Summary.

1. Phenylarsine has been condensed with butyraldehyde and benzaldehyde to give compounds consisting of two moles of aldehyde and one of arsine and probably having the following structure, $C_6H_5As(CHOH)_2R$.

2. The condensation products are stable toward dilute acid, alkali and water.

URBANA, ILLINOIS.

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

THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS. VI. THE EFFECT OF ACID HYDROLYSIS UPON TRYPTOPHANE.[1]

BY GEORGE E. HOLM AND ROSS AIKEN GORTNER.

Received Aug. 28, 1920.

In previous articles of this series[2] we have shown "that the formation of black acid insoluble humin in a normal protein hydrolysate is dependent upon the presence of tryptophane in the protein molecule." While tryptophane reacts readily with aldehydes to give a black insoluble product, it also undergoes some unknown reaction even when allowed to stand in an acid solution. This was noted by Abderhalden[3] who found that the mother liquor from a tryptophane preparation, which had been allowed to stand for a long time, slowly darkened, lost its ability to absorb bromine, gave no glyoxylic test, and finally deposited a dark brown product, soluble in acids and alkalies, and which when heated gave a strong indole odor.

Harries and Langheld[4] studied the effect of ozone upon hydrolytic products of proteins and found that tryptophane treated with ozone colored very dark, gave no glyoxylic reaction, reduced Fehling's solution in the cold, was precipitated by barium hydroxide, lead acetate, basic lead acetate, and liberated ammonia when treated with sodium hydroxide.

Van Slyke states[5] "tryptophane is known to be precipitated partially by phosphotungstic acid even from fairly dilute solution. When it is boiled with mineral acids, however, it is, to a large extent, at least, destroyed, the nature and fate of the products being unknown." In order to ascertain the behavior of tryptophane under the conditions of protein

[1] Published with the approval of the Director as Paper No. 208, Journal Series of the Minnesota Agricultural Experiment Station. Presented before the Biological Division of the American Chemical Society at the Spring Meeting, St. Louis, April 12–16, 1920.

[2] Gortner and Blish, THIS JOURNAL, 37, 1630–36 (1915); Gortner, *J. Biol. Chem.*, 26, 177–204 (1916); Gortner and Holm, THIS JOURNAL, 39, 2477–2501 (1917); 42, 821–827 (1920); Holm and Gortner, *ibid.*, 42, 632–40 (1920).

[3] Abderhalden, *Z. physiol. Chem.*, 78, 159–160 (1912).

[4] Harries and Langheld, *ibid.*, 51, 371–383 (1907).

[5] Van Slyke, *J. Biol. Chem.*, 10, 39 (1911).

hydrolysis he boiled pure tryptophane for 12 hours with 20% hydrochloric acid and studied the solution with regard to melanin (humin nitrogen), ammonia, amino nitrogen and total nitrogen, and also with regard to the amounts precipitated by phosphotungstic acid. He draws the following conclusions. 1. Tryptophane is responsible for none of the nitrogen estimated as ammonia, arginine or melanin. 2. Boiling with 20% hydrochloric acid does not alter the ratio 2 : 1 of total nitrogen to amino nitrogen in tryptophane. 3. It appears improbable that tryptophane affects the composition of the phosphotungstic acid precipitate under the usual conditions of analysis, but it is advisable, in the latter, as a precaution, to test a few drops of the solution of the bases for tryptophane.

Homer[1] later studied the effect of acid hydrolysis upon tryptophane and found that after 42 hours' hydrolysis with 25% sulfuric acid almost a complete recovery of the tryptophane was possible, but under the same conditions in a 21–24 hours' hydrolysis and in the presence of ferrous sulfate there was pigmentation and no recovery of tryptophane. She therefore, concludes that hydrolysis changes tryptophane to a compound which absorbs bromine, but does not precipitate with mercuric sulfate.

