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CHICAGO, ILL.

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

ON THE ORIGIN OF THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS. IV. HYDROLYSIS IN THE PRESENCE OF ALDEHYDES. III. COMPARATIVE HYDROLYSIS OF FIBRIN AND GELATIN IN THE PRESENCE OF VARIOUS ALDEHYDES.[1]

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In the earlier papers of this series, by Gortner and Blish,[2] Gortner,[3] and Gortner and Holm,[4] we have studied the effect of certain aldehydes upon the acid hydrolysis of proteins with especial reference to the origin and mode of formation of the "humin" fraction. Detailed observations were made using various carbohydrates, which under the conditions of the experiment would yield furfural and formaldehyde, with a few observations, where benzaldehyde was present. It was noted that the hydrolysis of a protein in the presence of an aldehyde markedly altered the nitrogen distribution, so much so that when formaldehyde was present in excess the resulting nitrogen distribution bore no resemblance to the values obtained in the absence of the aldehyde. The most noteworthy changes in the nitrogen distribution were in the ammonia and humin fractions, but the fact that an excess of formaldehyde over that required to cause maximum humin formation apparently unites with the α-amino groups so that they no longer react with nitrous acid, causes the remaining nitrogen fractions to lose all resemblance to those of a normal hydrolysate.

We have furthermore shown that in all probability the black insoluble humin nitrogen is derived from the interaction of tryptophane and an aldehyde. However, tyrosine also reacts with aldehydes to form com-

[1] Presented before the Biological Division at the Philadelphia meeting of the American Chemical Society, Sept. 2–6, 1919. Published with the approval of the Director as Paper No. 190, Journal Series of the Minnesota Agricultural Experiment Station.

[2] R. A. Gortner and M. J. Blish, "On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins," THIS JOURNAL, **37**, 1630–36 (1915).

[3] R. A. Gortner, "The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. II. Hydrolysis in the Presence of Carbohydrates and of Aldehydes," *J. Biol. Chem.*, **26**, 177–204 (1916).

[4] R. A. Gortner and G. E. Holm, "On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins. III. Hydrolysis in the Presence of Aldehydes. II. Hydrolysis in the Presence of Formaldehyde," THIS JOURNAL, **39**, 2477–2501 (1917).

pounds whose solubility depends upon the particular aldehyde present. Thus with formaldehyde the tyrosine-aldehyde compound is soluble in dil. hydrochloric acid and is thus readily separable from the tryptophane-aldehyde humin. On the other hand, certain experiments which we have reported seem to indicate that the tyrosine-benzaldehyde compound is insoluble in the various concentrations of hydrochloric acid and is therefore not separable from the tryptophane-benzaldehyde humin. It, therefore, seemed advisable to extend our observations so as to include a more extended study of the effect of other aldehydes, especially benzaldehyde, upon the nitrogen distribution of proteins when the aldehyde has been present during the process of acid hydrolysis.

Experimental.

No fibrin such as that used in our previous experiments could be obtained. A sample of commercial "fibrin from blood" was, therefore, purified as follows: The fibrin was finely ground and brought into solution with a 0.2% solution of sodium hydroxide. The solution was filtered through 4 double cheese cloths and precipitated with hydrochloric acid. This precipitate was washed to the absence of chlorides by decantation, dried at a low temperature and ground. This product, free from blood and other impurities, was used in subsequent hydrolyses. The gelatin used was of the same sample as that used in our earlier studies.

In order to obtain comparative data regarding the action of different aldehydes upon the same protein preparations, it was, therefore, necessary to repeat certain of the experiments with formaldehyde using this new

TABLES I AND II.—THE EFFECTS OF INCREASING AMOUNTS OF FORMALDEHYDE UPON THE ACID HYDROLYSIS OF GELATINE AND FIBRIN, RESPECTIVELY.

TABLE I.

