On concentrating the filtrate from this compound on a steam bath, a second crop of crystals was obtained, which was purified by repeated crystallization from hot water and finally identified as cyanuric acid. Yield, 0.7563 g. The following analytical data were obtained:

0.1035 g. of anhydrous substance required 0.0326 g. of sodium hydroxide for neutralization (thymolphthalein), and yielded 0.03375 g. of nitrogen. The molecular weight was found to be 127. The molecular weight calculated for cyanuric acid is 129.

Calc. for $C_3H_3N_3O_3$: 32.60. Found: 32.63% N.

0.03318 g. of anhydrous substance yielded 0.03405 g. of CO_2 and 0.00736 g. of H_2O (micro-combustion).

Calc. for $C_3H_3N_3O_3$: 27.90, 2.34. Found: 27.98% C; 2.48% H.

0.1020 g. of the copper ammonium salt gave 0.02255 g. of CuO.

Calc. for $Cu(C_3H_2N_3O_3)_2.2NH_3$: 17.96. Found: 17.66% Cu.

These data establish the identity of this compound as cyanuric acid.

Summary.

1. The so-called "tetracarbonimid" prepared by Scholtz by oxidizing uric acid with hydrogen peroxide in alkaline solution is in fact cyanuric acid.

2. The nitrogenous compound isolated from a number of soils and believed at first to be "tetracarbonimid" has been shown to be cyanuric acid.

3. Cyanuric acid has been isolated from the following soils: (1) 12 samples of sandy soils taken from different locations in Florida, (2) Norfolk sandy loam from Virginia, (3) lawn soil from the grounds of the Department of Agriculture, (4) Elkton silt loam from Maryland, (5) Scottsburg silt loam from Indiana, (6) Caribou loam from Maine, and (7) a Susquehanna fine sandy loam from Texas. It is apparent that cyanuric acid or its precursor is widely distributed in soils.

4. The results of Venable and Moore and our own agree in showing that the oxidation of uric acid in alkaline solution with hydrogen peroxide yields cyanuric acid as a product of this reaction.

WASHINGTON, D. C.

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

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.[1]

BY ROSS AIKEN GORTNER AND GEORGE E. HOLM.

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In the preceding papers of this series,[4,5]* the various hypotheses regarding the origin of the humin of protein hydrolysis have been discussed

[1] Published with the approval of the Director as Paper No. 84, Journal Series of the Minnesota Agricultural Experiment Station.

* References are to a bibliography at the end of this article.

and new data bearing on this subject have been presented. These data show that when the hydrolysis is carried out in the presence of carbohydrates or of aldehydes there is an increase in the humin nitrogen. It was likewise shown that when tryptophane was boiled with acid in the presence of carbohydrate practically all (86.5%) of the tryptophane nitrogen was converted into humin nitrogen. Gortner and Blish[5] therefore arrived at the apparently justifiable conclusion that "in all probability the humin nitrogen of protein hydrolysis has its origin in the tryptophane nucleus."

The suggestion was also made that the reaction involved was the condensation of an aldehyde with the —NH group of the tryptophane nucleus. The fact was noted that when tryptophane was added to pure zein, which contained no tryptophane and which gave only traces of humin nitrogen, there was a marked humin formation and that this humin formation reached a maximum, above which increasing additions of tryptophane did not increase the amount of humin. Obviously there were two factors involved, of which tryptophane was certainly one. If the other component of the reaction contained no nitrogen then the humin nitrogen would have an exclusive origin in the tryptophane molecule but if the second component was nitrogenous then the humin nitrogen would be derived from both tryptophane and the unknown component.

Since the publication of the second paper of this series[4] two articles have appeared bearing upon the subject. Roxas[10] boiled certain of the pure amino acids with carbohydrates in the presence of hydrochloric acid and reached the following conclusions: (*a*) "Alanine, leucine, phenylalanine, proline and glutaminic acid may be eliminated as factors in humin formation" and (*b*) "the following amino acids were responsible for humin formation, and in digestions with 20% hydrochloric acid plus sugar the proportion of their nitrogen disappearing was: Tyrosine, 15.0; cystine, 3.1; arginine, 2.33; lysine, 2.62; histidine, 1.84; tryptophane, 71.0%." Certainly these figures, excepting that for tryptophane, do not appear to indicate a chemical reaction but rather an imperfect washing of the humin resulting from the action of the acid on the carbohydrate. We have here a large mass of black, porous, amorphous, material apparently in much the same physical state as boneblack but with the difference that it is formed in the presence of the amino acid. It does not seem at all remarkable that it should adsorb or occlude a small part of the amino acid and hold it so tightly that it cannot be removed with a reasonable amount of washing. In addition, it is of interest to observe some of the analytical figures upon which the second conclusion is based. For example, 0.1915 g. of histidine when boiled in the presence of 0.720 g. of glucose and 20% hydrochloric acid gave 0.00077 g. humin nitrogen or 1.84% of the total nitrogen. This figure Roxas accepts as the correct

percentage but in another experiment where 0.4000 g. of histidine was boiled with 2.0 g. of glucose in the presence of 20% hydrochloric acid only 0.00080 g. of humin nitrogen was found or 1.16% of the total nitrogen. In this last experiment there is a greater proportion of carbohydrate to amino acid so that, if the reaction were chemical, one would expect an increase in the humin nitrogen. Likewise the amount of amino acid is much larger so that more reliable data should result, nevertheless the earlier 1.84% is accepted and the lower 1.16% is ignored. An analogous case occurs with the tyrosine figures where 6.87, 10.88 and 15.1% of the nitrogen remain in the humin in three successive experiments. Nevertheless the 15.1% is accepted as the correct figure. Certainly these tyrosine figures as presented in Roxas' paper do not indicate a purely chemical reaction but rather occlusion or adsorption. It is likewise difficult to understand just why glutaminic acid and phenylalanine, both of which gave (Expts. 13 and 17) 1.65% of "humin" nitrogen should be "eliminated as factors in humin formation" and histidine which gave 1.16 and 1.84% of humin nitrogen should be included among those "amino acids responsible for humin formation."

However, whether or not we agree that cystine, arginine, histidine and lysine are involved in the process of humin formation, and data presented later in this paper would seem to show that they are not so involved, it appears certain that tryptophane is one of the causes of the formation of the black humin. Tyrosine may likewise be somewhat reactive but to a much smaller degree. However, as one of us has demonstrated,[4] the humin nitrogen obtained by hydrolysis in the presence of carbohydrates cannot be regarded as typical humin nitrogen inasmuch as when increasing amounts of carbohydrate are added a maximum figure for humin nitrogen is never reached but instead there is a sudden initial rise for the first portion of carbohydrate followed by a much smaller rise for each succeeding increment. The primary rise is almost certainly due in part to a chemical reaction augmented by adsorption and occlusion while the later increases are probably more and more due to physical causes, the humin formed from the furfural or carbohydrate adsorbing or occluding the amino acids. In all probability boneblack would produce similar results.

Hart and Sure[6] have recently confirmed Gortner's conclusion that an accurate Van Slyke analysis cannot be obtained on protein material hydrolyzed in the presence of carbohydrates. However, they accept Roxas' view of the nature of humin formation in the statement "Roxas has therefore specifically demonstrated that the hexone bases, as well as tryptophane and tyrosine, are to be considered as taking part in humin formation." As we have stated above, this "demonstration" appears to us to be extremely doubtful.

Inasmuch as both physical and chemical combinations appear to take place when proteins are hydrolyzed in the presence of carbohydrates some other point of attack was sought. If furfural, formed from the carbohydrate by the acid was the reactive agent it appeared probable that other aldehydes could be used to replace the carbohydrate and at the same time largely eliminate the adsorption and occlusion phenomena. Certain preliminary experiments with furfural, benzaldehyde and formaldehyde have already been published,[4] together with a brief review of the literature. Since these preliminary experiments we have continued the investigation, especially with reference to the action of formaldehyde, benzaldehyde, salicylic aldehyde, acetaldehyde, propionic aldehyde and citral. Certain of the experiments dealing with formaldehyde are the subject of the present paper.