Johns and Jones[2] in studying the quantitative estimation of tyrosine by use of the Folin-Denis phenol reagent state "it is well known that tryptophane is decomposed by acid hydrolysis" and "the tryptophane was completely decomposed and its decomposition products gave no blue color with the reagent of Folin and Denis." The evidence which they submit in substantiation of the latter statement we have recently shown[3] to be in error, due to the fact that they decolorized their hydrolysate with "Norit" and that carbon adsorbs tyrosine, tryptophane and tryptophane decomposition products.

In the present study our interest is confined primarily to the "acid insoluble" and "acid soluble" humins formed on protein hydrolysis. Our previous studies[4] have shown that aldehydes, when present during the acid hydrolysis of a protein greatly increase the amount of acid-insoluble humin which may be formed, the amount of acid-insoluble humin rapidly increasing to a maximum, at which point the tryptophane is entirely converted into acid-insoluble humin. When the hydrolysis is conducted in the presence of an optimum amount of formaldehyde the nitrogen present in the acid-insoluble humin may be taken as a quantitative measure of the tryptophane nitrogen of the protein. Certain of our experiments have been taken as evidence that proteins contain some unknown component which reacts with tryptophane to form the small

[1] Homer, *J. Biol. Chem.*, **22**, 382–85 (1915).

[2] Johns and Jones, *ibid.*, **36**, 319–322 (1918).

[3] Gortner and Holm, THIS JOURNAL, **42**, 1678–1692 (1920).

[4] *Loc. cit.*

amount of insoluble humin which is present in a normal protein hydrolysate.[1] We therefore, wished to ascertain if the tryptophane molecule contributes to the "acid insoluble" or "acid" soluble humins when no aldehydes or other reacting compounds are present.

This paper reports the results of a study of the effect of prolonged boiling with 20% hydrochloric acid upon the tryptophane molecule, with especial reference to the nitrogen distribution.

Experimental.

The Method.—Five hundred milligrams of tryptophane was added to exactly 100 cc. of 20% hydrochloric acid. An aliquot of 2 cc. was then removed with a pipet and amino nitrogen determined upon this sample by the Van Slyke method. One cc. was also removed, diluted to 25 cc. volume, and a colorimetric determination of the tryptophane content made upon 5 cc. (representing one mg. of tryptophane), using the phenol reagent of Folin and Denis.[2,3] The flask was then weighed, fitted with, a reflux condenser and heated to boiling upon a sand-bath. At intervals of 12, 24, 36, 48, 96 and 144 hours, the flask was allowed to cool and was weighed. In case of a loss of weight, 20% hydrochloric acid was added at each interval to make up exactly for the acid lost by evaporation thereby insuring correct aliquots in each sample taken. The samples were taken at intervals as designated until the boiling had continued for 144 hours. The contents were then filtered, the residue washed thoroughly and nitrogen determined upon the residue by the Kjeldahl method. The filtrate was evaporated to dryness *in vacuo* and "soluble humin" and "ammonia" determined in the usual manner. The filtrate from these determinations was acidified, concentrated to about 35 cc., and precipitated by phosphotungstic acid in the usual manner. After a separation of "filtrates" and "bases" had been made, both "amino" and "total nitrogen" determinations were made upon each fraction.

This method enabled us to follow the rate of deaminization and also gave the amounts of tryptophane converted into the black "insoluble humin" and "acid-soluble humin." The amount of "acid-insoluble humin" formed is also indicated by the decrease in the tryptophane as

[1] For our evidence on this point, see Holm and Gortner, THIS JOURNAL, **42**, 632–40 (1920).

[2] *J. Biol. Chem.*, **12**, 239–43 (1912).

