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# The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. IX.[1] Hydrolysis in the Presence of Djenkolic and of Thiazolidine-4-carboxylic Acids

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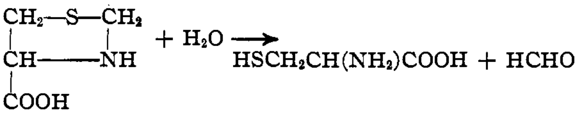
In a series of studies Gortner and co-workers have shown that: (1) the acid-insoluble humin of a protein hydrolyzate results from the condensation of an aldehyde with tryptophan,[2,3] or with other indole derivative[1]; (2) tyrosine and an aldehyde are factors in the formation of the acid soluble humin[3]; (3) the ammonia fraction steadily increases with added increments of aldehyde.[4] They concluded that proteins contain "an aldehyde of unknown origin" or a hitherto unknown component which, under the conditions of acid hydrolysis, yields an aldehyde to condense with the indole nucleus. Recently djenkolic acid has been found in the djenkol bean and is an aliphatic condensation product of formaldehyde with cysteine[5]; thiazolidine-4-carboxylic acid is a synthetic heterocycle produced from the same components. It seemed desirable to determine whether these compounds could yield their aldehyde component for humin formation.

The hydrolysis of djenkolic acid with 20% hydrochloric acid has not previously been noted. Van Veen and Hyman[5] reported that no cleavage occurred with varying concentrations of hydrochloric acid and for different durations of boiling since the original compound was recovered. However, when boiled with strong sulfuric acid, djenkolic acid cleaved to cysteine and formaldehyde which were identified as dibenzoylcystine and as the phloroglucinol derivative, respectively.

$$CH_2[SCH_2CH(NH_2)COOH]_2 + H_2O \longrightarrow 2HSCH_2CH(NH_2)COOH + HCHO$$

Schubert[6] has prepared thiazolidine-4-carboxylic acid by condensing cysteine and formaldehyde. Ratner and Clarke[7] identified formaldehyde by its dimedon derivative in a constant volume distillation of the compound with *N* hydrochloric acid, and demonstrated the equivalent amount of cysteine in the residue. No report has been made of the fate of thiazolidine carboxylic acid under the conditions of a protein hydrolyzate.



## Experimental

**Materials.**—Djenkolic acid was prepared according to du Vigneaud and Patterson.[8] The product gave a negative nitroprusside test and a benzoyl derivative, m. p. 84°. Thiazolidine-4-carboxylic acid was prepared by the procedure of Ratner and Clarke[7]: the product melted with decomposition at 196–197°. The tryptophan and the gelatin were from the lot used by Gortner and Holm.[3]

**Hydrolytic Cleavage with 20% Hydrochloric Acid.**—To determine whether djenkolic and thiazolidine-4-carboxylic acids would cleave to formaldehyde and cysteine under the conditions of acid hydrolysis of a protein, six solutions were prepared for treatment and analysis as indicated in Table I.

TABLE I

| Soln. number | | Hours boiled | Analyses Cystine (mg.) | Analyses Cysteine (mg.) | Polarogram limiting current (in cm.) at *E* of 1.7 v. |
|---|---|---|---|---|---|
| 1 | 0.01 *M* djenkolic acid | 0 | ... | ... | ... |
| 2 | .01 *M* djenkolic acid | 24 | 66.0 | 20.0 | 3.9 |
| 3 | .01 *M* formaldehyde and cystine | 24 | 82.5 | 18.8 | 4.2 |
| 4 | .01 *M* cystine | 0 | 120.0 | 0.0 | 4.0 |
| 5 | .033 *M* djenkolic acid[a] | 24 | 120.0 | 157.5 | 4.0 |
| 6 | .033 *M* thiazolidine-carboxylic acid[a] | 24 | 71.0 | 40.5 | 2.7 |

[a] Plus 0.075 g. tryptophan. All solutions made in 20% hydrochloric acid.

The polarographic analyses were made with a type X Heyrovsky micropolarograph for the automatic photographing of current–voltage curves. The solution for analysis was made with 1 ml. of the sample, 25 ml. each of *N* ammonium hydroxide and chloride, 10 ml. of 0.025 *N* cobaltous chloride plus distilled water to 250 ml. During all runs 0.005 of the full sensitivity of the galvanometer was maintained. The results are shown in polarograms 1 to 6 of solutions 1 to 6, respectively, Fig. 1.

The cystine and cysteine were deteremined by Sullivan's

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[1] For paper VIII of this series, see Burr and Gortner, THIS JOURNAL, **46**, 1224 (1924).

[2] Gortner and Blish, *ibid.*, **37**, 1630 (1915).

[3] Gortner and Holm, *ibid.*, **39**, 2477 (1917).

[4] Zeleny and Gortner, *J. Biol. Chem.*, **90**, 427 (1931).

[5] Van Veen and Hyman, *Rec. trav. chim.*, **54**, 493 (1935).

[6] Schubert, *J. Biol. Chem.*, **114**, 341 (1936).

