groups in *ortho* positions, may inhibit carbon polymerization sufficiently to give stability to the intermediate methylene derivative. Or, the conversion of $C_6H_5$—CHOH—$C(CH_3)_2$—COOE, into $C_6H_5$—C(COOE) $=C(CH_3)_2$ by dehydration with phosphorus pentoxide.[1] Methyl alcohol is not eliminated in this reaction, owing to the very considerable expense of energy required to separate the methyl group from the carbon atom, while the unilateral elimination of water is facilitated by the phenyl and the carbethoxyl group in decreasing the affinity between the carbinol-carbon and the hydrogen and hydroxyl. The great reservoir of free energy at the Δ-carbon in the intermediate structure, $C_6H_5 - \dot{\dot{C}} - C(CH_3)_2 -$ COOE, induces a rearrangement, in which the carbethoxyl, and not one of the methyl groups, migrates, because the energy required to separate the first group from carbon is less, and, because the heat of formation of the substance thus formed is probably greater than that of the isomeric ester.

The subject of organic rearrangement is far too comprehensive for treatment in a single paper, but it is believed that the analysis of typical reactions given above will suffice to enable the application of the affinity-energy-spatial viewpoint to other chemical transformations of this nature without difficulty.

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[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIOCHEMISTRY, MINNESOTA AGRICULTURAL EXPERIMENT STATION.]

## THE ORIGIN OF THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS. V.[2]

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Received January 12, 1920.

Our earlier contributions to the subject of humin formation[3] have led us to draw the conclusion that the formation of the black acid-insoluble humin on protein hydrolysis is due almost wholly to the interaction of tryptophane with some aldehyde or ketone and that no other known amino acid enters into the reaction to any appreciable extent. All of our earlier data have, however, involved the use of proteins, some of which were known to contain tryptophane, others from which this amino acid is supposed to be absent. By the addition of tryptophane to the latter, humin formation was markedly increased and we were unable to find any other group of compounds which would cause this increase excepting those con-

[1] Blaise and Courtot, *Bull. soc. chim.*, [3] **35**, 589 (1906).

[2] Published with the approval of the Director as Paper No. 193, Journal Series of the Minnesota Agricultural Experiment Station.

[3] THIS JOURNAL, **37**, 1630–1636 (1915); *J. Biol. Chem.*, **26**, 177–204 (1916); THIS JOURNAL, **39**, 2477–2501 (1917); *ibid.*, **42**, 632 (1920).

taining an indole nucleus. Nevertheless the fact remained that there was still a possibility of error in our chain of evidence due to the fact that it was necessary for us to assume that the proteins with which we were working either contained or did not contain certain amino acids. It was likewise possible that they contained substances which inhibited humin formation.

While a large mass of data has been accumulated regarding the nature of the protein molecule, there are many things still to be elucidated; and it is, therefore, unsafe to assume too much regarding the amino acid content of a protein, like gelatin for example, when the known amino acids actually separated total only a 44.6% recovery.[1] Our inadequate knowledge of the protein molecule is still further demonstrated by the recent discovery of Dakin,[2] who found 10.5% of β-hydroxyglutamic acid in casein. This finding is all the more remarkable because of the fact that it was found in such quantities in casein, a protein which has been more exhaustively studied than any other.

We recognized this weakness in our argument and stated:[3] "We are, therefore, firmly convinced that of all the known hydrolytic products of proteins, tryptoplane alone is concerned in the reaction which produces black insoluble humin, but that this reaction cannot take place without the presence of some as yet unidentified component of the protein molecule. There is, however, one further method of testing this statement, *i. e.*, to prepare all of the known amino acids in an absolutely pure form and then mix them in different combinations and boil these with hydrochloric acid both with and without the addition of aldehyde. If (*a*) pure tryptophane *plus* all of the other known amino acids does not produce a trace of insoluble humin when boiled with hydrochloric acid, but does produce humin when aldehyde is added and if (*b*) all the amino acids excepting tryptophane do not produce a trace of black insoluble humin either with or without the addition of aldehyde and if (*c*) tryptophane plus aldehyde produces the humin with the same ease when used alone or in the presence of any or all of the other pure amino acids, then our thesis will be definitely proven." We have now completed this experiment and this paper details the results.

## Experimental.

As indicated above, our problem was to ascertain the amino acid responsible for humin formation when a protein is hydrolyzed by strong acids. Inasmuch as one can never be sure of the exact quantity of each amino acid which is present in a given protein, we have prepared 15 amino

[1] R. H. A. Plimmer, "The Chemical Constitution of the Proteins. I. Analysis." Longmans, Green and Co., 1912, p. 61.

