5. Positive results of hydrogen sulfide formation may be obtained in eighteen hours.

6. No hydrogen sulfide formation is obtainable in as long a period as seventy-two hours from natural waters which are truly "clean," while much is formed in from twelve to twenty-four hours with contaminated waters.

7. The feces of domestic animals contain bacteria which are capable of producing hydrogen sulfide from a simple peptone medium in as large amounts as is the case of the bacteria from human feces.

8. The large amounts of hydrogen sulfide *rapidly* produced by organisms of sewage appears to be not due primarily to members of the *B. coli* group.

9. This group of hydrogen sulfide producing bacteria do not actively ferment carbohydrates. Hence testing for their presence is a valuable aid supplementing tests for gas producers and is of especial value in polluted waters in which the *B. coli* group is absent.

10. Some evidence has been obtained which apparently indicates that hydrogen sulfide is more rapidly produced in waters containing a mixed bacterial flora than by the isolated pure cultures alone.

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ON THE ORIGIN OF THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS.

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Introduction.

It is well known that, when proteins are subjected to hydrolysis by boiling acids, a blackening of the solution occurs, and that, when the boiling is continued for some time, black insoluble particles separate from solution. These compounds may be purified to a greater or a less degree by solution in alkali and reprecipitation by the addition of acid. They are, however, insoluble in all of the usual organic solvents and have never been obtained in crystalline form.

A very considerable amount of work has been done on these humins or "melanoidins" as Schmiedeberg[1] calls them. Schmiedeberg found that indol and skatol were formed in an alkali fusion and Samuley[2] by reduction obtained evidences of pyridine formation. Inasmuch as humin was not formed from proteins when the hydrolysis was carried out in the presence of stannous chloride, Samuley concludes that the formation of this dark-colored product is due to an oxidative process.

[1] *Arch. Exper. Path. u. Pharm.*, 39, 1–84 (1897).

[2] *Beitr. chem. Physiol. u. Path.*, 2, 355–88 (1902).

The question as to the nature of the mother substance from which these humins are formed is of the greatest importance. In a study of the nitrogen partition of proteins it often happens that several per cent. of the total nitrogen must be recorded as "humin nitrogen."[1] All of the other fractions obtained in the nitrogen partition can be correlated with known structures in the protein molecule, but the humin nitrogen has no known meaning other than that it varies in the different proteins.

The questions therefore remain: *Is the humin nitrogen derived from some known product of protein hydrolysis, or is it formed from some unknown constituent?* If it is formed from some known amino acid, can the reaction not be made quantitative and thus form a basis for the estimation of this amino acid?

In an attempt to answer the above questions we have performed a few experiments. We believe that we have answered the first question, and that we have secured some evidence which may help to answer the second question.

Osborne and Jones,[2] in a study of the hydrolysis products of proteins, state:

"There can be no doubt that this substance (humin) is a mixture of the secondary decomposition products formed from different constituents of the protein by the action of acids, for some of these, such as tryptophane, histidine, and carbohydrate, are known to yield colored products under such conditions. That the melanin originates chiefly from these substances is indicated by the fact that zein, which gives no reaction for tryptophane or carbohydrate and yields only a small amount of histidine, gives rise to the merest trace of humin when subjected to prolonged hydrolysis with acids."

Van Slyke[3] later took up one phase of this problem in so far as tryptophane was concerned. He boiled 0.9 g. of tryptophane for 12 hours with 100 cc. of 20% hydrochloric acid and concentrated the solution *in vacuo*. He states:

"The solution was clear, free from insoluble matter, and but very slightly colored..... *Tryptophane is responsible for none of the nitrogen estimated as ammonia, arginine, or melanin.*"

In view of Osborne's statement that zein yielded almost no humin and that tryptophane, was not present it seemed possible that Van Slyke might be in error as to his conclusions. It will be noted that he boiled the tryptophane alone, and not in the presence of other products of protein hydrolysis. It therefore seemed advisable to mix zein with definite amounts of tryptophane and to submit the mixture to acid hydrolysis and then secure the nitrogen partition on the hydrolyzed mixture.

[1] Owing to the similarity of this humin, both in color and solubility, to the naturally occurring melanins it is often referred to as "melanin" and its nitrogen as "melanin nitrogen." One of us (Gortner, 1912, *Science*, n. s. **36**, 52–3) has already pointed out that other criteria of relationship should be secured before such a confusing terminology is adopted.

[2] *Am. J. Physiol.*, **26**, 305–28 (1910).