Experimental.

The Materials.—In the following experiments two different proteins were used—fibrin and gelatin. The fibrin was from the same sample as that used in the earlier experiments[4] and the gelatin was of the best commercial grade obtainable, the sheets being practically colorless. Both proteins were used without further purification.

The amino acids used were in all instances prepared in this laboratory from protein material and were recrystallized until they had the theoretical nitrogen content.

The formaldehyde was added to the hydrolysates in the form of trioxymethylene. In this manner no dilution of the acid took place and the amount of aldehyde added could be controlled more exactly. The trioxymethylene was prepared from 40% formaldehyde in the usual manner.

The Method.—In general the methods for studying the nitrogen distribution as devised by Van Slyke[14,16] have been followed with minor alterations. Perhaps the most noteworthy alteration is that which we have employed in recording "humin" nitrogen. Instead of a single fraction of humin nitrogen as recorded by Van Slyke, we have separated the humin fraction into three portions: (*a*) acid-insoluble humin, (*b*) acid-soluble humin [pptd. by $Ca(OH)_2$], and (*c*) phosphotungstate humin (pptd. by phosphotungstic acid). The latter fraction has been recently recognized by Van Slyke.[17]

The "acid-insoluble humin" is obtained by filtering the hydrolysate when cool and washing the black residue until free from chlorides. A Kjeldahl determination then gives the nitrogen content. Following the removal of the acid-insoluble humin the acid filtrate is evaporated under diminished pressure until all of the hydrochloric acid which it is practicable to remove has been driven off. Water, alcohol and a calcium hydroxide suspension is then added as directed by Van Slyke and the ammonia distilled into standard acid at 40–45° under a pressure of less

than 30 mm. After the removal of the ammonia the residue in the distilling flask is filtered and the precipitate washed to the absence of chloride. A Kjeldahl determination of this residue gives the nitrogen retained in the "acid-soluble humin." The filtrate from the acid-soluble humin is concentrated and the diamino acids precipitated and washed as directed by Van Slyke.[15] The decomposition of the phosphotungstate precipitate was effected by means of barium hydroxide as in Van Slyke's earlier directions inasmuch as we found that reliable results could not be obtained on the aldehyde hydrolysates by using amyl alcohol-ether method recently proposed.[17] The precipitate of barium phosphotungstate was Kjeldahled and gave the "phosphotungstate humin." The remaining steps in the analyses were carried out according to Van Slyke's directions. All titrations were made with $N/14$ acid and alkali so that the figures obtained represent milligrams of nitrogen without the necessity of a calculation.

Hydrolysis of Fibrin in the Presence of Increasing Amounts of Trioxymethylene.

Three-gram samples of fibrin were boiled for 48 hours with 75 cc. of hydrochloric acid of 1.115 sp. gr. with additions of trioxymethylene

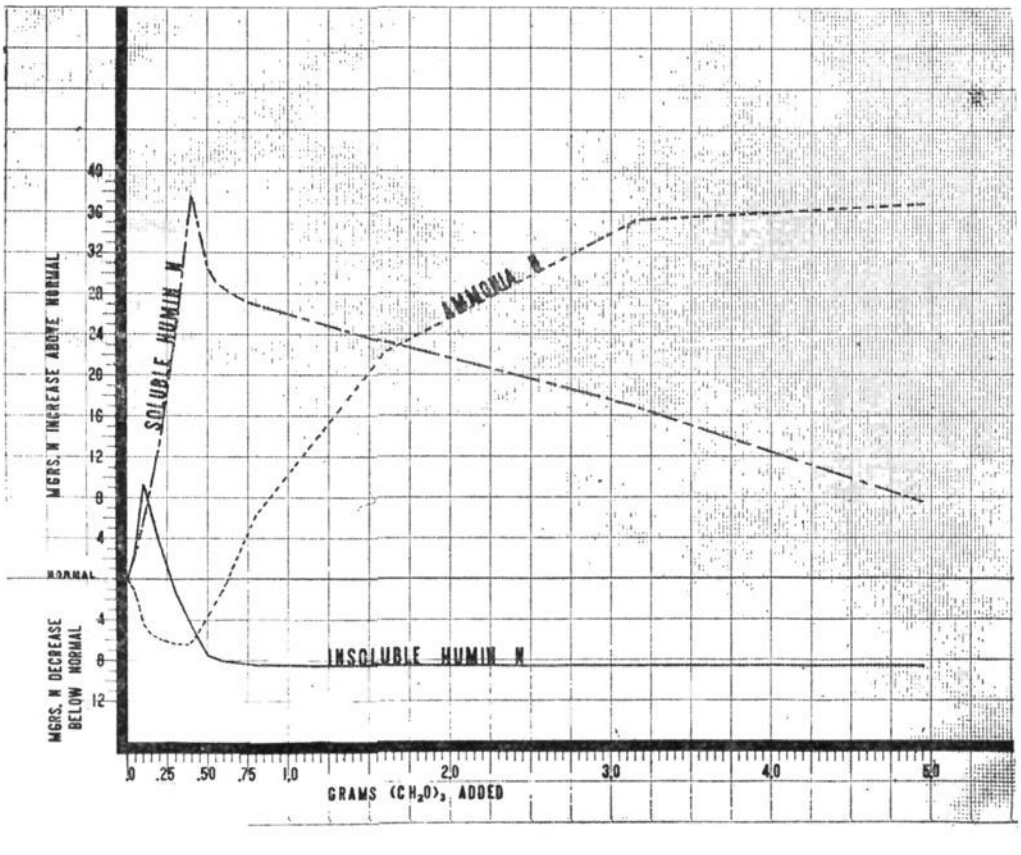

Fig. 1.—Showing the changes from the normal in the "insoluble humin," "soluble humin" and "ammonia" fractions of fibrin hydrolyzed in the presence of varying amounts of trioxymethylene.

ranging in amount from 0.05 g. to 15 g. In Table I are given the experimental data for acid-insoluble humin nitrogen, acid-soluble humin nitrogen and ammonia nitrogen. A graphic representation of these data is shown in Fig. 1, the base line representing the normal amount of nitrogen obtained when the hydrolysis was carried out without the addition of aldehyde and the various points on the curves indicating the increase or decrease in milligrams of nitrogen due to the aldehyde added.

TABLE I.—THE ACID-INSOLUBLE HUMIN, ACID-SOLUBLE HUMIN AND AMMONIA OBTAINED FROM 3 G. OF FIBRIN HYDROLYZED IN THE PRESENCE OF INCREASING AMOUNTS OF TRIOXYMETHYLENE.

G. trioxymethylene added.	Mg. acid-insoluble humin.	Mg. acid-soluble humin.	Mg. total humin.	Mg. ammonia.
None	9.60	6.22	15.82	46.23
0.050	13.10	8.74	21.84	45.20
0.075	17.40	10.60	28.00	43.90
0.10	19.02	11.49	30.52	42.02
0.15	16.00	15.75	31.75	41.00
0.20	13.45	19.00	32.45	40.85
0.30	8.37	29.45	37.82	39.92
0.40	3.27	43.81	47.08	41.60
0.50	2.05	36.80	38.85	43.10
0.60	1.78	Lost	...	45.50
0.80	1.03	Lost	...	51.60
1.60	1.08	29.90	30.98	68.20
3.20	1.07	22.85	23.92	81.85
4.80	1.05	11.73	12.78	82.78
15.00	1.05	13.50	14.55	118.85

This table and graph show that there are apparently a number of reactions taking place. With the addition of formaldehyde there is at first a rapid increase in the amount of insoluble humin nitrogen. This product is jet black in color until the maximum point on the curve has been reached. Beyond this point additional amounts of trioxymethylene cause the quantity of insoluble humin formed to rapidly diminish and the color changes from jet black to dark brown, the filtrate from the insoluble humin at the same time changing from the usual light yellow to a deeper and deeper red. The soluble humin rises rapidly to a higher maximum than that of the insoluble humin and then slowly decreases with additional amounts of trioxymethylene.

