Availability of Amino Acids

Fructose–Glycine as a Source of Nonspecific Nitrogen for Rats

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To study further the reasons for the decreased availability of amino acids in heated foods, fructose–glycine (FG) was prepared and tested as a source of nonspecific nitrogen for rats. Assayed microbiologically the compound had only 68% of the growth stimulating ability of glycine. As a source of nitrogen in nitrogen-deprived rats, it supported as good growth as equivalent amounts of glycine.

Heat treatment may alter the nutritive value of the protein of carbohydrate-containing foods (Liener, 1960). Such a change indicates some change in availability of its amino acids. To study the mechanism by which amino acids are made unavailable in carbohydrate-containing foods, fructose–methionine was prepared and its availability to *Leuconostoc mesenteroides P-60* and rats was determined (Horn *et al.*, 1968). For *L. mesenteroides* the compound had 80% of the growth-stimulating ability of methionine; for rats practically none of the methionine was available.

For maximal rate of growth, the rat needs supplemental nitrogen as well as 10 essential amino acids. Suitable supplemental nitrogen sources may be a single nonessential amino acid such as glycine or glutamic acid, an excess of essential amino acids, or a source of ammonia such as diammonium citrate (Rose *et al.*, 1949) or urea (Rose and Dekker, 1956).

Because nonessential as well as essential amino acids might be expected to form fructose–amino acids during heating of foods and thus cause a far greater loss of nutritive value than expected from consideration of availability of essential amino acids alone, fructose–glycine (FG) was synthesized and tested as a source of supplemental nitrogen for rats.

EXPERIMENTAL AND RESULTS

Preparation of *N*-Carboxymethyl-1-amino-1-deoxy-D-fructose (FG). A modification of the procedure of Heyns and Paulsen (1959) was used for preparing FG. Finely powdered glycine (10 grams, 0.133 mole) and glucose (100 grams, 0.555 mole) were suspended in 2 liters of absolute methanol and heated 2.5 hours under a reflux condenser. The resulting amber solution was concentrated *in vacuo* to a thick brown syrup which was dissolved in 300 ml of water and decolorized by two successive treatments with 20 grams of Darco G-60. Four such preparations were combined and diluted to a volume of 4 liters.

The product was isolated by absorption on a bed (16-cm diameter and 7.3 cm deep) of cation-exchange resin (Dowex 50W-X8, H^+ form, 100–200 mesh) supported in a 3-liter cylindrical glass funnel fitted with a coarse sintered-glass disk. The flow rate during absorption was about 25 ml per minute. The bed was washed with 3 liters of distilled water, fractions being collected in 250-ml volumes. Aliquots of these fractions were tested for the presence of amino acids by reacting with ninhydrin on a spot plate or filter paper (followed by heat), and for reducing sugar by triphenyl tetrazolium chloride (TTC) (Horn *et al.*, 1968). The tests indicated that all the amino acid was absorbed on the resin and that the free sugar was completely washed into the effluent. Absorbed compounds were eluted with 3 liters of 0.25*M* trichloroacetic acid (TCA) and then 3 liters of 0.5*M* TCA, collecting in 250-ml fractions. Several distinct slightly colored bands could be seen moving down the column. The first band eluted with 0.25*M* TCA appeared in fractions 6 to 11 and was not the desired product, but a side product, difructose–glycine. The major band, eluted with 0.5*M* TCA, was concentrated in eluate fractions 19 to 23 and contained the desired product which gave positive spot tests for both amino acid and fructose. Pooled fractions 19 to 23 were extracted repeatedly (*ca.* 10 times) in a large separatory funnel with small volumes of ether and the aqueous solution was concentrated *in vacuo* to a syrup, which was again extracted with ether to assure complete removal of TCA. The syrup was dissolved in *ca.* 75 ml of water and an approximately equal volume of ethanol, sufficient to yield a faint turbidity, was added. Crystallization was initiated by scratching with a glass rod and allowed to proceed at room temperature, and then in the cold. The colorless crystalline product was collected by filtration, washed first with cold 65% ethanol, then with absolute ethanol, and subsequently dried *in vacuo* over phosphoric anhydride.

The combined yield from two column purifications as above was 49 grams.

Anal. Calcd for $C_8H_{15}O_{17}N$: N, 5.90; neut. equiv., 237.2. Found: N, 5.81; neut. equiv., 235.8.