[3] *J. Biol. Chem.*, **10**, 15–55 (1911).

Experimental.

The purity of the material was the first consideration. The zein employed in the following experiments was from a sample kindly given us by Professor Osborne. A Kjeldahl determination of nitrogen gave only 15.25%. This figure is lower than that given by Osborne,[1] 16.13%, but it is probable that our sample contained moisture, inasmuch as we did not deem it advisable to dry it with the aid of heat.

The tryptophane was prepared by the tryptic digestion of casein and subsequent separation by the method of Hopkins and Cole.[2] The product was repeatedly treated with bone-black and recrystallized from dilute alcohol. It was obtained in the form of almost colorless nacreous plates.

Nitrogen found, 14.06%; calc., 13.72%. Amino nitrogen (by van Slyke's apparatus): found, 6.81%; calc., 6.86%.

Histidine-di-hydrochloride was prepared from cattle blood.[3]

Amino nitrogen found, 6.07%; calc., 6.14%.

The dextrose was from a sample prepared by the U. S. Bureau of Standards and contained no nitrogen.

The method of analysis was as follows: to the protein + the added substances 50 cc. of hydrochloric acid, sp. gr. 1.115, was added, and the mixture was boiled under a reflux condenser for 48 hours. The acid was distilled off under diminished pressure and ammonia nitrogen determined according to Van Slyke[4] by distilling with calcium hydroxide at 40–45° under a pressure of less than 30 mm.

The residue remaining in the distilling flask was filtered and the precipitate containing the humin washed with hot water to the absence of chlorides. A Kjeldahl nitrogen determination on the filter and its contents gave the humin nitrogen.

The filtrate from the humin was concentrated to about 100 cc., 10 cc. of concentrated hydrochloric acid and 7.5 g. of phosphotungstic acid were added and the mixture heated for a few minutes on a water bath and then allowed to stand in a cool place for 48 hours. The phosphotungstates of the bases were then filtered off and washed as described by Van Slyke.

The entire precipitate was used for a Kjeldahl nitrogen determination.

The results of the analyses may be found in the accompanying table.

When zein was hydrolyzed alone the solution did not become intensely black but when tryptophane had been added the black color developed sooner and was much more intense. Besides the black color there was a distinct reddish color in the hydrolysate from the zein + tryptophane, the filtrate from the humin, as well as from the phosphotungstate precipitate, being likewise darker in the case of added tryptophane.

In the ammonia determinations when tryptophane had been added in large amount there was an intense frothing toward the end of the distillation, after the alcohol had largely distilled off. In no other material have we obtained such a result.

[1] "The Vegetable Proteins," 1912, Longmans, Green & Co., N. Y.

[2] As described in "Handb. d. Biochem. Arbeitsmethoden," Vol. II, pp. 487–8.

[3] As outlined in "Handb. d. Biochem. Arbeitsmethoden," Vol. II, p. 505.

[4] *Loc. cit.*

TABLE I.—SHOWING THE WEIGHTS OF NITROGEN AND THE PERCENTAGE OF TOTAL NITROGEN OBTAINED IN THE VARIOUS FRACTIONS FROM THE DIFFERENT EXPERIMENTS.

Material hydrolyzed.	Ammonia N.	Humin N.	Basic N.	Nonbasic N.	Total N found.	Total N calc.
1 g. zein[1]	0.03164 g.	0.0007 g.	0.00489 g.	0.1184 g.	0.1546 g.	0.1525 g.
	20.75%	0.46%	3.21%	77.66%	102.08%	
1 g. zein + 0.25 g. tryptophane	0.0290 g.	0.0070 g.	0.01603 g.	0.1348 g. (calc.)	....	0.1868 g.
	15.52%	3.75%	8.58%	72.15% (calc.)	....	
1 g. zein + 0.125 g. tryptophane	0.0316 g.	0.0077 g.	0.0056 g.	0.1241 g.	0.1690 g.	0.1696 g.
	18.63%	4.54%	3.30%	73.14%	99.61%	
1 g. zein + 0.2312 g. histidine-dihydrochloride	0.0316 g.	0.0010 g.	0.0497 g.	0.1128 g. (calc.)	....	0.1951 g.
	16.20%	0.51%	25.48%	57.81% (calc.)	....	
0.5 g. zein + 0.5 g. dextrose	0.0154 g.	0.0014 g.	....	....	....	0.0762 g.
	20.20%	1.84%	....	....	....	
0.5 g. zein + 0.5 g. dextrose + 0.125 g. tryptophane	0.0158 g.	0.0154 g.	....	....	....	0.0933 g.
	16.93%	16.50%	....	....	....	
0.125 g. tryptophane + 0.5 g. dextrose	None	0.0148 g.	....	....	....	0.0171 g.
	....	86.56%	....	....	....	
Approx. 0.1 g. tryptophane	None	None	....	....	....	0.0137 g.
1 g. gliadin	0.0451 g.	0.0010 g.	0.0071 g.	0.1168 g. (calc.)	....	0.1700 g.
	26.54%	0.59%	4.17%	68.73% (calc.)	....	
1 g. gliadin + 0.25 g. dextrose	0.0454 g.	0.0016 g.	0.0068 g.	0.1162 g. (calc.)	....	0.1700 g.
	26.71%	0.94%	4.00%	68.37% (calc.)	....	
1 g. gliadin + 2 g. dextrose	0.0450 g.	0.0039 g.	0.0063 g.	0.1148 g. (calc.)	....	0.1700 g.
	26.47%	2.30%	3.70%	67.53% (calc.)	....	

