Probable Absence of Citrulline from Proteins. (17452)

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Several early investigators noted that carbon dioxide is one of the products of the acid hydrolysis of proteins. Dunn[1] found that as much as 0.81% of casein could be accounted for as carbon dioxide after 30 hours of acid hydrolysis. It has been generally supposed that this carbon dioxide is bound as a ureide in the protein molecule and the exact type of binding is uncertain. Fearon's[2] diacetyl monoxime test for ureide compounds is positive for many proteins. Wada[3] reported the isolation of citrulline from tryptic digests of casein, but this work has never been repeated. Since certain types of bacteria can convert arginine to citrulline, it is possible that Wada's citrulline may have been formed by bacterial action during the enzymatic hydrolysis.

This investigation was undertaken in order to obtain additional evidence as to the nature of the ureide groupings in proteins.

Methods and results. The proteins used in this study were all prepared in the laboratory. Casein was prepared according to the method of Van Slyke and Baker,[4] the crystalline globulins of watermelon seed and pumpkin seed by the method of Vickery,[5] arachin by the method of Johns and Jones,[6] amandin by the method of Osborne,[7] and myosin by the method of Dainty, Kleinzeller, *et al.*[8]

The 2 crystalline globulins were recrystallized twice after the initial isolation, and the arachin and amandin were twice dissolved and precipitated by dialysis after the initial isolation. The casein and myosin were used as originally isolated.

After preparation the proteins were air dried until completely equilibrated with regard to moisture content. They were analyzed for moisture and ash, and for nitrogen by the Kjeldahl method using mercury as the catalyst. The results of these analyses are given in Table I.

These proteins gave moderate to marked positive tests for ureide groups when tested under the conditions of Archibald's[9] citrulline determination. After they had been hydrolyzed for 16 hours or longer with 6N hydrochloric acid, none of them gave positive tests. It was thought that this might be due to destruction of any citrulline contained in the proteins during the hydrolysis, so the destruction of citrulline alone under the conditions employed for hydrolysis of the proteins was studied. One ml samples of solutions of citrulline (about 1 mg/ml) in 6N hydrochloric acid were sealed in small glass bomb tubes and heated in the oven at 110°C. At intervals samples were removed and cooled, and their citrulline content was determined by Archibald's method. Under these conditions citrulline is surprisingly resistant to acid hydrolysis; it is destroyed by a first order reaction whose half-life is approximately 26 to 28 hours, as is seen in Fig. 1. This is not conclusive evidence for the absence of citrulline from proteins since it is well known that cystine and threonine are much more easily destroyed when in peptide combination than when free. Other experiments in which paper chromatography was employed to identify the products of hydrolysis of citrulline showed that under these conditions ornithine was the only product formed in detectable amounts.

Since Wada's isolation of citrulline was

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TABLE I.
Analysis of Protein Samples.

Protein	Moisture, %	Ash, %	N, %	N, % M and A free
Casein	10.36	0.37	13.78	15.44
Myosin	10.04	1.03	15.03	16.90
Arachin	9.44	2.86	16.01	18.26
Amandin	9.38	0.43	17.45	19.35
Pumpkin seed globulin	10.23	0.15	16.47	18.38
Watermelon seed globulin	9.31	0.06	16.77	18.50