Amount $(CH_2O)_3$ used. G.	Acid-insoluble humin nitrogen. Mg.	Ammonia nitrogen. Mg.	Acid-soluble humin nitrogen. Mg.	Total amino nitrogen. Mg.
0.00	0.12	4.58	0.35	319.67
0.05	0.14	5.00	0.57	314.04
0.10	0.13	6.30	0.50	307.00
0.25	0.13	9.43	0.48	295.97
0.50	0.10	19.27	0.50	274.99
1.00	0.05	30.40	0.47	229.15
2.50	0.07	44.75	0.28	120.10
5.00	0.03	51.45	0.35	34.77

TABLE II.

0.00	6.45	34.65	5.87	341.32
0.05	10.70	30.35	10.95	..
0.10	14.25	29.50	16.55	313.56
0.25	2.65	26.47	29.60	292.54
0.50	1.10	30.50	27.90	261.49
1.00	1.40	38.70	25.95	236.50
2.50	0.95	51.32	19.90	117.12
5.00	0.97	60.80	11.00	37.69

material. Besides formaldehyde the following aldehydes were used: benzaldehyde, acetaldehyde, butyric and isobutyric aldehyde.

The method of procedure was the same as that used in the former experiments with two exceptions: (1) Hydrolysis was continued for 24 hours only; (2) besides insoluble humin nitrogen, ammonia nitrogen and soluble humin nitrogen, the total amino nitrogen was determined upon the filtrate from the soluble humin. This method of precedure gave an indication of the effect of various aldehydes upon humin formation and also showed their effect upon the α-amino nitrogen of the various amino acids not involved in humin formation.

Tables I to IX show the effects of the various aldehydes upon the acid

TABLES III AND IV.—THE EFFECTS OF INCREASING AMOUNTS OF BENZALDEHYDE UPON THE ACID HYDROLYSIS OF GELATINE AND FIBRIN, RESPECTIVELY.

TABLE III.

Aldehyde used. Cc.	Acid-insoluble humin nitrogen. Mg.	Ammonia nitrogen. Mg.	Acid-soluble humin nitrogen. Mg.	Total amino nitrogen Mg.
0.00	0.12	4.58	0.35	319.67
0.05	0.49	4.60	0.30	318.90
0.10	0.55	4.70	0.34	319.30
0.25	0.67	4.65	0.30	317.10
0.50	0.67	4.33	0.35	322.61
1.00	0.82	5.00	0.27	318.53
2.50	0.86	7.55	0.38	323.20

TABLE IV.

0.00	6.45	34.65	5.87	341.32
0.05	10.50	33.00	2.60	340.10
0.10	13.50	32.50	1.90	340.10
0.25	15.17	33.00	3.40	336.77
0.50	16.25	33.50	3.60	324.16
1.00	18.35	33.30	3.60	320.54
2.50	24.35	33.54	2.72	313.95
5.00	24.30	34.31	4.15	309.40 308.60

TABLE V.—THE EFFECT OF INCREASING AMOUNTS OF ACETALDEHYDE UPON THE ACID HYDROLYSIS OF GELATINE.

Aldehyde used. Cc.	Acid-insoluble humin nitrogen. Mg.	Ammonia nitrogen. Mg.	Acid-soluble humin nitrogen. Mg.	Total amino nitrogen. Mg.
0.00	0.12	4.58	0.35	319.67
0.05	0.47	4.60	1.78	Not determined
0.10	0.90	4.77	3.16	.....
0.25	1.60	5.10	4.26	.....
0.50	4.80	4.57	6.00	.....
1.00	12.10	4.32	6.56	.....
2.50	22.93	4.00	7.88	.....
5.00	41.75	6.00	6.10	.....

hydrolysis of fibrin and gelatin, 3 g. of protein being used in each experiment. The data for acid insoluble humin have been plotted in the form of curves and are shown in Fig. 1.

TABLES VI AND VII.—THE EFFECTS OF INCREASING AMOUNTS OF BUTYRIC ALDEHYDE UPON THE ACID HYDROLYSIS OF GELATINE AND FIBRIN, RESPECTIVELY.