[2] *Biochem. J.*, 12, 290–317 (1918).

[3] THIS JOURNAL, 39, 2498–9 (1917).

acids from various sources and by various methods and, after assuring ourselves of their purity, we have mixed them in certain combinations and subjected the mixtures to acid hydrolysis[1] both alone and in the presence of formaldehyde. We had hoped that we could make the experiment using all of the known amino acids, but at the time of making the experiments we did not have certain of the rarer ones, such as oxyproline, oxytryptophane, dioxydiamino-suberic acid, diaminotrioxy-dodecanoic acid and the new oxyglutamic acid. We did, however, have 15 amino acids at our disposal and inasmuch as the results which we obtained are so completely in accord with our earlier findings, we are convinced that the results would be unchanged even if the remaining amino acids had been included. Oxytryptophane would probably act like tryptophane, and the others would certainly react similarly to their analogues which we tested. The amino acids which we used, their source and the quantity which was present in each of the 4 hydrolyses, are given in Table I. In addition to the amino acids given in this table, tyrosine was added in Expts. I, II and III, and tryptophane was added in Expts. I and II. The plan of the experiment was as follows:

**Expt. I.**—To the 13 amino acids listed in Table I were added 0.1000 g. of tyrosine (7.74 mg. of amino nitrogen) and 0.1000 g. of tryptophane (6.86 mg. of amino nitrogen and 6.86 mg. of non-amino nitrogen). After adding 100 cc. of 20% hydrochloric acid the mixture was boiled for 24 hours, using a reflux condenser. This experiment corresponds to the hydrolysis of a protein of the composition shown in the second column of Table II. The hydrolysate was then analyzed more or less completely according to the Van Slyke method[2] with the modification that the humin fraction was estimated in 3 parts, as we have already suggested should be done.[3]

The data resulting from this analysis are shown in Table III.

**Expt. II.**—Expt. I (using all 15 amino acids) was repeated with the exception that 0.10 g. of formaldehyde (in the form of trioxymethylene) was added before the hydrolysis began. We have already pointed out[4] the fact that formaldehyde in small quantity markedly increases the amount of humin nitrogen formed in a protein hydrolysis when certain amino acids are known to be present. The percentage composition of the mixture hydrolyzed is given in Table II. The analytical data are shown in Table III.

[1] Perhaps "hydrolysis" is a misnomer in this instance inasmuch as the amino acids are no longer combined with each other in peptide linkages.

[2] *J. Biol. Chem.*, **10**, 15–55 (1911); **12**, 275–284 (1912).

[3] THIS JOURNAL, **39**, 2480 (1917).

[4] *Ibid.*, **39**, 2477–2501 (1917).

**Expt. III.**—To the 13 amino acids enumerated in Table I we added 0.1000 g. of tyrosine (7.74 mg. of amino nitrogen) and 0.10 g. of trioxymethylene. The mixture was then boiled with 100 cc. of 20% hydrochloric acid for 24 hours. It will be noted that Expt. III differs from Expt. II only in the fact that no tryptophane was present. The percentage composition of the mixture hydrolyzed here is given in Table II. The analytical data on this hydrolysate are shown in Table III.

**Expt. IV.**—To the 13 amino acids listed in Table I we added 0.10 g. of trioxymethylene and boiled with 20% hydrochloric acid for 24 hours. This experiment differs from Expt. II in that neither tyrosine nor tryptophane was present. The percentage composition of the mixture hydrolyzed is shown in Table II and the analytical data on the resulting solution are shown in Table III.

TABLE I.—SHOWING THE AMINO ACIDS USED IN THIS INVESTIGATION, THEIR SOURCES, THE QUANTITIES USED IN EACH EXPERIMENT, AND THE MILLIGRAMS OF AMINO NITROGEN AND NON-AMINO NITROGEN PRESENT.

| Amino acid. | Source. | Amount used in each experiment. G. | Total, N. Mg. | Amino, N. Mg. | Non-amino, N. Mg. |
|---|---|---|---|---|---|
| Aspartic acid | Kahlbaum | 0.1250 | 13.16 | 13.16 | ... |
| Arginine[a] | Gelatin and casein | 0.0562 | 18.10 | 4.53 | 13.57 |
| Alanine | Silk | 0.1250 | 19.66 | 19.66 | ... |
| Cystine | Human hair | 0.2500 | 29.13 | 29.13 | ... |
| Glutamic acid hydrochloride | Gelatin and casein | 0.2000 | 15.20 | 15.20 | ... |
| Glycine | Silk | 0.2500 | 46.65 | 46.65 | ... |
| Histidine dihydrochloride | Blood | 0.2500 | 46.05 | 15.35 | 30.70 |
| Leucine | Gelatin and casein | 0.1250 | 13.36 | 13.36 | ... |
| Lysine dihydrochloride | Gelatin and casein | 0.0300 | 3.84 | 3.84 | ... |
| Proline | Gelatin and casein | 0.0500 | 5.88 | .. | 5.88 |
| Phenylalanine hydrochloride | Gelatin and casein | 0.1250 | 8.69 | 8.69 | ... |
| Serine | Synthetic | 0.1250 | 16.68 | 16.68 | ... |
| Valine | [b] | 0.1000 | 11.96 | 11.96 | ... |
| Unknown[a] | | .. | 4.52 | 1.84 | 2.68 |
| Total | | 1.8112 | 252.88 | 200.05 | 52.83 |