The ammonia nitrogen, on the other hand, decreases sharply in amount with the first few additions of aldehyde, then rises again and continues at an ever-increasing amount until when 15 g. of trioxymethylene have been added the increase in ammonia nitrogen amounts to 72.62 mg. or an increase of more than 150% over the original ammonia nitrogen.

Comparative Van Slyke Analyses of Fibrin Hydrolyzed Alone and in the Presence of Increasing Amounts of Trioxymethylene.—In order to gather a further insight into the reactions involved analyses were made

by Van Slyke's method upon 3-g. samples of fibrin to which 0.10, 0.50 and 15.0 g. of trioxymethylene had been added. These amounts were taken because they correspond most nearly to the high and low points of the humin and ammonia curves in Fig. 1. The experimental data are given in Tables II and III.

The data presented in these tables indicate a series of very interesting chemical reactions. It is difficult to decide just how to present the discussion of these tables inasmuch as certain phases of the discussion depend upon experiments which will be presented later. Nevertheless it is necessary for the sake of continuity to consider some of the reactions at this point.

TABLE II.—SHOWING MG. OF NITROGEN OBTAINED IN VAN SLYKE ANALYSES OF 3 G. FIBRIN HYDROLYZED IN THE PRESENCE OF DIFFERENT QUANTITIES OF TRIOXYMETHYLENE.

	3 g. fibrin hydrolyzed alone.	3 g. fibrin + 0.10 g. $(CH_2O)_3$.	3 g. fibrin + 0.50 g. $(CH_2O)_3$.			3 g. fibrin + 15 g. $(CH_2O)_3$.		
			I.	II.	Av.	I.	II.	Av.
Total N taken	458.40	456.10	456.10	456.10	456.10	456.10	456.10	456.10
Ammonia N	46.50	42.00	43.00	43.20	43.10	118.00	119.70	118.85
Insol. humin N	9.60	18.85	2.10	2.00	2.05	1.05	1.05	1.05
Soluble humin N	6.22	12.35	36.90	36.70	36.80	15.00	12.00	13.50
Phosphotungstic humin N	1.40	3.80	11.10	6.00	8.55	11.00*	30.25*	20.63
Total N in filt. from bases	265.50	251.36	225.60	231.30	228.40	98.16	96.00	97.08
Amino N in filt. from bases	254.00	240.28	209.92	204.16	207.04	3.72	4.84	4.28
Non-amino N in filt. from bases	11.50	11.08	..	..	21.36	..	..	92.80
Total basic N	126.20	123.75	120.00	120.00	120.00	206.40	192.00	199.20
Amino N in bases	76.80	79.96	75.60	72.76	74.18	5.82	10.00	7.91
Non-amino N in bases	49.40	43.79	..	..	49.82	..	..	188.29
Arginine N	50.00	47.92	..	..	49.80	..	..	52.00
Total N recovered	455.42	452.11	..	..	438.90	..	..	450.31
Per cent N recovered	99.33	99.19	..	..	96.25	..	..	98.71

* Sample No. 1 was more strongly acidic (acetic acid) than was No. 2 and consequently gave less phosphotungstic humin N than did No. 2 since the ppt. of phosphotungstic humin is quite soluble in acetic acid. The solution containing the bases in Sample 1 was a much deeper red than that of Sample 2, showing that there was more of the red phosphotungstic humin in solution. In neither case, however, is it probable that all of the phosphotungstic humin was removed from the solution of the bases. In a sample hydrolyzed in the presence of 12 g. of $(CH_2O)_3$ where the phosphotungstic acid was shaken out with amyl alcohol-ether according to Van Slyke's modified direction,[17] 38.45 mg. of phosphotungstic humin were obtained. This represents more nearly the actual amount present. It was of interest to observe that the phosphotungstic acid cannot be removed from this humin either by amyl alcohol-ether or by barium hydroxide.

TABLE III.—SHOWING PERCENTAGES OF THE TOTAL NITROGEN OBTAINED IN THE VARIOUS FRACTIONS OF THE VAN SLYKE ANALYSES OF FIBRIN HYDROLYZED IN THE PRESENCE OF DIFFERENT QUANTITIES OF TRIOXYMETHYLENE.

	Fibrin hydrolyzed alone.	3 g. fibrin + 0.10 g. $(CH_2O)_3$.	3 g. fibrin + 0.50 g. $(CH_2O)_3$.	3 g. fibrin + 15 g. $(CH_2O)_3$.
Ammonia N	10.14	9.21	9.46	26.08
Insol. humin N	2.09	4.13	0.50	0.23
Soluble humin N	1.36	2.71	8.07	2.96
Phosphotungstic humin N	0.31	0.83	1.88	4.53
Total N in filt. from bases	57.89	55.14	50.11	21.30
Amino N in filt. from bases	54.29	52.72	45.42	0.94
Non-amino N in filt. from bases	2.50	2.43	4.69	20.36
Total basic N	27.52	27.14	26.33	43.70
Amino N in bases	16.75	17.54	16.28	1.74
Non-amino N in bases	9.67	9.61	10.93	41.31
Arginine N	10.90	10.51	10.93	11.40
Total N recovered	99.33	99.19	96.30	98.80

The Insoluble Humin.—The formation of this black product is almost instantaneous with the beginning of hydrolysis and when it is once formed it is not destroyed by boiling with 20% hydrochloric acid or with 20% hydrochloric acid + trioxymethylene. This latter statement would seem to be at variance with the data presented in Table I where it is shown that when no aldehyde is added 9.60 g. of insoluble humin nitrogen are obtained, when 0.10 g. of trioxymethylene is added 19.02 mg. are obtained, and when 15 g. of aldehyde are added only 1.05 mg. are obtained. However, the following experiments prove the point:

Experiment "A."—Three grams of fibrin were hydrolyzed for 48 hours with 20% hydrochloric acid + 0.10 g. of trioxymethylene. According to Table I, we should now have 19.02 mg. of insoluble humin in the hydrolysate. Without removing this insoluble humin 0.30 g. of additional aldehyde was added and the boiling continued for an additional 48 hours. The hydrolysate was analyzed at this point for insoluble and soluble humin nitrogen and for ammonia nitrogen. If the hydrolysis had been originally made in the presence of the 0.40 g. trioxymethylene we should expect only 3.27 mg. of insoluble humin nitrogen and we should expect the same figure if the insoluble humin were first formed and later broken down by the excess aldehyde. The analysis in Table IV shows that 19.65 mg. of insoluble humin nitrogen were present.

Experiment "B."—This experiment was carried out exactly like "A" for the first 48 hours. The insoluble humin was then filtered off and analyzed for nitrogen. The filtrate was concentrated, additional 20% hydrochloric acid was added together with 0.30 g. of trioxymethylene and the boiling continued for 48 hours more, at the end of which time analyses similar to those of "A" were made.

Table IV shows the figures obtained. These figures are further substantiated by the following experiment: The insoluble humin from a normal hydrolysis of 6 g. of fibrin was boiled for 44 hours with 12 g. of trioxymethylene in the presence of 20% hydrochloric acid. At the end of that period, the following figures were obtained: insoluble humin nitrogen 10.52 mg., soluble humin nitrogen 1.45 mg., "ammonia" nitrogen 2.40 mg. After this drastic treatment over 73% of the original nitrogen remains in the insoluble humin.

TABLE IV.—SHOWING THAT WHEN THE INSOLUBLE HUMIN HAS BEEN ONCE FORMED IT IS NOT DESTROYED BY AN EXCESS OF TRIOXYMETHYLENE.