done from a tryptic digest of casein it was decided to determine the amounts of citrulline present in enzymatic hydrolysates of the 6 proteins. Seven 1 g samples of each of the proteins were weighed out into 125 ml Erlenmeyer flasks. About 1 g of precipitated calcium carbonate was added to each flask, together with 50 ml of water. The samples were allowed to stand for 24 hours in the refrigerator in order for the protein to become thoroughly wetted. Ten mg of Difco, 1/110 trypsin were then added to each flask and to each of 7 enzyme blanks containing calcium carbonate and water. A known amount of citrulline was added to 3 of the samples of each protein and to 3 enzyme blanks; about 1 ml of xylene was added to each flask and all samples were incubated at 37°C. At intervals samples were withdrawn from one enzyme blank and from one sample (containing no added citrulline) of each protein and titrated by Northrup's[10] formol titration, modified in that the end points were determined with the aid of a Beckman pH meter. When the amino nitrogen contents had become constant in the pilot flasks an additional 10 mg of trypsin and 1 ml of an active Waldschmidt-Leitz[11] preparation of erepsin were added to each flask. Digestion and determination of the amino nitrogen in the pilot flask was continued; and when the amino nitrogen had become again constant an additional ml of erepsin was added to each flask. After one week's additional digestion the samples were removed from the incubator, filtered into 100 ml volumetric flasks, and made up to volume with the washings. Aliquots of these digests were taken for determination of total amino nitrogen content and of total ureide content. The results are shown in Table II. In each case a correction has been made for both amino nitrogen and citrulline content by subtracting the value found in the appropriate blank from that found in the protein digest. In no case was more than 0.09% of the protein accounted for as "citrulline" by the colorimetric determination; however, in all runs in which citrulline was added to the digests the recoveries in terms of color intensities were extraordinarily high, *i.e.*, the observed colorimetric values corresponded to from 300 to 500% of the amounts added. It was thought that a peptide with enhanced chromogenicity might have been formed during hydrolysis, but similarly high "recoveries" were obtained when the citrulline was added to the digests immediately before determination and even when the digests had been heated for half an hour on a boiling water bath to inactivate any residual enzyme.

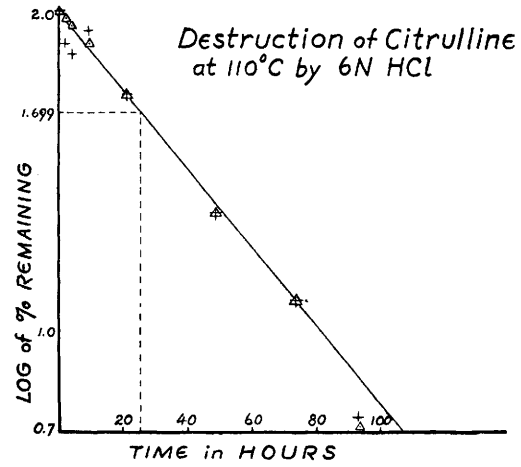

FIG. 1.

[10] Northrup, J. H., *J. Gen. Physiol.*, 1925-26, **9**, 767.

[11] Waldschmidt-Leitz, E., and Schaeffner, A., *Z. physiol. Chem.*, 1926, **151**, 31.

TABLE II. Citrulline Content of and "Recovery" from Protein Digests.

Protein	% N titrable as NH_2–N after acid hydrolysis (A)	% N titrable as NH_2–N after enzyme hydrolysis (B)	% digestion (100 B/A) (C)	% Citrulline in protein (M and A free) (D)	Citrulline added to digest, mg/100 ml (E)	Citrulline found, mg/100 ml (F)	% recovery (G)
Casein	11.80	8.01	67.8	0.093	4.675*	13.66	271
					3.260†	12.82	391‡
Myosin	12.76	8.79	69.0	0.089	4.675*	15.00	299
					3.260†	10.70	299§
Arachin	12.36	8.03	65.0	0.044	4.675*	15.56	320
					3.260†	11.20	336‡
Amandin	13.27	5.02	37.9	0.080	4.675*	18.14	368
					3.260†	15.08	435‡
Pumpkin seed globulin	12.01	8.13	67.6	0.072	4.675*	18.66	381
					3.260†	13.51	388§
Watermelon seed globulin	11.75	8.12	69.1	0.066	4.675*	17.02	346
					3.260†	11.02	316‡

* Present throughout digestion.
† 1 ml citrulline solution diluted to 25 ml with digest.
‡ Not boiled.
§ Boiled.

