# Availability of Amino Acids

## A Methionine-Fructose Compound and Its Availability to Microorganisms and Rats

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Previous evidence indicated that methionine in food proteins reacts with carbohydrates under the influence of heat to form complexes of obscure nature which are nutritionally inferior to methionine itself. In this study, to gain information which might elucidate the nature of this kind of complex, a model compound was synthesized from dextrose and methionine. Analyses and chemical properties prove that this compound is 1-deoxy-1-methionino-D-fructose (DMF). Assayed microbiologically, the compound had only 80% of the growth-stimulating ability of methionine. Assayed biologically with rats, practically none of the methionine was available.

Among the major sources of protein are foods high in carbohydrate. Before these are eaten they are generally heat-treated by either home cooking or commercial processing and are sometimes rendered nutritionally inferior to the unheated product. The decrease in nutritional value depends on the amino acid composition and the severity of the heat treatment.

It was recognized early that heated proteins sometimes did not give as good growth with animals as unheated proteins (Greaves *et al.*, 1938). Prolonged autoclaving of soybean and other carbohydrate-containing foods decreased the availability of many amino acids, and caused reduction in growth of rats, chickens, hogs, and microorganisms by amounts ranging from 10 to 60% depending on the heat treatment (Evans and Butts, 1949; Horn *et al.*, 1952; Waterworth, 1964). Waterworth (1964) found that for some processed fish meals the availabilities of seven amino acids studied varied considerably, although the amino acid contents were the same. Methionine availability varied from 33 to 70%, depending on the kind of meal studied. Bressani *et al.* (1963) studied the effect of heat on the nutritive value of black-bean (*Phaseolus vulgaris* L.) protein and found that cooking for 10 to 30 minutes at 15 p.s.i. (121° C.) was optimal. Longer cooking times decreased the nutritive value of the bean protein. The contents of lysine, methionine, and valine were not changed by cooking. Therefore, the decreases in nutritive value were due to changes in availability.

The hypothesis of the mechanism by which amino acids are made unavailable by heat, expressed by Evans and Butts in 1951, had changed little as discussed by Waterworth in 1964. The postulations of Evans and Butts (1951) were that three types of combination occur when proteins in foods are heated—a reaction of the protein-bound amino acid: with carbohydrate that destroys the biological activity of the amino acid; with some of the other constituents of the protein to form a linkage resistant to in vitro digestion with trypsin and erepsin but not to acid hydrolysis; or with carbohydrate to form a linkage with properties similar to the second type. Lysine inactivation is considered to be the first two types, arginine the first and third types, histidine all three types, and methionine the second and third types (Evans and Butts, 1949).

Waterworth (1964) pointed out that while the inactivation of lysine by reactions with reducing sugars during processing had long been known, the mechanism whereby other essential amino acids are bound is not so obvious.

Clandinin *et al.* (1947) showed that soybean flakes autoclaved for 4 minutes gave much greater growth than the same flakes autoclaved for 4 hours. They found on feeding these two soybean preparations to chicks that lysine and methionine were made unavailable by heat. By assaying with microorganisms they found that the methionine contents of acid-hydrolyzed overheated and unheated soybean meals were the same. They pointed out that microbiological assay of acid hydrolyzates cannot measure biologically available methionine.

Horn *et al.* (1952) demonstrated that various methods of processing cottonseed meals altered the nutritional value of the protein. Two methods of evaluating these meals were used for the 10 essential amino acids—the rat assay method which showed that the nutritional values of these meals for the rat were considerably altered by the processing; and the microbiological method combined with in vitro enzyme digestion which showed that changes in nutritional value were due to a change in the availability of

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[1] Retired

the amino acids. An acid and enzyme hydrolysis of each meal, raw and cooked, gave total and available amino acids, respectively. The availability of the enzyme digestion products to the microorganisms correlated well with the results of rat feeding. Methionine availability varied from 100 to 36%, lysine availability from 100 to 32%, and leucine availability from 100 to 77%. This work showed that microbiological assay coupled with in vitro digestion could be used to evaluate availability of amino acids in various compounds.

Methionine residues react when heated with carbohydrate. In one study with soybeans about 97% of the methionine was inactivated (Evans and Butts, 1949). Practically all was recovered as free methionine on acid hydrolysis. Besides the peptide linkage, methionine in protein contains a thioether group which may be an active site for reactions with carbohydrate. However, no evidence has been found that such a reaction takes place.