	Experiment "A." 0.10 g. $(CH_2O)_3$ for 1st 48 hrs. + 0.30 g. for 2d 48 hrs. Analysis at end of 96 hrs.			Experiment "B." 0.10 g. $(CH_2O)_3$ for 1st 48 hrs. + 0.30 g. for 2d 48 hrs. Analysis for insoluble humin N at end of 48 hrs. and remainder of analysis at end of 96 hrs.				
				48 hrs.		96 hrs.		
	I.	II.	Av.	I.	II.	I.	II.	Av.
Insol. humin N.	19.40	19.90	19.65	20.10	19.75	None	None	None
Sol. humin N...	7.50	8.00	7.75	...	...	7.20	7.30	7.25
Ammonia N....	55.00	57.30	56.15	...	...	57.55	57.00	57.27

These experiments prove conclusively that the insoluble humin is stable in the presence of 20% hydrochloric acid and trioxymethylene *when once formed* so that the low results for insoluble humin obtained where excessive amounts of aldehyde were added must be interpreted as having been due to the intervention of some reaction preventing the formation of the insoluble humin.

The Soluble Humin.—The data so far presented in this paper do not permit us to draw definite conclusions regarding the nature of the soluble humin but the almost straight line rise of the soluble humin curve in Fig. 1 indicates a definite chemical reaction. It will be later shown that in all probability the maximum point reached by this curve coincides closely with the amount of tyrosin nitrogen. One of us has already shown[4]* that tyrosin forms an "acid-soluble humin" when treated with formaldehyde in the presence of HCl and that the percentage of tyrosin N present in this acid-soluble humin decreases when excessive quantities of aldehyde are present. It is also shown later (Table IX) that when the maximum amount of insoluble humin is prevented from forming by excessive amounts of aldehyde a considerable part of the nitrogen which should appear in the insoluble humin fraction actually does appear in the soluble humin fraction.

The Phosphotungstic Humin.—When fibrin is hydrolyzed in the presence of trioxymethylene the fraction designated by Van Slyke[17] as "unadsorbed humin carried down by the basic phosphotungstates" increased with added amounts of trioxymethylene. This "humin" colors the filtrate from the soluble humin a more or less intense red, depending

* Gortner, *J. Biol. Chem.*, **26**, 200 (1916).

upon the quantity present, and is precipitated by phosphotungstic acid as a hard, dark-colored mass which is difficultly soluble in the sodium hydroxide solution, and which separates again more or less completely when the bases are concentrated. An exact quantitative separation of the fraction is extremely difficult since it is appreciably soluble in both acids and alkalies. The figures presented in the preceding tables do not represent the total amount actually present nor will duplicate determinations be found in good check. Probably a more accurate method for the separation of this product is afforded by the ether-amyl alcohol separation of Van Slyke. The dark red-brown precipitate of phosphotungstic humin is insoluble in the ether-amyl alcohol and can be separated approximately quantitatively, but even here a complete separation is effected only with considerable difficulty due to the solubility of the humin in the acid solution of the bases. This latter method was used in the experiment reported in the footnote to Table II. This phosphotungstic humin is the first substance to precipitate when the phosphotungstic acid is added for the precipitation of the bases and simultaneously with its precipitation the solution loses its red color, becoming the usual pale yellow. The phosphotungstates of the bases are later deposited upon this precipitate in the usual white granular form. Sufficient data have not as yet been obtained to warrant any definite conclusions as to the processes giving rise to its formation but certain of the figures strongly suggest the possibility that it is in part derived from those components which normally form the insoluble and soluble humins when the normal mode of reaction is prevented by an excess of aldehyde.

The Ammonia Fraction.—The figures for the "ammonia" presented in Table I and the "ammonia" curve in Fig. 1 confirm the earlier conclusions of one of us[4]* that "the ammonia nitrogen falls significantly when small quantities of formaldehyde are added, but rises very rapidly when larger quantities are present." The decrease in the amount of ammonia with small quantities of trioxymethylene must be due either to an effect of the aldehyde on the amide (—CO—NH_2) grouping or else to the removal of some structures which in the absence of the aldehyde break up to form "ammonia," but which when the aldehyde is present, react with the trioxymethylene to form insoluble humin, thus preventing the formation of the usual amount of ammonia.

The reactions involved in this ammonia formation are being studied in more detail, the results from which study will be reserved for a later paper. We wish only to point out at the present time that the second possibility mentioned above appears to be the more probable explanation of the decreased amount of ammonia formed.

The following experiment shows that the splitting off of ammonia from

* Gortner, *J. Biol. Chem.*, **26**, 197 (1916).

the amide linking is not appreciably affected by the presence of trioxymethylene:

A weighed amount of asparagine (0.40 g.) was boiled for 15 hours under a reflux condenser with 60 cc. of 20% hydrochloric acid and varying amounts of trioxymethylene. The solution was then evaporated to dryness and the ammonia determined as usual. Table V shows the results obtained.

TABLE V.—SHOWING THAT ADDED TRIOXYMETHYLENE DOES NOT DEPRESS AMMONIA FORMATION FROM ASPARAGINE.

Trioxymethylene added.	Mg. ammonia N obtained.
None	35.72
0.10 g.	36.00
0.50 g.	36.50
1.00 g.	39.10

The theoretical yield of amide nitrogen for $C_4H_8O_3N_2.H_2O$ is 37.32 mg. Although the ammonia obtained in the first three experiments is not equal to the theoretical amount added, the figures show that trioxymethylene does not depress ammonia formation from asparagine. The increase in ammonia where 1 g. of trioxymethylene was added shows that aspartic acid loses amino nitrogen under these conditions.

While, however, the decrease in the ammonia fraction in the fibrin hydrolysates may be due to the removal of compounds necessary for its formation, the sudden increase following the formation of the maximum amount of humin can only be ascribed to the formation of volatile alkaline compounds. In order to gain an insight into the nature of the product so formed, the following experiment was made:

Ten grams of fibrin were hydrolyzed in the usual manner and after the removal of the insoluble humin the "ammonia" was distilled off into standard hydrochloric acid, 0.1550 g. of ammonia nitrogen being obtained. The solution was then evaporated to dryness and yielded 0.5040 g. of hydrochloride. A nitrogen determination on this hydrochloride gave 26.95 and 27.00 or an average of 26.97% nitrogen, the theoretical for ammonium chloride being 26.17%. The platinum salt was prepared and was identical in crystal form and chemical properties with ammonium chloroplatinate. There seems to be no doubt but that this fraction consists of ammonium chloride with, at the most, only traces of other compounds.

The soluble humin and excess of calcium hydrate were filtered off from the residue remaining in the flask after the above ammonia distillation and the filtrate was concentrated to almost dryness *in vacuo* and sufficient hydrochloric acid added to make the volume of 100 cc. of 20% acid. Twenty-five grams of trioxymethylene were then added and the mixture boiled for 96 hours. The formaldehyde was removed by steam distilla-

tion and the "ammonia" again distilled off into standard hydrochloric acid in the usual manner. No note was made of the amount of nitrogen obtained. The solution of the hydrochloride was evaporated to dryness, leaving a quantity of a very deliquescent salt having a strong fishy odor. A nitrogen determination gave 18.85% of nitrogen in duplicate determinations. The chloroplatinate was much more soluble than is ammonium chloroplatinate, crystallized in tiny needles, melted at 203° (not cor.), contained 3.52% of nitrogen and 38.91% of platinum. The picrate was formed with difficulty, crystallized in silky needles and melted at 210–220° (not cor.). The filtrate from the platinum salt was made alkaline with NaOH and distilled. The distillate had a strong pyridine odor. Attempts to isolate a picrolonate from this distillate failed.

