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THREONINE DEFICIENCY IN HYDROLYSATES OF ZEIN PREPARED BY AUTOCLAVING*

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In a previous paper (Borchers and Berg, 1942) we showed that autoclaving zein with sulfuric acid longer than necessary for complete hydrolysis causes destruction and racemization; either of these might account for the failure of such a hydrolysate to promote growth in young rats when substituted for a hydrolysate, prepared by refluxing, in a diet which produced moderate growth. It seemed to us that this deterioration in the dietary protein might well be the result primarily of essential amino acid deficiencies which could be detected and overcome by appropriate supplementation. Because threonine is known to be present in zein in relatively small amount[1] and because its 2 asymmetric carbon atoms might render it more susceptible to configurational modification by racemization, a ready production of threonine deficiency was considered likely. This was confirmed; addition of threonine to an autoclave hydrolysate (heated with 10 per cent sulfuric acid for 8 hours at 165°) promoted about as rapid growth as had the reflux hydrolysate which it replaced. Longer heating at higher temperatures produced deficiencies not fully met by threonine alone.

The present communication records growth data which sub-

* Part of this paper was presented in abstract before the American Society of Biological Chemists at New Orleans in March, 1940 (Borchers and Berg, 1940).

Some initial observations were presented in a dissertation submitted by John R. Totter in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

[1] Private communication from Dr. W. C. Rose.

stantiate these observations and summarizes results obtained by analysis of representative hydrolysates for threonine as chemical methods became available.

EXPERIMENTAL

Feeding Studies—In an attempt to confirm and extend our observation that hydrolysis of zein in the autoclave may induce deficiencies not remedied by the usual supplements, four hydrolysates were compared for their effect on growth. These were prepared by refluxing with 25 per cent sulfuric acid solution for 18 hours (Hydrolysate R); by heating in the autoclave at 165° for 8 hours with 10 per cent sulfuric acid solution (Hydrolysate A-165); by autoclaving at 140° for 8 hours with 14 per cent sulfuric acid (Hydrolysate A-140); and by heating in the autoclave at 180° for 15 hours with 14 per cent sulfuric acid (Hydrolysate A-180). Hydrolysate A-140 was chosen because it showed nearly maximum free amino nitrogen and much less decomposition than Hydrolysate A-165; Hydrolysate A-180, because decomposition in it was extensive. The hydrolysates were rendered suitable for feeding by removing the sulfate ion exactly and concentrating to dryness, essentially as directed by Berg and Rose (1929). Unhydrolyzed zein served as a control. Each diet contained zein or zein hydrolysate 14.8 per cent, *l*(+)-lysine dihydrochloride 0.75, *l*(−)-tryptophane 0.2, *l*(−)-cystine 0.2, *l*(+)-histidine monohydrochloride 0.37, sodium bicarbonate 0.73, agar 2.0, salt mixture (Hubbell, Mendel, and Wakeman, 1937) 2.5, sucrose 15.0, starch 39.45, cod liver oil 5.0, and Crisco 19.0. The sodium bicarbonate was added to neutralize the hydrochlorides of lysine and histidine. The amino acids used here and for later supplementation were prepared in this laboratory; subsequent supplements replaced equal weights of the zein or of the hydrolysate. The vitamin B complex was fed separately in the form of two pills daily, each containing 0.02 mg. of crystalline riboflavin, 0.02 mg. of thiamine chloride, 0.1 mg. of nicotinic acid, 25 mg. of ryzamin-B, and enough starch to provide a consistency suitable for molding.

The experimental animals were litter mate rats weighing initially 44 to 64 gm.; they were housed in individual false bottomed cages; food and water were available at all times. To allow clear presentation of the growth data in a single chart

(Fig. 1), curves are given for only three of the five or six animals in each group; the records omitted were practically duplicates of those included. No striking difference was observed between average growth response on whole zein and on zein Hydrolysate R or A-140; individual growth rates varied widely, especially on unhydrolyzed zein. So far we have been only mildly successful in improving the nutritive value of whole zein by supplementing

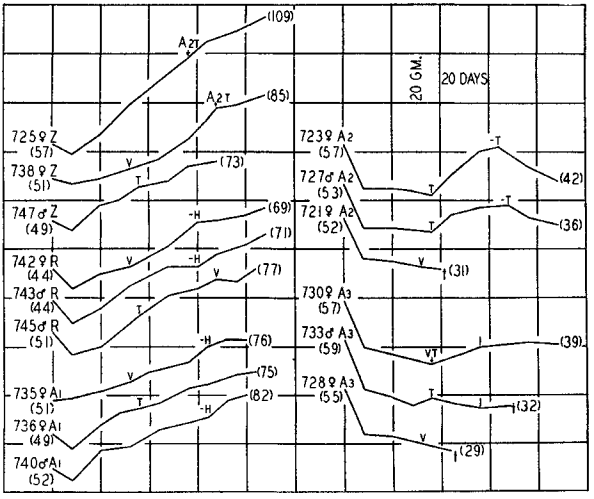

Fig. 1. Growth on zein and zein hydrolysates. Initial and final weights are given in parentheses. The daggers signify death. The symbols, Z, R, A_1, A_2, and A_3 represent basal diets containing zein, zein hydrolyzed under a reflux, and in the autoclave at 140°, 165°, and 180°, respectively. On the two upper left curves the A_2 which succeeds Z denotes replacement. Supplementation of the basal diet with *dl*-threonine (0.6 per cent) is indicated by T, with *dl*-valine (0.84 per cent) by V, and with *dl*-isoleucine (0.5 per cent) by I. Subsequent withdrawal is indicated by the minus sign. Removal of the histidine contained in the basal diet is represented by $-H$.