The speculations of workers in this field on amino acid combinations with carbohydrate cited above made it desirable to isolate and identify some of the amino acid complexes, especially those of methionine, which is a limiting amino acid in a considerable number of foods.

Before attempting to isolate a methionine-carbohydrate complex from a carbohydrate food, it was planned to set up model systems starting with free methionine and glucose, isolate the compound formed, and study its availability. A dipeptide of methionine, then a tripeptide, would then be used to study the combinations formed from these compounds and glucose.

This paper reports the synthesis and properties of a methionine-fructose compound obtained by the action of glucose on methionine and the availability of the methionine in this complex to microorganisms and rats.

## METHODS AND REAGENTS

**Carbohydrate (TTC) Test.** To 1 drop to 1 ml. of sample were added 4 drops of TTC reagent (3% methanol solution of 2,3,5 triphenyl-2*H*-tetrazolium chloride) and 3 drops of 6*N* NaOH. The mixture was shaken and allowed to stand at room temperature. A pink to brick-red color was a positive test.

**Ferricyanide Reduction Test for Ketose.** This is an adaptation of the test of Borsook *et al.* (1955). To 1 to 3 drops of sample were added 6 drops of 1% potassium ferricyanide solution; the mixture was shaken and allowed to stand at room temperature for 10 minutes; 6 drops of ferric sulfate reagent (1 gram of ferric sulfate in 15 ml. of phosphoric acid, diluted with water to 200 ml.) and 3 drops of phosphoric acid were added. A positive test was a blue color.

**Amino Acid (Ninhydrin) Test.** To 1 drop to 1 ml. of samples were added 4 drops of ninhydrin reagent (4% methanol solution of ninhydrin) and 1 ml. of propionate buffer (199.2 ml. of propionic acid added slowly with stirring to cool solution of 67.2 grams of NaOH in 150 ml. of water, then diluted to 400 ml. with water). The mixture was shaken and heated at 100° C. for 30 minutes. A positive test was a blue-violet color.

Methione was determined by both colorimetric (Horn *et al.*, 1946a) and microbiological (Horn *et al.*, 1946b) methods. *Leuconostoc mesenteroides* P-60 was used as assay organism in the latter in place of *Lactobacillus arabinosus*. Fructose was determined by the method of Ting (1956). A slight modification of the AOAC method (1965) for sugar products was used to determine ash. Sulfur, nitrogen, and molecular weight were determined by the Pregl bead-tube, micro-Kjeldahl, and carboxyl methods, respectively (Roth, 1937). Thin-layer chromatography was done on silica gel plates with 1-butanol–glacial acetic acid–water (80:20:20 by volume) as solvent (Stahl, 1965). Ion exchange chromatography was done by the Piez-Morris procedure (Piez and Morris, 1960) on a Phoenix amino acid analyzer. The compound was hydrolyzed by refluxing with 20% HCl for 6 hours.

**Nuclear Magnetic Resonance and Infrared Spectra.** The nuclear magnetic resonance spectrum of the ketose-methionine complex was scanned in heavy water, in perdeuterodimethyl sulfoxide, and in a deuteroethanol–heavy water mixture from 0 to 1 kiloherz (TMS reference compound).

For infrared analysis the complex was made into a potassium bromide pellet.

**Biological Feeding Tests.** To study the ability of the rat to utilize the methionine complex to supply its methionine needs, three experiments were conducted. Young rats were obtained from the supplier (Microbiological Associates, Inc., Walkersville, Md.), fed a commercial pelleted stock ration for one day, divided into groups, caged individually, and fed the experimental diets. Essential amino acids, except for cystine and methionine, were supplied in the diets in amounts to meet the requirements of the young rat (Rama Rao *et al.*, 1964). The nonessential amino acid pattern was that found in wheat (Womack, 1966). The amounts of amino acids in the diets (in per cent) were: arginine HCl, 0.50; histidine HCl·$H_2O$, 0.34; isoleucine, 0.55; leucine, 0.70; lysine HCl, 1.12; phenylalanine, 0.42; threonine, 0.50; tryptophan, 0.11; tyrosine, 0.30; valine, 0.55; alanine, 0.28; aspartic acid, 0.67; glutamic acid, 5.13; glycine, 0.43; proline, 0.86; and serine, 0.39. The various supplements replaced an equal amount of cornstarch. The calculated amount of nitrogen in the cystine-methionine-free diet was 1.55%, equivalent to 9.7% protein.