Obviously this second fraction does not consist of ammonium chloride but of the hydrochlorides of volatile amines which are so basic that they can be readily titrated with acid using the usual indicators. It is highly improbable that this fraction consists of a single compound. In the preparation of the platinum salt only 0.7016 g. of chloroplatinate containing 0.0247 g. nitrogen was obtained from 0.30 g. of the hydrochloride, whereas if all of the hydrochloride had been converted into the chloroplatinate the chloroplatinate should have contained 0.0565 g. nitrogen. In other words, 56% of the nitrogen in the hydrochloride did not form a relatively insoluble chloroplatinate.

Another proof that we are dealing with compounds other than ammonium chloride is shown by the molecular weight. If the formula of the chloroplatinate is represented by "X_2"$PtCl_6$, a platinum percentage of 38.91 would correspond to a molecular weight of 46 for "X" as contrasted with the expected 18 for (NH_4).

The above experiments show conclusively that the increase in the ammonia fraction is not due to the formation of ammonium chloride nor to amide linkings but to volatile alkaline compounds formed by the interaction of the amino acids and the aldehyde. The chemical configuration of these compounds is at the present time under investigation.

Changes in the Total Nitrogen and in the Amino Nitrogen of the Bases and Filtrate from the Bases.—The sudden decrease in the total nitrogen in the filtrate from the bases of 14.2 mg. on the addition of 0.10 g. trioxymethylene as contrasted with a loss of only 2.5 mg. from the bases and the increase of 9.25 mg. of insoluble humin and 6.1 mg. of soluble humin strongly suggests that the elements which go to form the humins are, in a large measure, derived from compounds which when insufficient aldehyde is present pass into the filtrate from the base. This conclusion is substantiated by the following experiment:

Ten grams of fibrin were hydrolyzed with 25% sulfuric acid for 24 hours. The humin was filtered off and barium hydroxide added to the filtrate

until it was almost neutral. The barium sulfate was filtered off, the filtrate made alkaline with barium hydroxide and the ammonia distilled off. The residue was filtered, the filtrate concentrated to about 300 cc., sulfuric acid added to 5% by volume and the bases precipitated with an excess of phosphotungstic acid. The bases were filtered off, washed and the phosphotungstic acid shaken out with amyl alcohol-ether. The phosphotungstic acid was likewise removed with amyl alcohol-ether from the filtrate of the bases and both solutions after quantitatively removing the sulfuric acid with barium hydroxide were made to 300 cc. volume. The filtrate from the bases contained 0.4830 g. nitrogen, of which 0.4189 g. was amino nitrogen while the solution of the bases contained 0.3840 g. nitrogen, of which 0.2044 g. existed as amino nitrogen.

Four aliquot portions of 50 cc. each were taken from each solution, hydrochloric acid added in sufficient quantity to make a 20% solution and the resulting solutions boiled for 48 hours in the presence of varying amounts of trioxymethylene. The experimental data are shown in Table VI. They show that at least the nitrogenous portion of the insoluble humin passes into the "filtrate from the bases" when insufficient aldehyde is present to produce maximum humin formation. They likewise show that the increase in the ammonia fraction, which we have shown above to be due to volatile amines, arises from both the bases and the filtrate from the bases. A similar conclusion apparently holds true for the soluble humin.

TABLE VI.—SHOWING THE EFFECT OF ADDED TRIOXYMETHYLENE UPON HUMIN AND AMMONIA FORMATION AND UPON THE AMINO NITROGEN OF SOLUTIONS OF THE "BASES" AND "FILTRATES FROM THE BASES."

Amino acids from	G. trioxymethylene added.	Mg. insoluble humin N.	Mg. soluble humin N.	Mg. ammonia N.	Mg. amino N.
Bases	None	None	Only a trace	Lost	34.85
Bases	0.05	None	1.30	2.40	32.75
Bases	0.20	None	1.40	2.50	30.85
Bases	0.40	None	2.10	2.90	26.19
Filtrate from bases	None	None	0.70	2.60	65.10
Filtrate from bases	0.05	2.80*	1.60	3.30	62.42
Filtrate from bases	0.20	2.55	1.75	5.25	58.10
Filtrate from bases	0.40	2.10	2.50	8.30	52.78

The decrease in amino nitrogen shown in Tables II, III and VI is due not only to the removal of compounds involved in humin formation but also to (*a*) the formation of volatile alkaline products which go to increase the "ammonia" fraction, (*b*) to the formation of basic compounds which are precipitated by phosphotungstic acid, and (*c*) probably to the formation of methylene linkings on the amino groups, $R - N = CH_2$. Tables II

* The maximum quantity of insoluble humin was not obtained as was indicated by a somewhat greenish color of this humin.

and III show that the "basic" nitrogen is greatly increased by hydrolysis in the presence of trioxymethylene, but the nature of the compounds causing this increase is still under investigation. That the methylene linking is another cause of the decrease in amino nitrogen is rendered probable by the work of Galeotti,[3] Löb,[8] Schiff[11,12,13], and Sörensen,[14] all of whom have formed methylene-amino compounds by the action of formaldehyde on amino acids in neutral solution. It is due to the formation of this class of compounds that Sörensen[14] found it possible to titrate amino acids with alkali using phenolphthalein as an indicator.

The Hydrolysis of Gelatin in the Presence of Trioxymethylene.—The preceding experiments with fibrin and trioxymethylene give a general indication as to the effect which the aldehyde has upon the analysis of a pure protein. However, they give no definite information as to which amino acids are involved in the formation of humin. That certain specific amino acids are the precursors of both the soluble and insoluble humin is indicated by the quantitative nature of the reaction with optimum concentration of trioxymethylene, while on the other hand, the "ammonia" reaction seems to be a general one for a certain class, if not for all, of the amino acids.

As has been previously stated, Osborne and Jones[9] expressed their belief from circumstantial evidence that the humin originates from histidine or tryptophane, or perhaps from both. Gortner and Blish[5] later found "that in all probability the humin nitrogen of *protein* hydrolysis has its origin in the tryptophane nucleus" and presented evidence to show that histidine was not involved. Roxas[10] has disputed this last finding but we have already criticized his data and the evidence presented in the following section will be found to uphold Gortner and Blish's conclusion as against that of Roxas.

Gortner later suggested[4] that, inasmuch as phenols are known to form resins with formaldehyde, it seemed probable that the soluble humin formed in the presence of formaldehyde might be due to tyrosine.

For the purpose of gaining some insight into which of the amino acids is involved in these reactions, we have hydrolyzed gelatin in the presence of trioxymethylene. Gelatin is a unique protein inasmuch as it contains no tyrosine, tryptophane or cystine and but a mere trace of histidine.* Analyses similar to those recorded for fibrin were made on 3 g. of gelatin using varying amounts of trioxymethylene. The hydrolyses in all instances were continued for 48 hours. The solutions, both those to which aldehyde had been added and those without aldehyde, were practically colorless at the conclusion of the hydrolysis, in marked contrast with the hydrolysates of the usual proteins. The data obtained are given in Table VII.

* Cf. Fischer, Levene and Aders[2] and Levene and Beatty.[7]

TABLE VII.—SHOWING THE EFFECT OF HYDROLYZING 3 G. OF GELATIN IN THE PRESENCE OF DIFFERENT AMOUNTS OF TRIOXYMETHYLENE.