TABLE VI.

Aldehyde used. Cc.	Acid-insoluble humin nitrogen. Mg.	Ammonia nitrogen. Mg.	Acid-soluble humin nitrogen. Mg.	Total amino nitrogen. Mg.
0.00	0.12	4.58	0.35	319.67
0.05	0.35	4.36	..	321.32
0.10	0.50	4.30	0.86	324.69
0.25	0.75	4.64	0.85	320.85
0.50	1.00	5.05	1.10	316.30
1.00	2.80	4.75	1.23	318.71
2.50	5.60	5.00	1.47	311.14
4.00	7.37	5.57	1.67	311.14

TABLE VII.

0.00	6.45	34.65	5.87	341.32
0.05	7.20	32.32	4.30	..
0.10	9.30	31.76	2.40	..
0.25	12.55	30.88	2.10	335.09
0.50	14.10	30.65	2.60	..
1.00	15.50	31.75	3.20	330.69
2.50	18.03	31.79	2.65	309.65
5.00	24.00	31.80	3.00	..

TABLES VIII AND IX.—EFFECTS OF INCREASING AMOUNTS OF ISOBUTYRIC ALDEHYDE UPON THE ACID HYDROLYSIS OF GELATINE AND FIBRIN, RESPECTIVELY.

TABLE VIII.

Aldehyde used. Cc.	Acid-insoluble humin nitrogen. Mg.	Ammonia nitrogen. Mg.	Acid-soluble humin nitrogen. Mg.	Total amino nitrogen. Mg.
0.00	0.12	4.58	0.35	319.67
0.05	0.27	4.40	1.11	320.50
0.10	0.33	4.15	1.40	320.60
0.25	0.55	4.53	1.40	319.77
0.50	0.74	4.06	1.42	320.77
1.00	0.89	4.65	1.70	314.00
2.50	2.57	5.32	2.08	312.22
5.00	3.23	6.05	1.80	307.7 307.14

TABLE IX.

0.00	6.45	34.65	5.87	341.32
0.05	7.37	31.70	6.35	338.45
0.10	9.42	31.66	5.12	337.18
0.25	11.23	30.00	4.25	328.41
0.50	13.57	30.00	4.35	332.10
1.00	12.97	30.95	4.55	328.50
2.50	20.80	31.00	4.15	..
5.00	23.10	30.85	4.00	..

Discussion.

The general reaction of formaldehyde upon these proteins has already been discussed in the paper by Gortner and Holm.[1]

Benzaldehyde gives an entirely different reaction. If benzaldehyde is added to gelatin or fibrin in a 20% hydrochloric acid solution and allowed to stand, a dark blue color develops in the case of the fibrin but only a faint blue color in the gelatin solution. This is undoubtedly the blue color produced by benzaldehyde and tryptophane in an acid solution, and serves as an indication of the presence of this amino acid. We believe that this reaction may be developed so as to serve for the quanti-