[1] Osborne gives the nitrogen partition of zein as ammonia N 18.4%, humin N 0.99%, basic N 3.0%, nonbasic N 77.5%. The amount of humin nitrogen varied in Osborne's determinations from 0.31% to 1.48% in different determinations. See Osborne and Harris, THIS JOURNAL, **25**, 323 (1903). Our higher percentage of ammonia nitrogen is probably due to a longer hydrolysis.

Discussion.

It will be seen from the table that Van Slyke's conclusion that tryptophane nitrogen is not transformed into humin nitrogen holds true only when tryptophane is boiled alone. When, however, tryptophane is added to a protein a very considerable portion of the tryptophane nitrogen passes into the humin fraction (approx. 0.0070 g. when tryptophane is added to 1 g. of zein). The remainder of the tryptophane nitrogen is distributed between the bases and the filtrate from the bases. In no case does added tryptophane cause an increase in the ammonia nitrogen.

From Experiments 2 and 3 it will be seen that the formation of humin from tryptophane had reached a maximum when 0.125 g. of tryptophane had been added, since no additional humin nitrogen was obtained when 0.25 g. of the amino acid was added. This observation shows, as does the fact that no humin is formed from the pure tryptophane, that the reaction involves not only tryptophane but also some other product of hydrolysis. We therefore boiled dextrose + tryptophane, zein + dextrose and zein + dextrose + tryptophane and found that when tryptophane was heated in the presence of a carbohydrate practically 90% of the tryptophane nitrogen remained in the humin fraction. It seems very possible that with a slightly longer hydrolysis, or with the altering of some of the conditions of hydrolysis that the reaction might have been made quantitative. It is, however, nearly enough quantitative for our present purpose, and we did not have sufficient material to work out the ideal conditions of the reaction.

It is well known that when carbohydrates are boiled with mineral acids a small amount of furfural is formed and it seems highly probable that the reaction involved in humin formation is a condensation of tryptophane with an aldehyde. Miss Homer[1] has prepared condensation products of tryptophane with various aldehydes and states:

"Indol derivatives by virtue of the —NH group in the nucleus will react with formaldehyde and trioxymethylene *in the presence of a condensing agent* to form substances of intense color and marked insolubility in ordinary solvents other than concentrated mineral acids."

When, however, the condensation takes place on the aliphatic $-NH_2$ group, colorless, crystalline compounds are formed.

From our experiments with zein + dextrose it appears certain that the humin nitrogen is not a result of an absorption phenomenon. The hydrolysate in this instance was jet-black and full of suspended flecs of solid humin formed by the decomposition of the carbohydrate, while the humin nitrogen was but slightly in excess of the experimental error, considering the relatively large amount of humin and lime to be washed free from nitrogen.

The fact that the hydrolysis of zein does give rise to some humin, and

[1] *Biochem. J.*, 7, 101–15 (1913).

that there is a slight increase in the humin nitrogen due to the addition of glucose may, however, be due to the presence of tryptophane in the zein molecule in such small quantity that it is difficult to detect with the color tests. For a long time gliadin was supposed to contain no lysine, but Van Slyke[1] found it to be present in this protein, and his findings were later confirmed by Osborne and Leavenworth.[2]

Osborne and Harris[3] state:

"Whether any of the above proteins wholly lack tryptophane could not be determined, as we were able to get a very slight reaction with a relatively large quantity of zein by cautiously adding the sulfuric acid up to one-half the volume of the glyoxylic acid. The color thus produced was, at the most, very slight and transitory."