In experiments 1 and 2 (Table II) rats were fed *ad libitum;* in experiment 3 the food furnished daily was restricted to the amount consumed by the rats receiving no methionine supplement. Scattered food was recovered daily and food intakes were corrected accordingly. In experiment 1 the diets fed to groups of five rats each for 10 days contained 0.2, 0.3, or 0.4% L-methionine or 0.8% of the methionine complex.

## EXPERIMENTAL AND RESULTS

**Preparation and Purification of Compound.** The method used for the synthesis of the methionine-carbohydrate complex was essentially that of Abrams *et al.* (1955). In a triple-necked flask were placed 18 grams of dextrose, 5 grams of L-methionine, and 200 ml. of absolute methanol. The mixture was stirred for a minute and its pH was adjusted to 4.0 with glacial acetic acid (usually 1 drop) using bromocresol green as outside indicator. Reaction was slow unless the mixture was acidified slightly. The

flask was heated under reflux with constant stirring in an oil bath maintained at 110° C. At half-hour intervals single drops of supernatant liquid were tested for ketose formation by the ferricyanide reduction test. The reaction was terminated when two equal successive dark blue tests were obtained.

After the reaction mixture had cooled, it was filtered through a sintered-glass funnel to remove undissolved dextrose or methionine, if any. The solids were washed with 100 ml. of methanol and the combined filtrate and washings were evaporated to a sirup in a rotary flash evaporator at 40° C. The sirup was dissolved in 100 ml. of distilled water, adjusted to pH 4.0 with hydrochloric acid, and 15 grams of Darco carbon was added. The mixture was stirred magnetically while being heated at 70° to 80° C. for 10 minutes. It was then filtered through sintered glass, the carbon being washed with distilled water.

The combined filtrate and washings from the carbon treatment were concentrated to 75 to 100 ml. on the rotary flash evaporator at 40° C. The concentrated solution was applied to an 8 × 25 cm. column of Dowex 50X4, hydrogen form, 50- to 100-mesh. Distilled water was passed through the resin at the rate of 72 drops per minute. Fractions of effluent were tested for carbohydrate by the triphenyl tetrazolium chloride (TTC) test, and all fractions yielding a positive test for carbohydrate were discarded. At this point elution with 1*N* hydrochloric acid was started. Fractions of 100 ml. were collected, each of which was tested with both TTC and ninhydrin. Those that were positive to both tests were retained for analysis by thin-layer chromatography.

The eluate fractions that showed evidence of containing both carbohydrate and amino acid were then tested by thin-layer chromatography on silica gel plates with butanol–acetic acid–water as solvent (Stahl, 1965). Fractions containing no free methionine were combined, adjusted with 6*N* sodium hydroxide to pH 6.8, and evaporated on the rotary flash evaporator at 40° C. to 150 ml. Sodium chloride that precipitated out during evaporation was filtered off through sintered glass and washed with small quantities of 95% ethanol until negative to the TTC test. Evaporation of combined filtrate and washings, filtration, and washing of the sodium chloride precipitate were repeated first with absolute methanol, then with 95% ethanol, until no further salt precipitation was evident upon evaporation.

The resulting alcohol solutions contained the compound essentially free of methionine and sodium chloride. These were evaporated to a sirup on the rotary flash evaporator at 40° C. The sirup was dissolved in a minimal quantity of absolute methanol. This solution was poured into 125 ml. of cold absolute ethanol and the mixture was kept in the refrigerator for 1 to 2 hours. The supernatant liquid was decanted off the precipitate that formed. The precipitate was washed several times with absolute ethanol by decantation.

The supernatant liquid and washings were combined and evaporated to a sirup on the rotary flash evaporator at 40° C. The sirup was dissolved in a small quantity of absolute methanol to which five times as much absolute ethanol was then added, thus forming a precipitate. The suspension was evaporated to a sirup or powdery sirup; and solution in absolute methanol, addition of absolute ethanol, and evaporation were repeated until a dry powder formed on the wall of the distillation flask. The dry powder was scraped off and saved.

The precipitate was dissolved in a small quantity of absolute methanol and was handled as were supernatant and washings until a dry powder was formed on the distillation flask wall. The powder was scraped off and combined with that from the supernatant and washings. The combined batch of compound was placed in a desiccator over concentrated sulfuric acid.