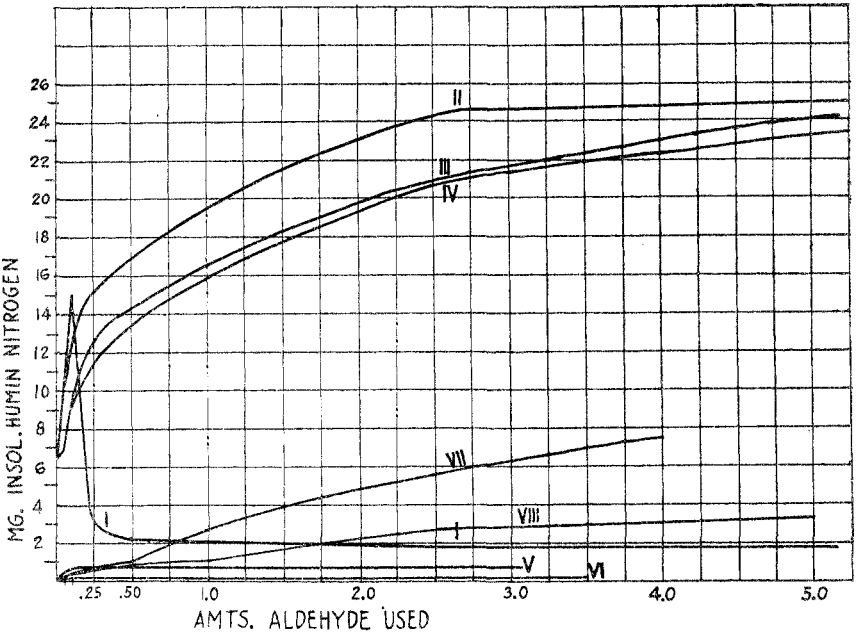

Fig. 1.

Graph showing the insoluble humin nitrogen curves for Tables I to IX inclusive.

I.—Fibrin-formaldehyde curve.
II.—Fibrin-benzaldehyde curve.
III.—Fibrin-butyric aldehyde curve.
IV.—Fibrin-isobutyric aldehyde curve.
V.—Gelatine-benzaldehyde curve.
VI.—Gelatine-formaldehyde curve.
VII.—Gelatine-butyric aldehyde curve.
VIII.—Gelatine-isobutyric aldehyde curve.

tative estimation of tryptophane colorimetrically, but the final experiments on this work have had to be delayed until the humin work reached a point where it could be suspended for a time. If a solution of fibrin and benzaldehyde in 20% hydrochloric acid is hydrolyzed, a black humin is formed. With increased amounts of benzaldehyde there is an increase in the amount of insoluble humin but no increase in ammonia or soluble

[1] *Loc. cit.*

humin. The insoluble humin rises to a constant value (see Curve II, Fig. 1) at which point a dark plastic mass floats in the benzaldehyde layer upon a remaining light straw-colored hydrolysate. Gortner[1] has shown that when fibrin is hydrolyzed with hydrochloric acid in the presence of benzaldehyde a large percentage of the nitrogen of both tryptophane and tyrosine remains in the "acid-insoluble" humin. The insoluble humin at the maximum point in this case therefore is undoubtedly derived from the tryptophane and tyrosine present in the gelatin and fibrin.

Gortner and Holm[1] have already shown that the insoluble humin obtained in the presence of formaldehyde is derived almost exclusively from tryptophane so that *by utilizing both the formaldehyde and benzaldehyde data one may estimate at least the minimal tryptophane and tyrosine content of a protein.* Thus in the case of fibrin our data indicate a content of 3.47% of tryptophane and at least 6.13% of tyrosine,[2] while in the case of gelatine[3] the values are 0.035% and 0.47%, respectively.

In the case of formaldehyde the decrease in α-amino nitrogen is probably due to formation of methylene linkages. Probably no such reaction occurs with benzaldehyde as may be seen in the case of gelatin hydrolyzed with an excess of benzaldehyde. *The total amino nitrogen value remains practically constant.* In the case of fibrin, however, the decrease in amino nitrogen is slightly more than can be accounted for by the removal of amino nitrogen through the formation of insoluble humin, when we attribute the formation of the humin to tryptophane and tyrosine. The data at present are insufficient to explain the slight additional decrease of amino nitrogen above that which is removed through the formation of humin.

The effects of acetaldehyde, butyric aldehyde and isobutyric aldehyde are somewhat similar. Acetaldehyde polymerized (?) readily in acid solution forming large masses of black amorphous material. In the case of gelatin it was possible to gain some idea of the amount of nitrogen in this black amorphous substance, while in the case of fibrin even small quantities of acetaldehyde produced such a large bulk of this amorphous material that no systematic study could be made. The difference in the amounts of "insoluble humin" formed by acetaldehyde with gelatin and

[1] *Loc. cit.*

[2] The figures for tryptophane are, we believe, a very accurate measure of the quantity of this amino acid which is present in the protein. The figures for tyrosine, on the other hand, are, in all probability, minimal values, for there appears to be a solubility factor, and possibly also an adsorption factor involved. We have not yet studied these phenomena in detail.