The high purity of this preparation was indicated by its emergence from the 60-cm column of a Phoenix amino acid analyzer as a single symmetrical peak eluted by the pH 3.25, 0.2*M* citrate buffer at approximately 35 minutes, and uncontaminated by the difructose derivative (elution time 23 minutes) or glycine (elution time 81 minutes). The OD 570/OD 440 ratio of FG was 6.3. The more rapid elution of FG reflects its reduced basicity (pK = 8.1) as compared with glycine (pK = 9.6). Previously, Anet (1957) had prepared FG by a different procedure and reported a pK of 8.4. When examined by paper chromatography in a butanol:acetic acid:water (12:3:5) system, the product appeared as a single zone at R_f = 0.16, at the identical position whether detected by ninhydrin or the TTC reagent. In this same system, glycine appeared at R_f = 0.26, difructose glycine at R_f = 0.09, glucose at 0.26, and fructose at 0.29.

AVAILABILITY STUDIES

Microbiological. FG was assayed microbiologically for glycine using *L. mesenteroides P-60* as the assay test organism. It yielded only 68% of the growth given by an equimolar amount of free glycine.

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Table I. Food Intakes, Weight Gains, and Serum Glycine Levels of Young Rats Fed Diets Furnishing No Nonspecific Nitrogen (NN) or NN from Glycine or Fructose–Glycine (FG)

Group	No. of Rats	Food Intake,[a] G	Weight Gains,[a] G	Glycine/Ml Serum, μMoles
No NN (control)	8	47.5	9.4 ± 0.68[b]	0.2
20 mg NN (glycine)	8	48.6	15.5 ± 0.60	1.4
20 mg NN (FG)	5	46.9	15.6 ± 0.75	0.4
40 mg NN (glycine)	8	44.4	15.2 ± 1.00	4.6
40 mg NN (FG)	5	47.4	12.6 ± 1.54	0.7

[a] Totals for 7 days. [b] S.E. of mean. Gains of the control group are significantly less ($P < 0.01$) than of the next three groups.

Rat Feeding Trials. Earlier work in this laboratory (Womack, 1969) indicated that young rats fed restricted amounts of food containing barely adequate amounts of essential L-amino acids responded with increased weight gains when various sources of supplemental nitrogen were added to the diet. Thus it was possible to test the availability of FG as a source of supplemental or nonspecific nitrogen (NN) in the rat. Specific pathogen-free male rats approximately 4 weeks old were fed a nitrogen-free diet for 4 days, divided into groups, and then were given 7 grams of the experimental diets daily for one week. Each diet contained essential L-amino acids in amounts sufficient to meet requirements and supplied 60 mg of essential amino acid nitrogen per day with either no NN or 20 or 40 mg of NN from glycine or from FG. The daily food allotment supplied either 107 or 215 mg of glycine or 334 or 689 mg of FG. Other dietary ingredients and the composition of the amino acid mixture have been described (Womack, 1969).

Weight gains of rats fed 20 mg of NN from glycine were significantly greater ($P < 0.01$) than those of rats given no source of NN (Table I) but were not further increased when 40 mg of NN from glycine were given. Rats fed both levels of FG had diarrhea during the entire feeding period. Despite this condition, gains of the rats fed the lower level of FG were equal to those of rats fed the same level of nitrogen in the form of glycine and significantly higher than those of rats with no source of NN in the diet. While gains of some rats fed the higher level of FG were as good as those fed the lower level, others did not grow as well and the mean of the group was not significantly different from that of the group given no NN. Nevertheless, the animals fed the lower level of FG were able to utilize the nitrogen for growth despite diarrhea.

On the morning of the final day of the growth study, each rat was given 2 grams of food. One hour after the animals had been fed they were injected with sodium amytal, the body cavity was opened, and blood was taken from the heart. Protein was precipitated from the serum by addition of an equal volume of 20% sulfosalicylic acid. Equal volumes of serum from animals of each group were combined and amino acids determined using a Technicon amino acid analyzer. It was evident (Table I) that ingestion of FG caused some increase in blood glycine. However, the increases were very much smaller than when equivalent quantities of glycine were eaten.

