations of Rats Trained to Accept a Single Daily Feeding.

	Fasting level	Basal diet			Imbalanced diet*		
		1 hr	3½ hr	6½ hr	1 hr	3½ hr	6½ hr
	μmole/100 ml						
lys + orn	29.2	27.6	36.0	30.3	66.6	57.0	62.0
arg	9.9	7.4	6.7	7.6	12.0	16.4	15.0
leu	9.2	4.5	5.1	5.5	13.0	11.4	11.1
ileu	6.0	4.5	5.4	5.2	8.8	8.8	6.9
val	9.8	8.8	9.8	8.5	40.3	40.0	30.4
thr	45.2	42.0	40.0	47.6	52.3	71.2	58.0
met	5.2	3.0	2.4	2.8	27.36	24.0	24.0
phe	3.7	3.8	2.9	3.0	16.41	15.2	11.1
tyr	4.2	2.2	4.8	4.4	10.17	8.6	8.7
taurine	39.4	19.2	24.2	22.8	60.0	36.0	34.2
asp	2.7	2.6	2.9	1.7	3.12	2.2	2 9
aspargine	22.0	49.6	27.6	31.0	27.12	43.6	56.8
glu	61.2	25.6	32.7	15.6	30.0	27.6	27.6
pro	21.0	26.6	21.0	20.2	21.2	21.2	28.2
gly	53.6	49.6	56.8	60.8	45.6	54.4	72.0
ala	48.8	118.0	119.2	94.8	110.8	114.0	89.6

* See "c," Fig. 1 for composition of amino acid mixture used for creating imbalance.

amino acids were higher in blood from protein-depleted rats than in blood from rats trained to eat for only one hour a day (Table I).

Discussion. Effects of amino acid deficiencies on appetite were reported years ago (18-21). Rose(19) pointed out that "growing animals lose the desire to eat when the food is not suitable for tissue synthesis, but regain it when all the components required for anabolism are made available". The overall effects of an amino acid imbalance are comparable to those of an amino acid deficiency in that both depress food intake and, therefore, rate of tissue formation and protein synthesis. These effects are prevented by a supplement of the amino acid that is most limiting for growth(2,4). That the appetite of an animal is depressed if it has an inadequate supply of the essential building blocks for tissue proteins is almost self-explanatory. It is more difficult, however, to understand why an amino acid imbalance, due to addition to the diet of a quantity of amino acids that cannot be used for protein synthesis, depresses growth and food consumption when intake of the amino acid (or acids) that is limiting growth remains unaltered.

The depressed food intake of protein-depleted rats ingesting an imbalanced diet (Fig. 1) cannot be attributed to low palatibility of the diets(8,10) nor to delayed stomach-emptying as was suggested by Shahinian *et al.* (22). No delay in stomach-emptying oc-

TABLE II. Effect of Amino Acid Imbalance Due to Addition of Methionine and Phenylalanine on Plasma Amino Acid Concentrations of Protein-Depleted Rats Fed for 2 Hours.

Amino acid	Fasting level	Basal diet			Imbalanced diet*		
		1 hr	3½ hr	5½ hr	1 hr	3½ hr	5½ hr
	μmoles of amino acids/100 ml plasma after feeding period of 2 hr						
leu	15.8	6.0	6.2	5.6	4.8	4.5	4.2
ileu	10.5	3.7	4.4	4.2	4.0	3.8	3.4
val	14.8	7.8	8.0	7.6	5.6	4.8	4.5
his	7.8	8.2	7.0	6.8	5.9	5.6	5 5
met	4.4	3.4	2.8	3.4	13.2	12.8	11.8
phe	4.1	4.1	4.1	3.8	16.6	15.5	13.4
tyr	4.3	7.0	4.5	4.9	16.4	15.4	15.0

* See "b," Fig. 1.

curred as a result of an amino acid imbalance caused by addition of methionine and phenylalanine to a 6% fibrin diet in rats trained to accept a large single meal (11.8 g) in 2 hours (8). In other studies the more severe imbalance induced by the amino acid mixture lacking histidine also did not delay the rate of stomach-emptying. The rate of food intake would, therefore, appear to be influenced by some systemic effect of the unsatisfactory balance of amino acids in the diet.