[3] Gelatin is supposed to contain neither tyrosine nor tryptophane. Possibly these amino acids are absent from extremely pure gelatin, but we have as yet found no sample from which we believe either amino acid to be entirely absent.

fibrin indicated, however, that the reaction is not a simple polymerization with simultaneous adsorption or occlusion of amino acids, but that other reactions are involved. As yet no means of identifying the nature and extent of these reactions has been devised. Butyric and isobutyric aldehydes, like acetaldehyde, formed black, insoluble residues when boiled in a 20% hydrochloric acid solution, but do so much less readily than does acetaldehyde. Their effect upon the hydrolysis of gelatin and fibrin is apparently somewhat analogous to that of benzaldehyde. They affect chiefly the acid-insoluble humin, increasing it to an approximately constant value, forming a dark layer floating upon a straw-colored solution. The effect in the case of gelatin shows the part that polymerization plays in increasing the insoluble humin nitrogen. With benzaldehyde this fraction remains constant but with butyric and isobutyric aldehydes it increases very slowly with increased amounts of aldehyde, but the increase is far from the amounts and proportionalities it should maintain if it were purely a chemical reaction. In short, the behavior of acetic, butyric and isobutyric aldehydes resembles more or less closely that of furfural, which Gortner[1] has already studied, reaching the conclusion that, inasmuch as both physical and chemical reactions are involved, no exact interpretation of the humin nitrogen figures can be made.

Difficulty was experienced in the determination of the α-amino nitrogen in hydrolysates where acetic, butyric and isobutyric aldehydes had been used. There was always a tendency to a formation of large amounts of gas when aliquots were treated with nitrous acid in the Van Slyke[2] apparatus. This may be accounted for by a small amount of aldehyde which may remain in loose combination with the amino acids and when treated with nitrous acid forms some non-absorbable gas. That all the gas evolved upon treatment with nitrous acid is not nitrogen and oxides of nitrogen is shown by the fact that the gas from samples of hydrolysates in which acetaldehyde, butyric or isobutyric aldehydes had been used would not come to constant volume over water for several hours, after the evolved gases had been shaken with the fresh alkaline permanganate.

These results, in so far as humin formation is concerned, only serve to confirm our conclusions which were drawn from the earlier data, *i. e.*, that "the formation of black acid-insoluble humin in a protein hydrolysate is due to a combination of tryptophane with some as yet unidentified aldehyde or ketone, and that the only part which any of the other known amino acids have in humin formation is to (perhaps) furnish some of their nitrogen to the humin fraction through either adsorption or occlusion." It may appear to some that the accuracy of this statement depends largely

[1] *Loc. cit.*

[2] D. D. Van Slyke, "The Quantitative Determination of Aliphatic Amino Groups. II," *J. Biol. Chem.*, 12, 275–284 (1912).

on the nature of the hypothetical aldehyde or ketone which we postulate must be present (or formed) in a protein hydrolysate before humin formation can take place. Thus from the above data it would appear probable that if the hypothetical aldehyde or ketone present in a protein preparation were similar to formaldehyde the acid-insoluble humin will be derived solely from tryptophane, excluding a small but negligible amount of other amino acids, not chemically concerned in humin formation which may be adsorbed on the tryptophane humin. If, however, the hypothetical compound were an aromatic aldehyde or one of the aliphatic aldehydes (other than formaldehyde) it would appear that both tryptophane and to a certain extent tyrosine should be involved in acid-insoluble humin formation.[1] All that we know of the nature of this hypothetical aldehyde is derived from experiments which have already been reported where tryptophane was added to zein, by Gortner and Blish[2] and to gelatin, by Gortner and Holm,[2] respectively. In the zein experiment figure 1 g. of zein hydrolyzed alone yielded 0.7 mg. of humin nitrogen; hydrolyzed in the presence of 0.125 g. tryptophane, it yielded 7.7 mg. of humin nitrogen, and when 0.25 g. tryptophane was added 7.0 mg. of humin nitrogen was obtained, showing that the unknown component of the protein had been exhausted by the addition of 0.125 g. tryptophane so that none remained to react with a larger amount of tryptophane. Osborne and Liddle[3] have found 3.55% of tyrosine in zein, so that it is obvious, in the light of our present knowledge, that tyrosine plus the hypothetical aldehyde of zein cannot produce any appreciable quantity of black, insoluble humin, but that tryptophane plus the unknown factor does produce such humin.