The product was a light tan, amorphous, hygroscopic powder. Attempts to crystallize it were unsuccessful. By the procedure described, a yield of 46% of theoretical was obtained. (An additional 42%, of lesser purity, was obtained from the Dowex 50 eluates which had shown evidence by thin-layer chromatography of containing methionine.) When tested by ion exchange chromatography three peaks were obtained: a small unidentified peak occurring shortly after the emergence of the buffer front; a large peak, the methionine-fructose compound, occurring somewhat ahead of the aspartic acid position; and a small methionine peak which represented 0.8% of the compound. The unidentified small peak could not be quantified but appeared to be negligible in amount. By thin-layer chromatography the methionine-fructose compound had an $R_f$ of 0.23; that of the unidentified impurity, 0.06 (methionine-0.37). A melting point determination showed the compound to decompose in the range from 80° to 120° C.

**Analyses of Complex.** The analytical data are given in Table I.

The molecular weight value of 312 denotes but one sugar and one methionine residue.

The reducing reactions of the complex indicate the presence of a ketose rather than an aldose. Ketoses and aldoses can be differentiated by the speed and temperature required for them to reduce TTC and ferricyanide. The rapid reduction of these reagents at room temperature by the methionine complex shows the presence of a ketone group and indicates that the sugar is in the form of a ketose. When the total reducing sugars in the methionine complex were determined by the method of Ting (1956), the value for sugar was found to be the same at 55° as at 100° C. Since the compound reduced all the ferricyanide at 55°, this again indicates all the sugar to be in the form of a

**Table I. Analytical Data on 1-Deoxy-1-methionino-D-fructose**

| Analyses | Found,[a] % | Theoretical, % |
|---|---|---|
| Ash | 5.24 | ... |
| Nitrogen | 4.60 | 4.50 |
| Sulfur | 9.88 | 10.30 |
| Methionine (colorimetric) | 49.0 | 47.6 |
| Methionine (microbiological) | | |
| Unhydrolyzed | 37.8 | 47.6 |
| Hydrolyzed | 44.9 | 47.6 |
| Fructose | 58.4 | 52.4 |
| Molecular weight | 312 | 311 |

[a] Corrected for moisture and ash.

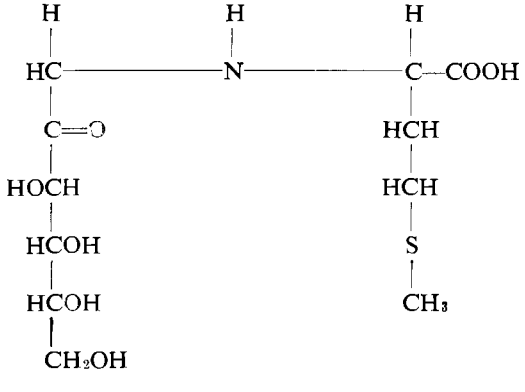
ketose and none in the form of an aldose. When heated with acid, the methionine complex was stable, requiring 20% hydrochloric acid for 6 hours before giving a good yield of methionine. This is a property of amino acid fructoses in contrast to aldoses (Hodge and Rist, 1953).

Tests for a free amino group were made on the methionine complex with sodium 1,2-naphthoquinone-4-sulfonate (Feigl, 1947) and compared with free methionine under the same conditions. The complex gave a negative test in contrast to the positive test given by free methionine. Primary amino groups were also tested by Van Slyke's micromethod (Roth, 1937). Free methionine gave a quantitative yield of nitrogen, the complex only the blank. When the compound was tested for the amino group by nuclear magnetic resonance, the proton signals associated with the amino group could not be observed, probably because of the relaxation effect of the nitrogen. Tested by infrared the strong ionic carboxyl band, usually found between 1600 and 1560 $cm.^{-1}$, is shifted to a higher frequency (1625 $cm.^{-1}$) in the spectrum of this preparation, obtained as a potassium bromide pellet. The two bands characteristically found in all primary amino acids in the 1600- to 1500-$cm.^{-1}$ region, and absent from secondary amino acids, were not found in the spectrum of this preparation, indicating that the amino group exists as the $>NH_2^+$, as expected. The 3600- to 2500-$cm.^{-1}$ region was characterized by a broad band, superposed on which were several sharper bands (in the 3100- to 2900-$cm.^{-1}$ region). Though not particularly diagnostic, this region was nevertheless characteristic of the stretching vibrations of the OH hydroxyl bond as found in carbohydrates and the N—H bond found in amino acids. All these tests suggest that the nitrogen of the complex occurs in secondary form and that the carbohydrate is joined to this nitrogen.