Grams trioxymethylene added.	Mg. insoluble humin N.	Mg. soluble humin N.	Mg. ammonia N.
None	0.25	1.70	5.88
None	0.25	Lost	5.56
0.05	0.20	2.40	5.72
0.05	0.40	Lost	5.53
0.10	0.20	1.75	6.95
0.20	0.17	2.65	8.64
0.30	0.18	2.30	12.98
0.40	0.15	2.30	15.80
0.60	0.10	3.00	Lost
1.00	0.10	Lost	33.71
2.00	0.11	2.46	50.51
2.00	Lost	2.45	50.60
3.00*	0.10	0.65	48.80

A comparison of these data with those of Table I and Fig. 1 shows very marked differences. Instead of very prominent curves showing marked chemical action with the formation of the humins, we have here no indication of reaction in the humin figures and only a steady rise in the "ammonia" fraction. This rise is similar to that found for fibrin and has been discussed previously. We have come to regard the insoluble and soluble humin formed from gelatin as due to physical causes, *i. e.*, the insoluble humin nitrogen represents a portion of the nitrogen contained in amino acids which splashed up upon the sides of the flask and which were thus subjected to greater heat so that a partial carbonization took place; the soluble humin nitrogen we regard as occluded in and adsorbed by the particles of calcium hydroxide and thus filtered off. The amounts of each of these substances is very small in one instance amounting to a maximum of only 0.09% of the total nitrogen and in the other to only 0.76%. We believe that our contention as to their nature is at least as valid as is the other possibility that they represent the products of chemical reactions. If this be true, and it almost certainly is with regard to the insoluble humin, no other conclusion seems possible but that the humins arise from one or more of the four amino acids which gelatin lacks, *i. e.*, cystine, histidine, tyrosine and tryptophane, all of which are present in fibrin. The absence of a drop in the early part of the ammonia curve again confirms the earlier conclusion that this reaction in the fibrin hydrolysates was not due to any interference of the aldehyde with the amide linkings but to the removal of some compound in the humin reactions which normally breaks down, either in whole or in part, to form volatile alkaline products. The lack of compounds with which the aldehyde unites to form humin causes an immediate increase in "ammonia" when small amounts of trioxymethylene are added to gelatin.

* 24-hour hydrolysis. This accounts for the lower "ammonia" figure.

The Hydrolysis of Gelatin Plus Certain Amino Acids in the Presence of Trioxymethylene.—Inasmuch as gelatin produces negligible quantities of humin both when hydrolyzed alone and in the presence of trioxymethylene, and inasmuch as certain amino acids normally present in proteins which produce humin are lacking from gelatin, the addition of these amino acids to a gelatin hydrolysate might be expected to cause humin formation. The four lacking amino acids, all of which were absolutely pure, were therefore added to gelatin and 48-hour hydrolyses made as is indicated in Table VIII. Three grams of gelatin were used in each experiment and where amino acids were used the following amounts were added: cystine 0.10 g., tyrosine 0.10 g., histidine 0.10 g., tryptophane 0.075 g.

TABLE VIII.—SHOWING THE EFFECT OF ADDED AMINO ACIDS UPON HUMIN FORMATION WHEN GELATIN IS HYDROLYZED IN THE PRESENCE OF TRIOXYMETHYLENE.[1]

Trioxymethylene added, g.		Gelatin alone.	Gelatin + cystine + histidine.	Gelatin + cystine + histidine + tyrosine.	Gelatin + cystine + histidine + tyrosine + tryptophane.
None		0.25	0.40	0.20	2.05
0.05		0.30	0.30	0.18	12.50
0.075	Mg. insoluble humin..	*	0.10	0.05	13.35
0.10		0.20	0.15	0.10	12.15
0.60		0.10	0.10	0.03	6.60
None		1.70	0.60	1.90	4.50
0.05		1.90	1.30	7.30	4.55
0.075	Mg. soluble humin....	*	1.30	7.10	6.94
0.10		1.75	1.20	8.30	6.90
0.60		3.28**	1.10	8.70	11.90
None		5.72	5.30	7.02	7.70
0.05		5.62	5.30	*	5.80
0.075	Mg. "ammonia".....	*	6.05	5.90	7.00
0.10		6.95	6.00	*	7.40
0.60		Lost	16.80	*	15.00

The analytical figures are strikingly significant. *There is no increase in the black insoluble humin due to the addition of three of the amino acids, cystine, histidine or tyrosine, but there is a marked insoluble humin formation when tryptophane is added.* These experiments convincingly demonstrate the correctness of the conclusion of Gortner and Blish[5] that the humin of protein hydrolysis arises from the tryptophane nucleus. It will be noted that there is an appreciable amount (2.05 mg.) of insoluble humin formed when tryptophane is added to gelatin and when no aldehyde has been added. That most of this 2.05 mg. is true humin nitrogen

[1] The added tyrosine nitrogen amounted to 7.74 mg. and that of the tryptophane to 10.29 mg.

* Not determined.

** Undoubtedly too high. See Table VI.

is demonstrated by the blackening of the solution for no blackening took place when gelatin was hydrolyzed alone or in the presence of the three other amino acids. This observation is similar to that which Gortner and Blish made when they obtained an increase of 6.3 mg. of humin nitrogen by adding tryptophane to zein. *Obviously the black humin formation in protein hydrolysis is dependent upon two factors, one of which is tryptophane or some other indole or pyrrole (see later) derivative, and in all probability the other is an aldehyde or ketone.* A small amount of this aldehyde or ketone must be normally present in proteins and we suggest the possibility that an amino aldehyde is a normal constituent of the protein molecule. Some idea as to the amount of this unidentified constituent may be gained from the insoluble humin nitrogen figures of Table VIII for whereas only 10.29 mg. of tryptophane nitrogen were added a maximum of 13.25 mg. of insoluble humin nitrogen was obtained. The excess of approximately 3 mg. of nitrogen we believe to belong to the hypothetical aldehyde or ketone.

The figures for soluble humin are equally striking. *The addition of cystine and histidine produces no increase in either insoluble or soluble humin nitrogen but the addition of tyrosine and trioxymethylene causes a sharp increase in soluble humin.* This soluble humin is not black but a reddish yellow. *It is therefore not the true humin of protein hydrolysis.* The addition of 7.74 mg. of tyrosine nitrogen causes an increase of 7.00 mg. of soluble humin nitrogen. That the reaction is not exactly quantitative is easily explained, due to the fact that the tyrosine humin is appreciably soluble and a part passes into the filtrate from the humin.

The addition of cystine and histidine to gelatin causes no significant alteration of the ammonia figures but the addition of tryptophane produces an ammonia curve similar to that in Fig. 1. Here again our earlier contention receives additional experimental confirmation, *i. e.*, that the decline in the ammonia curve, caused by the addition of small amounts of aldehyde, is due to the removal of some substance (tryptophane) in the insoluble humin which, when no aldehyde or insufficient aldehyde is present, partially breaks down to form volatile alkaline products [NH_3(?)]. Whether or not tyrosine contributes nitrogen to this ammonia fraction cannot be decided from the data of Table VIII inasmuch as certain of the ammonia determinations were not completed. There seems to be good evidence however that the figure of 7.02 mg. ammonia nitrogen for the hydrolysate of gelatin + cystine + histidine + tyrosine is too high for one of us has shown* that tyrosine boiled with 20% hydrochloric acid and small quantities of formaldehyde produces only traces of ammonia. In the same paper it is shown that a sufficient amount of ammonia is formed

*Gortner, *J. Biol. Chem.*, 16, 200 (1916).

from tryptophane to account for the form of the ammonia curve shown in Fig. 1.

Table VIII likewise furnishes data as to the sequence of the different reactions. From the data in the last column, it will be observed that the increase in insoluble humin with the addition of aldehyde is much more rapid than the increase in soluble humin and that the ammonia fraction is the last to show an increase. The trioxymethylene apparently reacts first with tryptophane to form insoluble humin, then any uncombined aldehyde reacts with tyrosine to form soluble humin and when enough aldehyde is present to combine with both, the amino groups of the remaining amino acids are attacked and volatile basic compounds are formed. The completeness of these reactions is influenced however by the fact that an excess of formaldehyde prevents the formation of the maximum amount of insoluble humin. *This series of hydrolyses is now exactly analogous to that of fibrin. The insoluble humin can be increased to a maximum; excess of trioxymethylene prevents maximum insoluble humin formation and causes a maximum formation of soluble humin, while a further excess over that necessary to combine with tryptophane and tyrosine reacts to increase the ammonia fraction.*

The behavior of gelatin plus different amounts of tryptophane hydrolyzed in the presence of an excess of trioxymethylene.—In order to determine the accuracy of our conclusion that when an excess of aldehyde prevents insoluble humin formation a portion of the nitrogen which under optimum conditions would appear in this fraction actually does appear in the soluble humin, we hydrolyzed gelatin in the presence of 3 g. of trioxymethylene plus increasing amounts of tryptophane. The results are given in Table IX. It will be observed that there is an increase in both the insoluble and the soluble humin nitrogen due to the addition of each increment of tryptophane, the tryptophane nitrogen recovered in the insoluble humin rising from 7% to 20% of the added tryptophane nitrogen and the soluble humin nitrogen accounting for from 33 to 57% of the added tryptophane nitrogen.