In the gelatin experiment 3 g. of gelatin hydrolyzed alone gave 0.25 mg. of insoluble humin nitrogen. When 0.10 g. of tyrosine was added 0.20 mg. of insoluble humin nitrogen was found, but when 0.075 g. of tryptophane was added the insoluble humin nitrogen amounted to 2.05 mg. Thus in both zein and gelatin the unknown factor in humin formation reacts only with tryptophane and not with tyrosine in forming humin nitrogen. In other words, its acts very similar to the formaldehyde of our experiments. For this reason we believe that, of the amino acids, tryptophane only furnishes any appreciable part in the formation of the black acid-insoluble humin.

[1] Formaldehyde does not differ radically from the other aldehydes for here the greater part of the humin formed by tyrosine is estimated in the "acid-soluble humin" fraction, that is, it is soluble in acid but relatively insoluble in the presence of calcium hydroxide.

[2] *Loc. cit.*

[3] T. B. Osborne and L. M. Liddle, "Notes on the Analysis of Edestin and Zein" *Am. J. Physiol.*, 26, 295–304 (1910).

Summary.

In the experiments reported in the preceding pages fibrin and gelatin were hydrolyzed in the presence of formaldehyde, benzaldehyde, acetaldehyde, butyric aldehyde and isobutyric aldehyde with especial reference to the formation of the black, insoluble humin of protein hydrolysis and with incidental reference to the formation and composition of the "soluble humin" and the "ammonia" fractions. The following conclusions are evident:

1. The data in those experiments where formaldehyde was used confirm the earlier conclusions of Gortner and Holm.[1]

2. When proteins are hydrolyzed in the presence of increasing amounts of benzaldehyde the acid-insoluble humin nitrogen rises rapidly to a maximum and the reaction appears wholly chemical. This increase is undoubtedly due to the presence of both tryptophane and tyrosine, since fibrin and gelatin both give an increase in insoluble humin nitrogen over the maximum insoluble humin nitrogen formed in the presence of formaldehyde approximately equal to the sum of the increase of the insoluble and soluble humin in the latter case.

3. The "ammonia" and "soluble humin" are not significantly altered when proteins are hydrolyzed in the presence of benzaldehyde.

4. The action of butyric and isobutyric aldehydes upon protein hydrolysis is analogous to the action of benzaldehyde with the exception that the former polymerize (?) readily permitting perhaps of some adsorption or occlusion of other nitrogen compounds.

5. Acetaldehyde polymerizes (?) too readily in acid solution to give consistent and reliable results.

6. The total amino nitrogen in the filtrate from the soluble humin rapidly falls with the addition of increasing amounts of formaldehyde. This decrease is probably due to the formation of methylene linkages. On the other hand, hydrolysis in the presence of benzaldehyde, butyric and isobutyric aldehydes causes at the most only a slight decrease in the amino nitrogen figures over that which is removed by the soluble and insoluble humin.

7. Our data confirm the conclusion that the formation of the black acid-insoluble humin in a normal protein hydrolysate (only protein and acid present) is dependent upon the presence of tryptophane in the protein molecule, and the only part which any of the other known amino acids has in such humin formation is to (perhaps) furnish an insignificant amount of nitrogen to the humin fraction through either adsorption or occlusion.

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[1] *Loc. cit.*