[a] Our arginine was prepared by the Kossel separation. Inasmuch as we could not use the nitrate or picrolonate to separate it because of the nitrogen contained in these salts, we tried to prepare a pure hydrochloride but experienced difficulty in procuring sufficient crystalline material. We therefore used our arginine fraction in this work, analyzing the solution for arginine by Van Slyke's method, and found that while it was largely arginine it also contained a small amount of some other amino acid. This is certainly one of the bases for the arginine was precipitated twice by phosphotungstic acid before the silver separation was made. This residual nitrogen is reported in the table as "unknown."

[b] We are indebted to Dr. C. O. Johns of the Bureau of Chemistry for this sample of valine.

TABLE II.—SHOWING THE PERCENTAGE COMPOSITION OF THE MIXTURE OF AMINO ACIDS ANALYZED IN EXPTS. I–IV INCLUSIVE.

| | Percentage of each acid present in | | |
|---|---|---|---|
| | Expts. I and II. | Expt. III. | Expt. IV. |
| Aspartic acid | 6.65 | 7.03 | 7.45 |
| Arginine | 2.99 | 3.16 | 3.35 |
| Alanine | 6.65 | 7.03 | 7.45 |
| Cystine | 13.31 | 14.05 | 14.89 |
| Glutamic acid | 8.53 | 9.01 | 9.55 |
| Glycine | 13.31 | 14.05 | 14.89 |
| Histidine | 9.05 | 9.56 | 10.13 |
| Leucine | 6.65 | 7.03 | 7.45 |
| Lysine | 1.06 | 1.12 | 1.19 |
| Proline | 2.66 | 2.81 | 2.98 |
| Phenylalanine | 5.45 | 5.76 | 6.10 |
| Serine | 6.65 | 7.03 | 7.45 |
| Valine | 5.32 | 5.62 | 5.96 |
| Unknown[a] | 1.06 | 1.12 | 1.19 |
| Tryptophane | 5.32 | .. | .. |
| Tyrosine | 5.32 | 5.62 | .. |
| Total | 99.98 | 100.00 | 100.03 |

[a] Calculated as 1/2 lysine and 1/2 histidine.

TABLE III.—SHOWING CERTAIN OF THE ANALYTICAL DATA OBTAINED IN EXPTS. I TO IV.

| | Expt. I. | Expt. II. | Expt. III. | Expt. IV. |
|---|---|---|---|---|
| Ammonia N | 1.80 | 0.80 | 3.90 | 4.50 |
| Insol. humin N | 0.00 | 13.10 | 0.00 | 0.00 |
| Sol. humin N | 0.20 | 5.00 | 5.40 | 0.20 |
| Phosphotungstic acid humin N | 5.30 | 3.98 | 3.20 | 3.15 |
| N in bases | 84.50 | 60.08 | 89.80 | 77.00 |
| Amino N in bases | 46.44 | 13.10 | 28.65 | 13.22 |
| N in filtrate from bases | 179.20 | 186.00 | 150.08 | 165.50 |
| Amino N in filtrate from bases | 173.46 | 177.42 | 145.80 | 160.87 |
| Total N recovered | 271.00 | 268.96 | 252.38 | 250.35 |
| Per cent. N recovered | 98.78 | 98.04 | 96.84 | 99.00 |

**Color Observations.**—After the completion of the 24 hours' boiling there were marked differences in the appearances of the solutions in the flasks. No. II was an intense black color, being caused by solid black particles suspended throughout the solution. No. I contained no solid particles but had a dark red-brown color. No. III was light red-brown but contained no solid particles, while No. IV was a clear light straw-colored solution.