Routinely in our studies, in order to resolve glutamine, the column is held at 30° C for the first 4 hours (Oreskes *et al.*, 1965). In the chromatograms we invariably see a small unidentified prethreonine peak (eluted at *ca.* 33 minutes before threonine and equivalent to *ca.* 0.03 to 0.04 μmole of amino acid per ml of serum). In the serum of rats fed FG, this peak was much higher than in serum of rats fed the diet containing glycine—0.09 μmole for the group fed the lower level of FG and 0.15 for those fed the higher level. When FG was added to the serum of the rats fed FG, it and the unidentified compound were eluted as a single peak. Thus it would appear that a small amount of FG, or some similar compound, is a normal constituent of rat serum. In any case, amounts found in the blood when FG was fed were small as compared to the amounts of glycine found when glycine was fed (see Table I).

Excretion Experiments. Rats weighing 450 to 500 grams received 1-ml volumes of an aqueous solution of FG containing 100 mg (0.42 mmole), either by intraperitoneal injection or by stomach tube. Separate control animals received an equivalent molar amount of glycine. Following the administration of the compounds, the animals, which had been on a stock diet, were transferred to metabolism cages for collection of urine and feces. Urine (under toluene) and feces were collected for approximately 20 hours, during which the animals received water only. Collected samples were then stored in the frozen state until examined by chromatography.

Urines were spotted on large sheets of Whatman No. 1 filter paper using total volumes of approximately 20 μl. Development was for an overnight period in the butanol:acetic acid:water system described above. Duplicate chromatograms of each series of urines were set up together with reference spots of FG, glycine, and glucose. One chromatographic sheet was then sprayed with ninhydrin reagent for detection of amino acids and the other with TTC reagent for detection of sugar, reagents being as mentioned earlier. FG initially appeared as a greyish zone and then turned to the lavender color given by most amino acids. Besides the characteristic color response with ninhydrin, it was easy to recognize FG by the bright red zone with TTC reagent which coincided with the ninhydrin zone. Results from these experiments clearly demonstrated that FG was rapidly excreted in the urine by those rats which received it by intraperitoneal injection, but it was not detectable by these tests in urines of those receiving the compound by stomach tube.

Fecal extracts, prepared by suspending the feces in a small volume of water, grinding with the end of the stirring rod until uniformly dispersed, and then centrifuging, were examined by paper chromatography, in the same manner as the urines. There was no evidence of excretion of FG in the feces of the animals, regardless of the manner of administration. (Similar injection studies, with identical results, were carried out with fructose–methionine.)

DISCUSSION

The excitement which formerly surrounded the subject of fructose–amino acids has diminished with the recognition that these compounds do not serve as intermediates in protein synthesis (Abrams *et al.*, 1955; Borsook, 1958). Nevertheless, interest in fructose–amino acids has continued as a result of their involvement in nonenzymic browning reactions (Hodge, 1953; Reynolds, 1965). The presence of fructose–amino acids in stored purees of apricots and peaches (Anet and Reynolds, 1957) was demonstrated. Isolation of several fructose–amino acids was reported, first in amorphous form (Anet and Reynolds, 1957; Gottschalk, 1952; Abrams *et al.*, 1955) and then several, including FG, were isolated from liver in crystalline form (Heyns and Paulsen, 1959). Previous syn-

theses of crystalline FG have been reported (Anet, 1957; Heyns and Paulsen, 1959).

Other than the previous report on fructose–methionine (Horn *et al.*, 1968), we are unaware of feeding experiments with purified fructose–amino acids. As previously reported, the essential amino acid methionine was not replaceable for rat growth by fructose–methionine, a result expected from the observation (Borsook *et al.*, 1955) that fructose–leucine with ^{14}C-label in the amino acid moiety was not incorporated into protein in an *in vitro* system. The present observation that FG is as effective as glycine in supporting growth of the young rat fed a nitrogen-deficient diet suggests that fructose–amino acids may be metabolized in such a way that the nitrogen, but little or none of the amino acid moiety *per se* can be utilized. The intestinal flora may play a role, since digestive enzymes would be expected to result in the release of utilizable amino acids.

The excretion experiments indicate that fructose–amino acids are not rapidly metabolized. However, the small increases of glycine and of the prethreonine compound in blood sera of rats fed FG, as well as the appearance of the latter compound in all rat sera examined by column chromatography, suggest that the utilization of fructose–amino acids deserves further investigation.

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