[7] Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

[8] Du Vigneaud and Patterson, *J. Biol. Chem.*, **114**, 533 (1930).

method,[9] using the appropriate amino acid as standard.

Analysis of the humin nitrogen from solutions 5 and 6 gives an independent measure of the extent to which formaldehyde is liberated and can condense with tryptophan. These figures are incorporated in Tables II and III and are indicated by the fact that no gelatin was included in the hydrolyzate.

**Humin Formation in the Presence of Gelatin.**—The formaldehyde precursors (djenkolic and thiazolidine-4-carboxylic acids) were next added to 3 g. of gelatin and 0.075 g. of tryptophan. The usual Van Slyke nitrogen determinations were made of the humin and ammonia fractions. For small amounts of humin nitrogen the micro-Kjeldahl method of Pregl[10] was modified; for larger values the semi-micro method of Cavett[11] was used with the boric acid modification of Meeker and Wagner.[12] The results are reported in Tables II and III.

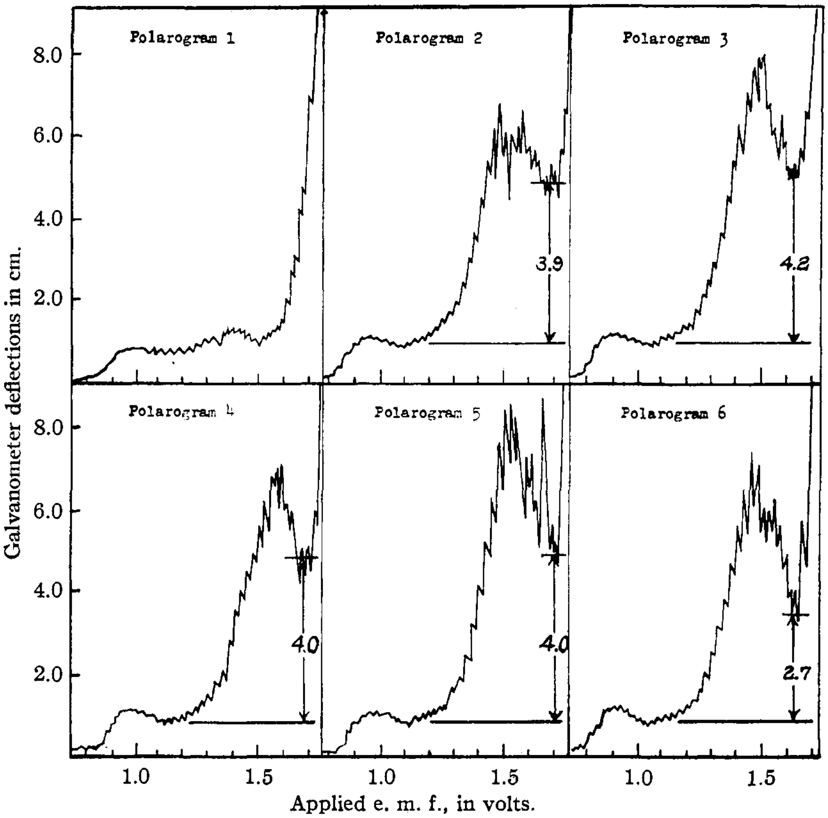

Fig. 1.

## Discussion

**The Hydrolytic Cleavage of the Formaldehyde Precursors.**—Three lines of data indicate that djenkolic acid and thiazolidine-4-carboxylic acid hydrolyze to formaldehyde and cysteine (or cystine) under the conditions of a Van Slyke protein analysis. The sums of the cystine plus cysteine values reported in Table I indicate that djenkolic acid yields 86 mg., whereas an equivalent synthetic mixture (solution 3) yields 101.3 mg.; in other words, the djenkolic acid is cleaved to the extent of 85%. The cystine plus cysteine values from solutions 5 and 6 indicate a partial cleavage of the two formaldehyde precursors also in the presence of tryptophan.

The polarograms furnish additional evidence of

TABLE II

THE EFFECT OF DJENKOLIC ACID ON NITROGEN DISTRIBUTION

3 g. gelatin plus 75 mg. tryptophan

| Djenkolic acid added, mg. | Insoluble humin N, mg. | Soluble humin N, mg. | Ammonia N, mg. |
|---|---|---|---|
| 0[a] | 0.10[a] | 1.20[a] | 2.80[a] |
| 726[a] | .05[a] | 1.18[a] | 3.00[a] |
| 0 | 2.98 | 4.20 | 1.03 |
| 363 | 9.07 | 1.97 | 3.00 |
| 436 | 7.76 | 2.34 | 2.85 |
| 532 | 8.24 | 2.62 | 3.02 |
| 601 | 7.26 | 2.17 | 3.42 |
| 726 | 8.66 | 1.94 | 3.17 |
| 968 | 7.66 | 2.12 | 3.77 |
| 726[b] | 9.73[b] | 1.49[b] | 0.00[b] |

[a] No tryptophan added. [b] No gelatin added.