After the removal of the "soluble humin" by calcium hydroxide certain differences were still apparent. No. I was still a fairly dark red-brown solution, No. II was distinctly red-brown, but much lighter than No. I, No. III was a still lighter red-brown and No. IV was a very pale yellow,

Upon the addition of phosphotungstic acid all color was removed from each of the solutions,[1] the resulting filtrates being the pale straw color usually obtained. The basic phosphotungstates were white in Expts. III and IV, but distinctly grayish in Expts. I and II. This color was retained by the barium phosphotungstate precipitate which was buff colored in the case of Expts. I and II and practically white in the case of Expts. III and IV. The nitrogen remaining in this precipitate has been reported in our tables as "Phosphotungstic acid humin."[2]

## Discussion.

**The Acid Insoluble Humin.**—It is this fraction with which we are principally concerned. It will be noted by reference to Table III that *the only experiment which yielded any insoluble humin nitrogen was that in which both tryptophane and aldehyde were present and that the nitrogen secured in that fraction amounted to 95.5% of the tryptophane nitrogen which had been added. There can be no further doubt, therefore, but that the black insoluble humin of protein hydrolysis is derived from tryptophane.* We are still in doubt as to the exact nature of the chemical reaction by which humin is produced in the hydrolysate of a purified protein. Whether or not this reaction is identical with that caused by the deliberate addition of an aldehyde to the hydrolysate can only be determined when we know the structure of the resulting products formed in the two cases, and we are continuing our investigations along that line.

**The Acid Soluble Humin.**—We have presented certain evidence in our earlier paper[3] which indicated that the "soluble humin" was derived from tyrosine. It will be noted that in our present experiments *only those hydrolysates which contained both tyrosine and aldehyde yielded appreciable amounts of soluble humin.* The recovery was not quantitative due to the appreciable solubility of this soluble humin. Another method of demonstrating that the soluble humin is derived from tyrosine would be to determine the amount of tyrosine remaining in the "filtrate from the bases" in each experiment. Folin and Denis[4] have proposed a method for the colorimetric estimation of tyrosine. We have utilized this method in estimating the tyrosine remaining in our "filtrates from the bases" and find the following figures for Expts. I, II and III after correcting the readings by subtracting the value of such color as was developed in the filtrate from the bases in Expt. IV where no tyrosine was added:

[1] The color was the first material to precipitate when phosphotungstic acid was added and this portion of the precipitate did not redissolve when the flask containing the precipitated bases and solution was warmed on the water bath.

[2] For a discussion of this fraction see Gortner and Holm, THIS JOURNAL, **39**, 2485 (1917).

[3] *Ibid.*, **39**, 2485 (1917).

[4] *J. Biol. Chem.*, **12**, 245–251 (1912).

| Expt. | Tyrosine added. Mg. | Tyrosine found. Mg. |
|---|---|---|
| I | 100 | 98.93 |
| II | 100 | None |
| III | 100 | None |

These figures, taken in conjunction with the figures for soluble humin nitrogen, prove conclusively that that fraction is produced by the interaction of tyrosine and the aldehyde.

**The Phosphotungstic Acid Humin.**—Our present series of experiments gives no clue as to the origin of this fraction inasmuch as it is present in all experiments in about equal amount. It may represent only an adsorption phenomena.

**The Ammonia Fraction.**—This fraction presents certain very interesting figures. It has been commonly supposed that cystine is quite readily deaminized by acid hydrolysis, but our data do not confirm this belief. The 1.80 mg. of ammonia nitrogen in Expt. I represents pure deamination. The larger figures in Expts. III and IV represent the increase in volatile amines, etc., due to the presence of formaldehyde in the hydrolysate[1] while the *decrease* in ammonia in Expt. II is an exact duplication of our findings when a protein containing tryptophane (fibrin) was hydrolyzed in the presence of formaldehyde.[2] Further work must be done before this interesting phenomena is elucidated.

## Summary.

In order to secure further evidence on the nature of humin formation, 15 pure amino acids were mixed in various proportions and boiled with 20% hydrochloric acid both in the presence and in the absence of formaldehyde. The following conclusions are evident:

1. The black insoluble humin is derived from tryptophane, and when the proper amount of aldehyde is present is a quantitative measure of the tryptophane present.

2. The "soluble humin" formed in the presence of formaldehyde is derived from tyrosine. It is not a quantitative measure of the tyrosine, due to the appreciable solubility of the resulting humin.

3. No evidence was secured as to the nature of the "phosphotungstic acid humin."

4. Cystine was not readily deaminized under the conditions of the experiments.

5. When both tryptophane and formaldehyde are present in the proportions necessary for the maximum formation of insoluble humin there is less deamination, as measured by ammonia formation, than when either the aldehyde or the tryptophane is absent from the hydrolysate.

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[1] Gortner and Holm, THIS JOURNAL, **39**, 2486 (1917).

[2] Gortner and Holm, *loc. cit.*, p. 2481, Fig. 1.