TABLE IX.—SHOWING THE EFFECT OF THE ADDITION OF DIFFERENT AMOUNTS OF TRYPTOPHANE TO GELATIN HYDROLYZED IN THE PRESENCE OF AN EXCESS OF TRIOXYMETHYLENE.

Gelatin, g.	Tryptophane N added, mg.	Trioxymethylene added, g.	Insoluble humin N, g.	Soluble humin N, mg.	Ammonia N, mg.
3.0	None	3.0	0.07	5.00	59.15
3.0	6.86	3.0	0.45	7.25	56.50
3.0	13.72	3.0	1.90	10.80	55.75
3.0	17.15	3.0	3.45	14.85	57.70

The reactions involved in these experiments are however apparently too complex for analysis, but we have produced direct evidence that, when an excess of aldehyde is present tryptophane nitrogen does not appear

quantitatively in the insoluble humin as it does when only a small amount of aldehyde is present, but that a portion of the nitrogen appears in the insoluble humin, a larger portion in the soluble humin, and the remainder in the filtrate from the humin.

The Behavior of Indole when Boiled with Trioxymethylene in the Presence of HCl.—In order to ascertain what groupings were responsible for the insoluble humin formation the following experiments were made:

Three grams of gelatin were hydrolyzed in the presence of 0.10 g. of indole with and without the addition of aldehyde and the usual fractions determined. Indole was likewise boiled with hydrochloric acid without the addition of either protein or aldehyde. The data are given in Table X.

TABLE X.—SHOWING THE EFFECT OF THE ADDITION OF INDOLE TO A GELATIN HYDROLYSATE BOTH WITH AND WITHOUT THE ADDITION OF TRIOXYMETHYLENE.

Gelatin added, g.	Indole added, g.	Trioxymethylene added, g.	Insoluble humin N, mg.	Soluble humin N, mg.	Ammonia N, mg.
None	0.05	None	None	3.05	1.00
None	0.10	0.10	11.70	Not detd.	Not detd.
3.00	None	None	0.25	1.70	5.72
3.00	0.10	None	5.80	5.45	4.50
3.00	0.10	0.10	14.40	1.75	4.80

It will be seen that when no aldehyde is present no insoluble humin is formed from indole alone. Van Slyke[15] has made the same observation as regards tryptophane and his results were confirmed by Gortner and Blish.[5] *When however aldehyde is added the indole nitrogen is quantitatively converted into insoluble humin nitrogen* (indole nitrogen added 11.97 mg., insoluble humin nitrogen formed 11.70 mg.). The figures for gelatin hydrolyzed with indole are similar to those of gelatin + tryptophane. Black insoluble humin nitrogen is formed from the hydrolysis of gelatin and indole without the addition of aldehyde, just as was the case when tryptophane was added, indicating the presence of an aldehyde grouping in the gelatin molecule; and when aldehyde is added the maximum insoluble humin nitrogen exceeds the added indole nitrogen by 2.43 mg. just as it exceeded the added tryptophane by 3.06 mg. Perhaps this excess represents occluded or adsorbed nitrogen, but we are inclined to regard at least a part of it as the nitrogen belonging to the hypothetical aldehyde.

The fact that indole forms a black insoluble humin when boiled with hydrochloric acid and aldehyde is significant, not for its direct bearing upon protein analysis, for insofar as we know unsubstituted indole does not exist as such in the protein molecule, but it does indicate that the condensation of tryptophane with aldehyde to form insoluble humin involves only the indole nucleus of tryptophane and not the α-amino

propionic acid side chain. This seems to disprove the elaborate formulas of Roxas[10] in which the α-amino group of tryptophane is involved.

The Behavior of Pyrrole and Pyridine when Boiled with Trioxymethylene in the Presence of Hydrochloric Acid.—Pyrrole when boiled with hydrochloric acid polymerizes to tri-pyrrole.[1] In the presence of trioxymethylene it reacts, forming a dark condensation product similar to the insoluble humin of protein hydrolysis. When 0.10 cc. of pyrrole was boiled with hydrochloric acid for 48 hours without the addition of aldehyde, 8.35 mg. of insoluble humin nitrogen were obtained, whereas when 0.10 g. of trioxymethylene was added 13.00 mg. of nitrogen remain in this fraction. Pyridine treated in a similar manner failed in either instance to form any trace of insoluble humin. These experiments are interesting for they tend to show that Gortner and Blish's hypothesis that the =NH grouping in tryptophane is responsible for humin formation is incorrect. Certainly the imino group in pyridine will not react and it appears possible that the reaction involves rather the α-hydrogen atom of the indole molecule.

Is the α-Amino Group of Tryptophane Involved in Humin Formation?—As we have already suggested, the preceding experiments indicate a combination of the aldehyde with the indole nucleus of tryptophane rather than with the α-amino group of the aliphatic side chain such as was suggested by Roxas.[10] Roxas bases his conclusions as to the part which the α-amino group plays in humin formation upon the observation that when 0.1514 g. of tryptophane was boiled with 1 g. of glucose and the resulting humin treated with nitrous acid in Van Slyke's apparatus, only 1.90 mg. out of a total of 13.82 mg. or 27.4% of the theoretical reacted as free amino nitrogen. It appeared to us possible that this inactivity of the α-amino group might be due to secondary reactions and not to the major reaction through which humin is formed. The following experiments were therefore undertaken:

Weighed quantities of tryptophane, each containing 10.29 mg. of nitrogen, were boiled with hydrochloric acid in the presence of trioxymethylene and after 48 hours' boiling the insoluble humin was filtered off. The filtrate from the insoluble humin was Kjeldahled and the difference between the amount of nitrogen found in the filtrate from the humin and the amount of tryptophane nitrogen taken was regarded as the nitrogen of the insoluble humin.

The insoluble humin was finely ground with dilute sodium hydroxide and introduced into Van Slyke's amino nitrogen apparatus. This afforded a measure of the quantity of the α-amino nitrogen of tryptophane which was not involved directly in the reactions which produce humin. The data are shown in Table XI.

TABLE XI.—SHOWING THE DISTRIBUTION OF NITROGEN IN TRYPTOPHANE WHEN BOILED WITH TRIOXYMETHYLENE IN THE PRESENCE OF HYDROCHLORIC ACID.

Tryptophane added, g.	Trioxymethylene added, g.	Black insoluble humin N, mg.	—NH_2 nitrogen in the insol. humin, mg.	Humin N existing as free —NH_2—N, %.	Color of filtrate from humin.	Total N in filtrate from humin, mg.
0.075	0.010	5.89	2.32	39.3	Bluish	4.40
0.075	0.015	8.19	3.97	48.4	Brown	2.10
0.075	0.020	8.69	4.25	48.8	Faint yellow	1.60
0.075	0.025	9.04	3.70	40.9	Faint yellow	1.25
0.075	0.050	9.14	2.23	24.4	Yellow-brown	1.15
0.075	0.075	8.49	1.74	20.4	Brown	1.80
0.075	0.100	7.69	1.31	17.1	Brown	2.60

The figures here presented prove that the α-amino group is not involved in the primary reaction by which the black insoluble humin is formed. The point of maximum humin formation probably coincides closely with the addition of 0.020 g. trioxymethylene and the humin found here contains 48.8% of its nitrogen in the amino form as contrasted with the theoretical 50% of the original tryptophane. Beyond this point the amino nitrogen decreases, due probably to the formation of methylene combinations with the excess of aldehyde and to deamination and formation of volatile alkaline products such as have been demonstrated earlier in this paper.