it with essential amino acids; normal rate of growth has not yet been obtained. Addition of threonine to any one of these diets produced no distinct growth acceleration.

On Hydrolysate A-165 all animals showed marked loss in weight; when threonine was added, growth began promptly at a rate approximately the same as that observed on pure zein or on Hydrolysate R or A-140. When the diet containing Hydrolysate A-165,

to which threonine had been added, was fed to rats which had previously received whole zein, growth continued without interruption at a rate only slightly reduced. Hence, under the experimental conditions obtaining, threonine effectively met the nutritional deficiency induced by the autoclaving. Tests with a casein hydrolysate prepared like Hydrolysate A-165 gave similar results.

Rats on Hydrolysate A-180 also showed marked losses in weight; when threonine was added, three of the four animals still living responded by growing, but at a rate decidedly below the average for the series receiving Hydrolysate A-165. Lack of adequate threonine was evidently not the only nutritional deficiency induced by this more drastic autoclaving treatment; additional supplements of isoleucine or valine caused no further improvement.

Differences in growth response on the hydrolysates thus correlated well with differences in optical rotation and in amino nitrogen content, as summarized in Table I. The extent of amino acid destruction was indicated also by ammonia content. In Hydrolysate A-165, 28 per cent of the nitrogen was found in this form before removal of the sulfate and evaporation to dryness. The ammonia content of Hydrolysates R, A-140, and A-180 was not determined, but hydrolysates prepared under the same conditions contained about 19, 22, and 30 per cent of ammonia nitrogen, respectively, before concentration.

Estimations of Threonine Content—When the method of Block and Bolling (1939) became available for estimating threonine, we undertook to apply it to determine the degree of threonine destruction in the hydrolysates fed. This method is empirical and unsuited to casual application. After considerable study several convenient modifications were found possible. These consisted chiefly of the elimination of the 4° bath, the preparation of the aldehyde-free glacial acetic acid-lead tetraacetate mixture in a single step, omission of the precipitation of the sulfate ion from the hydrolysates, and the use of aqueous standards of threonine. Results obtained upon incorporating these changes in the procedure were essentially the same as those obtained with the unmodified method. In our experience the rate of aeration seemed especially significant; since both too slow and too rapid speeds yielded less than maximum color and no set rate was suitable for all analyses,

it was necessary to establish an optimum rate for each test. After some experience with the method as a whole, maximum errors on separately run aliquots were reduced to 5 per cent or less.

Publication of the data obtained with the Block and Bolling procedure has been delayed to allow simultaneous presentation of results obtained with the recent method of Shinn and Nicolet (1941). Their procedure is relatively simple. It requires no standard and reagents obtainable on the market are employed. For our initial tests periodic acid was not immediately available; substitution of potassium periodate in N sulfuric acid and modification of the other solutions, to permit neutralization of the extra acidity and to keep the final total volume approximately the same, gave results essentially identical with those obtained later with the procedure as published. An average of 96.8 per cent of the theoretical yield of acetaldehyde has been produced from several samples of threonine; this agrees with the findings of the originators.

Data obtained by applying the two threonine methods to the same samples of threonine and allothreonine[2] and to the same hydrolysates of casein and zein are summarized in Table I.

The Shinn and Nicolet procedure yielded as much acetaldehyde from allothreonine as from threonine. Identical yields from the *dl* forms of these amino acids were obtained also by Martin and Synge (1941) whose procedure is similar in principle but produces less acetaldehyde. In the Block and Bolling procedure less than half as much color was produced from allothreonine as from the standard *dl*-threonine during 1 hour of aeration; in the 2nd hour an additional 10 per cent could be accounted for. Calculations based on the response of threonine and allothreonine to the two methods suggest that the mixture of threonine and allothreonine

[2] The nomenclature is that of Meyer and Rose (1936) who designated natural threonine *d*(−) because it is structurally analogous to *d*(−)-threose. We are indebted to Dr. H. E. Carter for the samples of *l*(+)-threonine and *l*-, *d*-, and *dl*-allothreonine used in these studies. The synthetic mixture of *dl*-threonine and *dl*-allothreonine and the samples of *d*(−)- and *dl*-threonine were prepared in this laboratory by Dr. C. D. Bauer who used the synthetic and resolution procedures of West and Carter (1937). All of the samples were carefully dried to remove any trace of alcohol, since such might undergo conversion to acetaldehyde and thus be a source of error.