The dietary amino acid imbalances are reflected, and evidently exaggerated, in the blood amino acid patterns, to a degree depending upon severity of the imbalance. Similar effects on blood amino acid pattern have been observed when incomplete proteins have been fed alone(23,24) or added to diets containing some other protein(25). If the influx of amino acids into the blood stimulates cellular uptake and protein synthesis, the concentration of the amino acid in shortest supply would fall much lower in extracellular fluids than would the concentrations of those in relative excess. This is roughly what Longenecker and Hause(26) and Morrison *et al.*(27) observed when they fed animals proteins deficient in one or more amino acids. Thus the effects of dietary deficiencies and imbalances on blood amino acid pattern would also appear to be similar.

The initial effect of an amino acid imbalance that is responsible for the fall in food intake and the accompanying changes remains elusive. If amino acid imbalances affect food intake directly, as suggested by observations on food consumption and food preference of protein-depleted rats(16), the immediate stimulus may be the altered blood amino acid pattern itself. This would provide an alternative to the hypothesis that the effect on food intake is secondary to some change in amino acid metabolism(3) but both hypotheses require thorough testing.

Summary. Effects of adding a mixture of methionine and phenylalanine or a mixture of indispensable amino acids lacking histidine to diets containing 6% of fibrin on rate of food intake and on the blood amino acid pattern of rats have been determined in short term experiments. Amino acid imbalances induced by both of these procedures caused a reduction in rate of food consumption within a few hours after the feeding period. Feeding diets containing additional methionine and phenylalanine to protein-depleted rats caused a fall in concentration of the group of amino acids (leucine, isoleucine, valine and histidine) limiting growth. Similarly, a rapid fall in concentration of the most limiting amino acid, histidine, occurred in the blood of rats trained to accept single daily feeding and then fed diets containing an amino acid mixture lacking histidine.

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Histochemical Studies of Succinic Dehydrogenase in *Trypanosoma cruzi* and *Leishmania leproide*. (27567)

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Biochemical evidence accumulated in recent years shows that the Krebs cycle intermediates are utilized by the culture forms of *Trypanosoma cruzi*(1,2); moreover the presence of some of the enzymes involved, *viz.*, succinic dehydrogenase(3,4) and isocitric dehydrogenase(5), has been demonstrated by the manometric technic. Studies on the metabolism of leishmania are fragmentary; nevertheless, utilization of substrates of oxidative metabolism by the leptomonad form (in *L. tropica, donovani, brasiliensis*) has been reported(1,2,6).

Though the histochemical technic does not permit an accurate measure of enzyme activity, it offers the advantage of localizing the enzyme site in the cell and the possibility of applying it for *in vivo* and drug action studies. Therefore, we made an effort to develop a method which would permit the demonstration of succinic dehydrogenase in *T. cruzi* and leishmania. By this method we have demonstrated histochemically the presence of succinic dehydrogenase in *T. cruzi* obtained from cultures and infected mice, as well as in the leptomonad form of a variety of *L. brasiliensis*. We have also studied the effect of a drug (Spirotrypan) on the enzyme in *T. cruzi*.

Material and method. Two- to six-week-old cultures of a local strain of *T. cruzi*, grown at 26°C in the liquid medium described by Warren(7), were used. For *in vivo* experiments *T. cruzi* was collected, with a slight modification of the method described by Anderson *et al.*, for *T. equiperdum*(8), from the blood of mice at the peak of infection. For investigation of leishmania we utilized a strain employed previously for chemotherapeutic studies(9), a variety of *L. brasiliensis*, called by Pifano "*L. leproide*"(10). One- to 2-week-old cultures in Davis medium were used.

As incubation medium for staining, one of the more recent tetrazolium salts, 2,2'-di-p-nitrophenyl - 5,5' - diphenyl - 3,3'(3,3' - dimethoxy - 4,4' - biphenylene) ditetrazolium chloride (Nitro blue tetrazolium or "nitro-BT"), shown by Tsou *et al.*(11) to be a very effective histochemical reagent for succinic dehydrogenase, was selected. However, when this reagent was used as previously described (12-14) no reaction was obtained. Yet, we succeeded in staining the parasite by introducing an electron carrier in the staining medium, with a modification of the technic of Farber and Louviere for succinic dehydrogenase in mammalian tissue(15). The staining medium which finally led to the best results consisted of:

	ml
Sodium succinate, 0.2 M	.75
Nitro-BT, 3 mg/ml	.5
Brilliant cresyl blue, 0.5 mg/ml	.1
Phosphate buffer, 0.2 M, pH 7.4	.5
Sodium bicarbonate, 0.6 M	.15
Calcium chloride, 0.004 M	.3
Water	.7
	3.00

Demonstration of the enzyme. About 2 ml of *T. cruzi* culture was usually washed 2-3 times by centrifuging for 5 minutes with sub-