Since these extraordinarily high "recoveries" were not obtained in the enzyme blank, substances other than the proteins or their digestion products are eliminated as a cause for the high "recovery." Amino acids cannot cause this effect since an acid hydrolysate of casein added to citrulline and put through the colorimetric determination of citrulline did not give high results. Similar but less markedly high "recoveries" were obtained when commercial peptones were added to citrulline solutions, so that it would appear that the presence of partial digestion products of the proteins in a citrulline solution markedly enhances the chromogenicity of the citrulline in the diacetyl monoxime reaction.

Discussion. Although the results obtained in this study are not absolutely conclusive in demonstrating that citrulline is not present in proteins, they nevertheless strongly indicate that it is absent or present only in very small amounts in the 6 proteins studied. Citrulline might be more readily destroyed by acid when in peptide combination than when in the free state, but unless this difference were very great, small amounts of it should survive acid hydrolysis. Acid hydrolysates were completely negative for citrulline by the diacetyl monoxime reaction. Furthermore, if significant amounts of citrulline were present, it should have been recovered in the enzymatic digests. The exact nature of the ureide compound present cannot be determined from these results. The virtual elimination of citrulline as a possibility enhances the probability that the original ureide may be either a cyclic type of structure or more probably simply carbamic acid present as a constituent of the peptide chain and hydrolyzed and spontaneously decomposed during enzymatic hydrolysis.

Summary. 1. Casein, amandin, arachin, myosin, and the crystalline globulins of pumpkin seed and watermelon seed give marked to moderately strong diacetyl monoxime reactions for citrulline when tested directly but their acid hydrolysates give completely negative results.

2. Citrulline itself is relatively slowly destroyed by treatment with 6N hydrochloric acid at 110°C.

3. Only very small amounts of ureide com-

pounds are found in enzymatic hydrolysates of the 6 proteins; however, when citrulline is added to such hydrolysates, its "recovery" in terms of colorimetric units corresponds to several times the theoretical value. The reason for this anomalous result has yet to be determined.

4. It is probable that citrulline is either absent or present only in minimal quantities in these proteins.

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Comparative Study of Lipids in Whole Carcasses of Arctic and Non-Arctic Fish.* (17453)

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Information concerning lipid content and metabolism in cold-blooded animals is relatively meager.[1] Concerning these matters in Arctic poikilotherms, information is non-existent. A survey was, therefore, made of the lipid content of whole fish, *Pygosteus pungitius*,[2] the Arctic 9-spined stickleback. The results were evaluated in comparison with similar analyses made on the common guppy *(Girardinus guppyi)*.

Material and methods. Whole sticklebacks or guppies were carefully weighed and then dropped into a mixture consisting of 3 parts of pure ethanol and 1 part benzine. After 24 hours the fish were ground with sand and the lipids extracted according to Bloor's method.[1] Total fatty acids were estimated using the method of Bloor as modified by Snell.[3] Lipid phosphorus was estimated by a modified Youngburg technic;[4] from the latter results phospholipids were calculated as lecithin. Measurements of cholesterol were based on methods developed by Bloor.[5] Since all the procedures are colorimetric, a Klett-Summerson photometer was used to measure the various color intensities. Ten sticklebacks were analyzed. Two groups of 5 each guppies were tested: one group was fed ordinary commercial fish food; the other, chopped earthworms.

Results. The results are summarized in Table I, which shows that there is a mean value of 4.88% fatty acids, 0.32% cholesterol, and 1.87% phospholipids in the Arctic stickleback.

Table I also shows that the group of guppies fed on commercial fish food have the following mean lipid values: fatty acids, 8.87%; cholesterol, 0.56%; phospholipid, 2.31%. Guppies fed on chopped worms have lipid values practically identical with those fed commercial fish food.

Discussion. The results indicate that there is about the same amount of cholesterol in the guppies on either diet, but that there is less cholesterol in the Arctic fish than in the guppies. Total fatty acids are higher in the guppies, on either diet, than in the Arctic stickleback. The phospholipid content of all

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