[3] We have recently (THIS JOURNAL, **42**, 1678–1692 (1920)), studied the colorimetric values obtained by adding the phenol reagent to tryptophane solutions, and have shown that the reagent cannot be used on protein hydrolysates to obtain reliable data because of the fact that more than one reactive compound may be present. Our criticisms of the use of this reagent for quantitative work do not apply in the present instance, because all of the color developed in these solutions can be definitely ascribed to the indole nucleus.

determined colorimetrically. The effect of acid hydrolysis upon the precipitability of tryptophane by phosphotungstic acid is also shown.

The Experimental Data.—The color changes observed in the solution were as follows, before the boiling began the solution of the tryptophane in the acid was perfectly colorless; at the end of 12 hours' boiling the solution was transparent but dark red-brown; at the end of 24 hours' boiling the solution was a deep brownish-black and was no longer transparent (depth of liquid about 4 cm.); while the formation of dark, amorphous particles floating in the liquid was first noted after 48 hours' boiling.

The changes in amino nitrogen due to the prolonged boiling with hydrochloric acid are shown graphically in the upper curve of Fig. 1. This curve shows that boiling with 20% hydrochloric acid causes a gradual decrease in amino nitrogen, more rapid at first than during later periods.

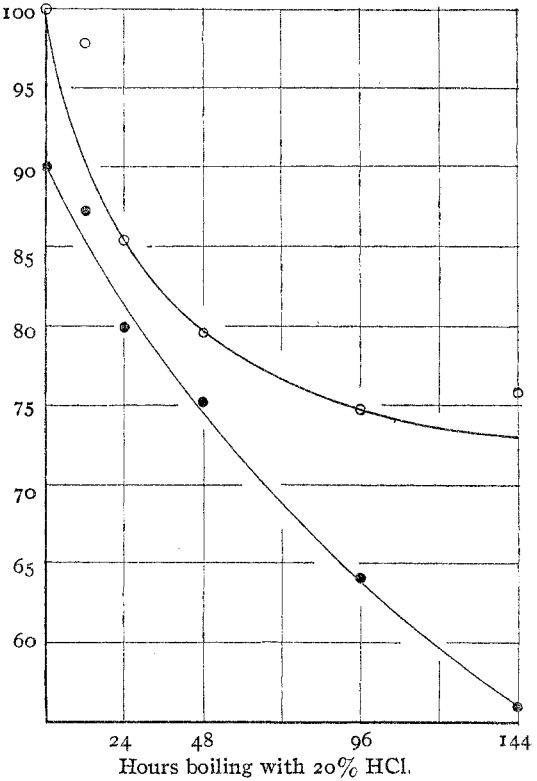

Fig. 1.—Showing (upper curve) the decrease in amino nitrogen (deaminization) in percentage of original amino nitrogen, and (lower curve) the decrease in color values of a tryptophane solution with increasing length of boiling with 20% hydrochloric acid.

A colorimetric determination at each interval serves to indicate the amount of tryptophane which has been destroyed or the amount or portion of the indole nucleus in which the α-position[1] has so been changed or combined that no oxidation is possible. It may indicate even to a greater extent than humin formation or deaminization the total changes in the indole nucleus. The lower curve in Fig. 1 shows the colorimetric values at the different

[1] That it is the α-position of the indole nucleus of tryptophane which reacts with the phenol reagent is indicated by the fact that α,β-di-phenyl indole did not produce the blue color when added to the phenol reagent. Of course, the failure to produce the blue color with the diphenyl compound may have been due to the insolubility of the indole.

periods, in terms of tyrosine color equivalents (tyrosine, one mg. in 100 cc. of final volume being used as a standard).

At the end of 144 hours' hydrolysis 72 cc. of the solution remained which represented 49.39 mg. of nitrogen upon which the separations of the different fractions were made as has already been described.

Table I (Sample 1) shows the distribution of nitrogen in each fraction.

TABLE I.—THE DISTRIBUTION OF NITROGEN IN TWO SAMPLES OF TRYPTOPHANE BOILED FOR 144 HOURS WITH 20% HYDROCHLORIC ACID.