Free carboxyl is present, since the compound can be titrated easily with standard alkali.

The presence of an —S—$CH_3$ group is shown by the ease with which methionine in the complex can be determined colorimetrically with nitroprusside. This reaction is negative for methionine sulfoxide, homocystine, and cystine. A compound like glycyl methionine, having the amino group blocked, gives a positive test.

The analytical values obtained for methionine, fructose, nitrogen, sulfur, and molecular weight, recorded in Table I, together with the reactions given above are consistent with theoretical values for a compound of the structure:



or 1-deoxy-1-methionino-D-fructose (DMF).

**Availability Studies.** The compound was assayed microbiologically for methionine with *Leuconostoc mesenteroides* P-60 as assay test organism (Horn *et al.*, 1946b). It yielded only 79.5% of the growth expected from an equimolar amount of free methionine. When the compound was hydrolyzed with hydrochloric acid and assayed in the same way, 94% of the growth obtainable from an equivalent amount of methionine resulted.

Assayed biologically with rats, practically all the methionine was unavailable. In the rat assay the level of 0.8% DMF fed in experiment 1 (Table II) was selected on the basis that the compound was about half methionine, and that the methionine it contained was about 75% utilized by microorganisms. The failure of the rats to eat the diet (Table II) in amounts necessary for growth could indicate that the methionine of the compound was not available to the rat, was toxic, or had an adverse effect on food intake. Experiments 2 and 3 indicated that the compound was not toxic and had no adverse effects on food intake. The slightly better gains and food intakes of the rats fed the combination of 0.3% methionine plus 0.8% DMF in experiment 2 may have been due to the small amount of free methionine (1.72%) in the sample of DMF.

## DISCUSSION

The reaction of the free amino group of the amino acid with carbohydrate suggests that in carbohydrate-containing foods all the end amino groups of the peptide chain could form carbohydrate complexes when heated. However, there are too few of these groups to account for the decreased availability of amino acids in foods. Evidence has accumulated over the past 50 years indicating that to a greater or lesser degree perhaps all the amino acids are made unavailable at the same time. This would mean that some group common to all the amino acids in the peptide chain must react with carbohydrate. The only active group common to all the amino acids in protein that could

**Table II. Average Daily Weight Changes and Food Intakes of Rats Fed Diets Containing L-Methionine (M), 1-Deoxy-1-methionino-D-fructose (DMF), or M plus DMF[a]**

| Supplement to Diet, % | Av. Weight Change, Grams | Av. Food Intake, Grams per Day |
|---|---|---|
| | Experiment 1 | |
| 0.2 M | 4.0 | 5.6 |
| 0.3 M | 14.4 | 6.8 |
| 0.4 M | 17.6 | 7.2 |
| 0.8 DMF | −7.4 | 3.8 |
| | Experiment 2 | |
| 0.3 M | 18.5 | 7.5 |
| 0.3 M + 0.8 DMF | 21.8 | 8.6 |
| | Experiment 3 | |
| None | −16.5 | 3.6 |
| 0.8 DMF | −13.5 | 3.7 |
| 0.1 M | −12.5 | 3.8 |
| 0.2 M | −10.5 | 3.8 |

[a] For numbers of rats per group and length of feeding trials, see text. Average initial weight, expt. 1, 55 grams; expt. 2, 55–56; expt. 3, 67–69. Amino acids used were all L-form.

possibly react with carbohydrate would be the imide group. If the hydrogen of the imide group were replaced by a carbohydrate group, two possibilities could occur: The peptide bond could no longer be broken by the body enzymes, leaving large peptides containing many amino acids undigested, or the peptide bond could be broken but the carbohydrate residues on the nitrogen would make the amino acid unavailable to the animal body. The proposed studies on di- and tripeptides, heated with sugar, should throw some light on this question.

The diminished availability of methionine in DMF to microorganisms (20%) compared to the unavailability of the methionine to rats (100%) is disappointing in view of the need for a rapid method for studying availability. However, in earlier work on cottonseed proteins (Horn *et al.*, 1952) the microbiological method gave satisfactory results when compared with the results of animal feeding. The difference in availability to the microorganism between compounds of the type described here and the complexes formed in heated carbohydrate foods after proteolytic enzyme digestion, suggests that the complexes obtained in the food hydrolyzates are different from those of the simple DMF type. A microorganism with more fastidious requirements than the organism used here is necessary for a rapid availability test on all types of amino acid complexes.

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