Mann[4] in a discussion of the humin formed from carbohydrates states:

"If in addition to the carbohydrate, ammonia or other nitrogenous substances are in solution, then the humins combine with the ammonia and thereby become nitrogenous."

We do not believe that this reaction, if it takes place at all, can be any great factor in the formation of the humin nitrogen of protein hydrolysis, for our analyses show a variation in the amount of ammonia nitrogen which is certainly not much greater than the experimental error. Thus we have 0.0154 g. of ammonia *N* from 0.5 g. of zein hydrolyzed in the presence of 0.5 g. of dextrose, and 0.0316 g. of ammonia *N* from 1 g. zein when hydrolyzed alone, a difference of only 0.0008 gram *N* calculated on a 1 g. basis. Again there is a loss of only 0.0001 g. ammonia *N* when 1 g. of gliadin was hydrolyzed in the presence of 2 g. of dextrose. In this instance there is an increase in the humin nitrogen and it is of interest to note that this increase comes from the bases and the filtrate from the bases in the ratio of approximately 4:6, almost the identical ratio (38.7:61.3) which Van Slyke[5] finds for the distribution of tryptophane between the bases and the filtrate from the bases.

Experiment 4 indicates that histidine has no part in the formation of humin nitrogen, since there was no increase in color over that produced when the zein was hydrolyzed alone, and there was no significant increase in the humin nitrogen, the histidine nitrogen being quantitatively recovered in the bases.

We believe, therefore, that we have shown that, in a large measure at least, the humin nitrogen represents a portion of the tryptophane nitrogen, and that if sufficient carbohydrate be present the humin nitrogen can be regarded as an almost quantitative determination of the tryptophane nitrogen. What reaction takes place when no carbohydrate is present

[1] *J. Biol. Chem.*, **10**, 15–55 (1911).

[2] *Ibid.*, **14**, 481–7 (1913).

[3] THIS JOURNAL, **25**, 853–5 (1903).

[4] "Chemistry of the Proteids," p. 88, 1906, MacMillan & Co., N. Y.

[5] *Loc. cit.*

and humin nitrogen is formed, as in Experiments 2 and 3, we are unable to state. Various possibilities present themselves, such as the oxidation of tryptophane itself to indol aldehyde, which would in turn unite with more tryptophane to form a deeply colored, insoluble compound,[1] or possibly traces of some other amino acid may be oxidized to the corresponding aldehyde. Isham and Vail[2] have recently shown that ether is readily oxidized to aldehyde by atmospheric oxygen at 110°. A contamination of the protein with a small amount of ether, or of the aldehyde formed from the ether during extraction, would account for this humin formation, if tryptophane were present. We regret that lack of material prevented our trying further experiments in this direction.

Summary.

1. We have shown that in all probability the humin nitrogen of protein hydrolysis has its origin in the tryptophane nucleus.

2. When tryptophane is boiled with mineral acids in pure solution no humin is formed, but when tryptophane is added to a protein, or when carbohydrates are present, an abundance of humin is formed. This humin contains nitrogen which can belong to no amino acid other than tryptophane.

3. The reaction involved in humin formation is probably the condensation of an aldehyde with the —NH group of the tryptophane nucleus.

4. When an abundance of carbohydrate is present nearly 90% of the tryptophane nitrogen remains in the humin nitrogen fraction.

It is suggested that this property be utilized to determine the approximate quantity of tryptophane in proteins.

5. The addition of histidine causes no increase of humin nitrogen, the histidine being quantitatively recovered in the bases. Histidine, therefore, can be eliminated as a factor in the formation of humin nitrogen.

6. Adsorption of ammonia by non-nitrogenous humins formed from carbohydrates is not an important factor in the formation of humin nitrogen.

7. These findings allow us to assign a distinct value to humin nitrogen determinations.

ST. PAUL, MINN.

NOTE.

On Rapid Organic Combustions.—The use of cerium dioxide as contact substance for rapid organic combustions, in place of the more expensive platinum of Dennstedt, has recently been recommended.[3] In trying out the method in this laboratory, it has been found that it is not entirely

[1] Homer, *Biochem. J.*, **7**, 116 (1913).

[2] THIS JOURNAL, **37**, 902 (1915).

[3] Julius Bekk, *Ber.*, **46**, 2574 (1913).