TABLE III

THE EFFECT OF THIAZOLIDINE-4-CARBOXYLIC ACID ON NITROGEN DISTRIBUTION

3 g. gelatin plus 75 mg. tryptophan

| Acid added, mg. | Insoluble humin N, mg. | Soluble humin N, mg. | Ammonia N, mg. |
|---|---|---|---|
| 0[a] | 0.10[a] | 1.20[a] | 2.80[a] |
| 333[a] | .05[a] | 1.74[a] | 3.07[a] |
| 0 | 2.98 | 4.20 | 1.03 |
| 166 | 8.25 | 2.31 | 3.00 |
| 200 | 6.68 | 2.47 | 2.85 |
| 244 | 7.56 | 2.50 | 3.56 |
| 333 | 7.91 | 1.94 | 3.73 |
| 443 | 8.12 | 3.24 | 4.69 |
| 333[b] | 9.17[b] | 1.90[b] | 0.00[b] |

[a] No tryptophan added. [b] No gelatin added.

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(9) Block, "The Determination of Amino Acids," Burgess Publishing Co., Minneapolis, Minn., 1938.

(10) Pregl, "Quantitative Organic Microanalysis," J. and A. Churchill, London, 1938.

(11) Cavett, *J. Lab. Clin. Med.*, **17**, 79 (1931).

(12) Meeker and Wagner, *Ind. Eng. Chem., Anal. Ed.*, **5**, 396 (1933).

a breakdown with the production of cysteine (or cystine). The unboiled djenkolic acid gives a curve similar to that reported by Smith and Rodden.[13] When the djenkolic acid is boiled (solution 2) cystine is produced as evidenced by the fact that the current–voltage maximum comes at 1.5 volts, as is true for cystine (solution 4) and as found by Brdicka.[14] The limiting current intensity of the synthetic solution (number 3) indicates the values which would be attained if the djenkolic acid of solution 2 were completely hydrolyzed and gives effect to any influence of formaldehyde. By comparing the limiting current values for solutions 2 and 3, approximately 90% of the djenkolic acid is converted to its equivalent of cystine.

The third line of evidence is revealed by boiling the formaldehyde precursors with tryptophan. Table II reports 9.73 mg. of acid insoluble humin nitrogen resulting from the addition of 726 mg. of djenkolic acid; Table III records 9.17 mg. under similar conditions except that 333 mg. of thiazolidine carboxylic acid supplies the formaldehyde. These values represent 94.5 and 89.1%, respectively, of the tryptophan nitrogen converted to humin by formaldehyde and agree very well with the value of 95.5% obtained by Gortner and Holm[3] when trioxymethylene was added in an amount equivalent to the formaldehyde content of our two acids.

**Hydrolysis of Gelatin in Presence of the Formaldehyde Precursors.**—This run was made as a final test of the availability of the formaldehyde of djenkolic and thiazolidine-carboxylic acids for humin formation. Gelatin was hydrolyzed in the presence of varying amounts of the aldehyde precursors. The results are reported in Tables II and III.

The results substantiate the preliminary findings that acid hydrolysis of djenkolic and thiazolidine-carboxylic acids yield formaldehyde, and that this aldehyde then behaves in the same manner as that added as trioxymethylene. By the addition of appropriate amounts of the formaldehyde precursor the insoluble humin nitrogen varies in a manner previously observed by Gortner and Holm[3] for gelatin and fibrin with trioxymethylene. However, in the latter case 75 mg. of trioxymethylene was required whereas we found that 38 mg. of equivalent formaldehyde in the form of the two precursors was needed. The rate of liberation of the aldehyde may be the reason for this variance. It should be noted that the same amount of insoluble humin is produced and that our values supply just a slight excess over 3 moles of aldehydes to 1 mole of tryptophan, a ratio postulated by Burr and Gortner[1] from studies using indole and trioxymethylene.

In the absence of a compound liberating formaldehyde but in the presence of tryptophan the acid soluble humin is higher than in the presence of a formaldehyde compound. Since gelatin is deficient in tyrosine these results might have been anticipated, and the lowered values in the presence of formaldehyde may be due to the formation of more insoluble humin.

## Summary

1. The two compounds, djenkolic and thiazolidine-4-carboxylic acids, are hydrolytically cleaved to formaldehyde and cysteine (or cystine) after hydrolysis for twenty-four hours with 20% hydrochloric acid. This has been supported by evidence of polarographic analysis, Sullivan's colorimetric analysis and condensation of the derived formaldehyde with tryptophan. This finding with respect to djenkolic acid is contrary to that reported by other workers.

2. Djenkolic acid, a naturally occurring compound, or thiazolidine-4-carboxylic acid added to gelatin and tryptophan and hydrolyzed for twenty-four hours has provided a possible factor of an "aldehyde of unknown origin" to bring about humin formation.

3. It has been found that a lesser amount of formaldehyde as obtainable from these precursor compounds is needed to bring about the maximum insoluble humin than when formaldehyde is added as trioxymethylene.

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(13) Smith and Rodden, *J. Research Natl. Bur. Standards*, **22**, 669 (1939).

(14) Brdicka, *Collection Czechoslov. Chem. Commun.*, **5**, 238 (1933).