That a similar condition to the above holds for the normal humin of fibrin hydrolysis is shown by the determination of amino nitrogen on a sample of this humin. The humin obtained from 3 g. of fibrin was treated in a Van Slyke apparatus and gave 3.16 mg. of amino nitrogen for 9.60 mg. of insoluble humin nitrogen, or approximately 33% of the total. This is somewhat low but we do not know how much deamination takes place in the 48-hour hydrolysis. When *3 g. of fibrin were hydrolyzed in the presence of 0.10 g. of trioxymethylene, so as to obtain maximum humin formation and form a total of 19.02 mg. of insoluble humin nitrogen, we obtained 9.80 mg. of amino nitrogen, or 102% of the theoretical yield, a conclusive demonstration that the α-amino group is not involved.*

We are unprepared at present to prove the structure of the humin molecule; but are inclined to the view that when its structure has been elucidated it will be found to be closely related to that of indigo blue.

Discussion.

In order to preserve the continuity of the experimental work, it has been necessary to discuss most of the data at the time of their presentation. We wish, however, to emphasize again the major observations with respect to the nature and mechanism of humin formation.

We have been able, by the addition of appropriate quantities of aldehyde, to increase the formation of the *black insoluble humin* in a fibrin

hydrolysate to a maximum approximately 100% above the normal, then by further additions of aldehyde we have found that the maximum formation of insoluble humin is inhibited and that the formation of *soluble humin* is increased to a maximum, and that further additions of aldehyde cause rapid decrease in the amount of insoluble humin N and soluble humin N while the ammonia fraction steadily increases.

We have likewise shown that when gelatin is hydrolyzed in a similar manner only the ammonia fraction is increased. Obviously then the increases in the insoluble and soluble humins in fibrin hydrolysates must be due to the presence of amino acids in fibrin which are lacking in gelatin. These amino acids are cystine, histidine,[1] tyrosine and tryptophane.

We have therefore hydrolyzed gelatin plus these different amino acids and have shown that the addition of histidine and cystine do not influence the figures obtained for either fractions of the humin, that the addition of tyrosine markedly increases the formation of soluble humin but does not influence that of the insoluble humin and that with the addition of tryptophane the insoluble humin is immediately increased with a somewhat less marked increase in the soluble humin. The curve for insoluble humin in gelatin to which tryptophane and aldehyde have been added is identical in form to that for fibrin hydrolyzed in the presence of trioxymethylene so that we are forced to conclude that the formation of black insoluble humin in a protein hydrolysate is due to the interaction of tryptophane and some, as yet unidentified, aldehyde or ketone. The fact that the addition of tryptophane to zein and gelatin causes black humin formation we regard as at least strongly indicative of the presence of an aldehyde grouping in the zein or gelatin molecule, and the fact that the maximum amount of insoluble humin is not formed in a fibrin hydrolysate to which no aldehyde has been added indicates that there is not sufficient aldehyde normally present to combine with all of the tryptophane.

We have gone further and have shown that the primary reaction of humin formation does not involve the α-amino group of tryptophane but only the indole nucleus and we have produced good evidence to show that it is the α-position on this nucleus which is reactive rather than the imino group.

We are therefore firmly convinced that *of all the known hydrolytic products of proteins, tryptophane 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

[1] Histidine is not entirely absent from gelatin but is present only to the extent of 0.4%.

form and then mix them in different combinations and boil these with HCl 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 HCl but does produce humin when aldehyde is added and if (*b*) all of 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 + 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. The preparation for this final set of experiments is already far advanced and we hope to be able to publish the data in the near future.

In addition we are planning to ascertain whether or not the reactions outlined in this paper may not be utilized for the quantitative estimation of the tryptophane and tyrosine content of proteins.

Summary.

In the experiments reported in the preceding pages we have studied the reactions which take place when proteins are hydrolyzed in the presence of formaldehyde with especial reference to the formation of the black insoluble humin of protein hydrolysis and with incidental reference to the composition of the "soluble humin" and "ammonia" fractions. The following conclusions are evident:

1. When proteins are hydrolyzed in the presence of trioxymethylene and the resulting hydrolysate analyzed by Van Slyke's method, the nitrogen distribution is so altered as to bear no resemblance to the analysis conducted in the absence of aldehyde.

2. When a protein containing tyrosine and tryptophane is hydrolyzed with increasing amounts of trioxymethylene, the figures for both insoluble and soluble humin N are rapidly increased to a maximum after which there is a sharp decrease in the nitrogen curve of these fractions. The ammonia fraction on the other hand decreases with the smaller additions of trioxymethylene and then rises rapidly for larger additions of aldehyde.

3. When both tyrosine and tryptophane are absent from a protein, hydrolysis in the presence of trioxymethylene produces no change in the insoluble or soluble humin nitrogen and only a steady increase in the ammonia fractions.

4. We have shown that the rise in the insoluble humin curve, and the formation of black insoluble humin is due to the presence of tryptophane in the hydrolysate and we believe that the maximum point on the insoluble humin N curve coincides closely with the amount of tryptophane nitrogen present in the hydrolysate.

5. An excess of trioxymethylene largely inhibits the formation of insoluble humin but does not break down insoluble humin which has once been formed.

6. Contrary to the belief of Roxas, histidine and cystine are not involved in the formation of black insoluble humin, and we have expressed the belief that tryptophane alone, of all the known hydrolytic products of proteins, is involved in this reaction.

7. We believe that the formation of black 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 has in humin formation is to (perhaps) furnish some of their nitrogen to the humin fraction through either adsorption or occlusion.

8. The α-amino group of the aliphatic side chain of tryptophane is not involved in the primary reaction by which black insoluble humin is formed. The primary reaction concerns only the indole nucleus, inasmuch as the same reaction takes place when tryptophane is replaced by indole and it appears probable that it is the α-position of the indole nucleus which is reactive.

9. The soluble humin nitrogen of proteins hydrolyzed in the presence of trioxymethylene is largely derived from tyrosine. However, the maximum point of the soluble humin curve includes some tryptophane nitrogen. We believe that it is possible to distinguish the soluble humin formed from tryptophane from that derived from tyrosine for there is a sudden drop from the maximum insoluble humin nitrogen when additional aldehyde is added and then, on the further addition of aldehyde, the curve flattens and becomes approximately a straight line. The sudden drop we believe to be due to the non-formation of soluble humin from tryptophane due to the presence of an excess of aldehyde and the straight line drop to the deamination of the tyrosine humin. If this be true an extension of the deamination curve until it intercepts the rising soluble humin curve should indicate the proportion of the soluble humin nitrogen due to tyrosine.

10. The sudden initial drop in the ammonia fraction is probably due to the removal of some compound (tryptophane) in the insoluble humin which, when no aldehyde is present, contributes nitrogen to the "ammonia" fraction.

11. The sudden rise in the "ammonia" curve with larger additions of trioxymethylene is not due to the formation of ammonia but to the deamination of amino acids and the formation of volatile alkaline compounds, the nature of which is still under investigation.

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OBSERVATIONS ON THE McLEAN-VAN SLYKE IODOMETRIC METHOD FOR THE TITRATION OF SMALL AMOUNTS OF HALIDES, IN ITS APPLICATION TO CHLORIDES.

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For the purpose of determining small amounts of chlorides more accurately than could be done by the Volhard method, McLean and Van Slyke devised an iodometric method.[1] To the chloride solution they added an excess of a standard solution of silver nitrate in nitric acid, and titrated the excess of silver nitrate with potassium iodide, in an aliquot part, using sodium nitrite and soluble starch as an indicator. To regulate the acidity they added trisodium citrate. They showed the method to be satisfactory for the determination of halides, and it was found

[1] THIS JOURNAL, 37, 1128 (1915).