obtained by synthesis according to the method of West and Carter (1937) contained 96 per cent of these amino acids, of which 35 or 36 per cent was threonine. This is in general agreement with the observation made by West and Carter that the mixture of α-bromo-

TABLE I

Analysis of Threonine and Allothreonine and of Casein and Zein Hydrolysates

	α_D*	$[\alpha]_D^{30}$†	N found‡	Threonine found	
				Block-Bolling method	Shinn-Nicolet method
	degrees	*degrees*	*per cent*	*per cent*	*per cent*
d(−)-Threonine		−27.4	11.03	100.0	96.4
l(+)-Threonine		+28.1	11.53	98.7	97.7
dl-Threonine			11.71	101.5	96.6
d-Allothreonine		+7.9	11.64	47.5	
l-Allothreonine		−8.2	11.52	43.0	
dl-Allothreonine			11.52	43.0	96.9
Threonine-allothreonine mixture, as synthesized			10.98	65.0	93.1
Casein Hydrolysate R				3.5	3.2
" " A-165				1.0	0.7
Zein Hydrolysate R	+2.6		72	2.4	2.20
" " A-140	+2.0		69	1.9	1.65
" " A-165	+0.5		60	0.5	0.65
" " A-180	+0.3		58	0.15	0.25

* The rotations of the zein hydrolysates were read in 1 dm. tubes before neutralization of the sulfuric acid and are based on concentrations of 27.5 mg. of total nitrogen per cc.

† In the determination of the specific rotations of threonine and allothreonine, 2 per cent aqueous solutions (0.2 gm. in 10 cc.) were polarized in 2 dm. tubes.

‡ The nitrogen figures for threonine and allothreonine represent total nitrogen by the micro-Kjeldahl method; calculated nitrogen is 11.76 per cent. The nitrogen figures for the zein hydrolysates refer to amino nitrogen expressed as per cent of total nitrogen. The latter determinations were made prior to removal of the sulfuric acid used for hydrolysis.

β-methoxy-*n*-butyric acids prepared in their method of synthesis contains from 30 to 40 per cent of the precursor of *dl*-threonine.

The data for the threonine content of the zein and casein hydrolysates in Table I are not corrected for alanine, of which zein contains almost 10 per cent and casein about 2. The Block and Bolling procedure, applied to 2 mg. samples of alanine (ap-

proximately the amount present in the zein samples analyzed), yielded a little less than 2 per cent of the color obtainable from a molecular'y equivalent amount of threonine. Correction on this basis would lower the estimated threonine content of zein Hydrolysate R from 2.4 to about 2.2 per cent, but would have little effect on the estimate on casein Hydrolysate R. Just what correction should be applied to the analyses of the autoclaved hydrolysates is uncertain because the extent to which alanine may have been destroyed is not known. In the Shinn and Nicolet procedure only 0.18 per cent as much iodine was required to titrate the blank from 40 mg. samples of commercial *dl*-alanine as would have been needed had a molecularly equivalent weight of threonine been analyzed. Hence correction for alanine in this method seems pointless. Calculations to compensate for the production of but 97 per cent of the theoretical amount of acetaldehyde might well be applied, but this has not been done. Correction of the data which Martin and Synge (1941) obtained by applying their method to zein, and which they estimate to be 30 to 35 per cent low, gives a threonine content of 2.16 to 2.24 per cent.

Despite discrepancies in the figures for the threonine content, with both the Block and Bolling and the Shinn and Nicolet methods decreases of the same order are shown in the autoclave hydrolysates: about 20 per cent in Hydrolysate A-140, 70 per cent in Hydrolysate A-165, and 90 per cent in Hydrolysate A-180. Lack of information concerning the degree of alanine destruction prevents drawing definite conclusions concerning allothreonine production. That there may be such in Hydrolysates A-165 and A-180 is suggested by the lower estimate with the Block and Bolling than with the Shinn and Nicolet procedure.

Pure threonine also undergoes marked destruction when autoclaved in sulfuric acid solution; preliminary tests have been inadequate in scope to clarify interrelationships between destruction and racemization and allothreonine formation. Preparation of an adequate supply of the optical isomers of threonine for use in such studies is in progress.

SUMMARY

Feeding tests show that sulfuric acid hydrolysates of zein prepared under a reflux or under relatively mild conditions in the autoclave possess about the same capacity as zein to support

growth when incorporated in diets supplemented with lysine, tryptophane, histidine, and cystine. More drastic autoclave treatment produces hydrolysates which do not support growth under similar conditions unless threonine is added; still more severe autoclaving induces deficiences which cannot be met by threonine supplementation alone.

Threonine and allothreonine produce equal amounts of acetaldehyde in the procedure of Shinn and Nicolet; in the method of Block and Bolling considerably less color is produced with allothreonine than with threonine. Assays of the several hydrolysates of zein and casein according to either method show that threonine is destroyed as the conditions of hydrolysis become more severe. Pure threonine also suffers marked destruction when autoclaved with sulfuric acid under conditions which destroy it in zein.

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