	Sample I.		Sample II.			
Fraction.	Nitrogen. Mgr.	Fraction of total nitrogen. %.	Nitrogen. Mgr.	Fraction of total nitrogen. %.	Tryptophane as tyrosine equivalents (colorimetric). Mgr.	Tryptophane actually present (calc. from N.) Mgr.
Total	49.39	.....	34.30	.....	.....	.....
"Acid-insoluble" humin N	4.14	8.38	1.05	3.06	.....	.....
Ammonia N	12.62	25.55	6.42	18.71	.....	.....
"Acid-soluble" humin N	19.78	40.04	9.80	28.57	7.40	71.43
"Phosphotungstic acid humin" N	0.45	0.91	1.25	3.64	.....	.....
Total N in "bases"	5.27	10.66	11.90	34.69	70.00	86.74
Amino N in "bases"	(2.09)	.....	5.25	.....	.....	.....
Total N in "filt. from bases."	6.32	12.78	5.00	14.57	20.45	36.44
Amino N in "filt. from bases"	(2.80)	.....	(3.23)(?)	.....	.....	.....
Total recovered	48.58	98.32	35.42	103.24		

In order to verify these results and also to investigate more closely the nature of the "soluble humin" in its relation to color formation with the phenol reagent, we boiled 250 mg. of tryptophane for 144 hours with 20% hydrochloric acid. At the end of that time the solution was filtered, concentrated and the separations made as in the earlier experiment with the following exceptions: the soluble humin was filtered off and, after thorough washing, was dissolved in dil. hydrochloric acid and made up to 100 cc. volume. Both a total nitrogen determination and a colorimetric determination were made upon this dark solution. Colorimetric determinations were also made upon the "bases" and "filtrates from the bases." Table I (Sample 2) shows the results which were obtained.

Discussion.

While the figures for the nitrogen distribution as shown in Table I are not in exact agreement, they indicate clearly that the nitrogen distribution of tryptophane is altered very markedly by boiling the amino acid with 20% hydrochloric acid. Moreover, the fact that the 2 samples were boiled with 20% hydrochloric acid for equal periods of time and yet exhibit a difference in nitrogen distribution, indicates the ease with which the hydrolysate of a protein containing tryptophane may be altered by slightly varying the conditions of hydrolysis.

We are undecided as to whether or not some of the changes which take place are due to oxidizing reactions. The work of Abderhalden and others would tend to indicate this. Our experimental conditions were such that this could easily be the case, for all hydrolysates were freely open to air. We have also stated[1] that "the vigor of boiling seems to influence both ammonia and insoluble humin formation." This difference (since the 2 samples were not boiled at the same time) might, and we believe does, account for the differences in the nitrogen distribution. However, as stated above, we are interested in possible changes in the nitrogen distribution of tryptophane under conditions comparable with those obtained in the acid hydrolysis of a protein over extended periods of time, whether they be due to oxidizing effects, or directly due to the effect of the acid, or to both.

The data for the acid-insoluble humin show that no detectable amount was formed during the first 24 hours. At the end of that time a few black amorphous particles could be seen in the dark reddish-brown liquid. At 48 hours there were a great number to be seen and these increased in amount with prolonged boiling. Our figures show that, at the end of 144 hours' boiling, from 4 to 8% of the tryptophane nitrogen appeared in this fraction. The figures for the ammonia fraction substantiate the evidence that we have already put forth in a former publication[2] where we state "a part of this (ammonia fraction) must be due to the breaking up of tryptophane." Fig. 1 indicates that deaminization proceeds quite rapidly at first as is the case in a protein hydrolysate.[3] This rate diminishes as the concentration of unaltered tryptophane in the solution decreases. The concordant results obtained for ammonia in each of the 2 hydrolysates appear to indicate that acid concentration and length of hydrolysis are alone responsible for the deaminization. The "soluble humin" nitrogen in these experiments is probably an intermediate product in "acid-insoluble" humin formation. No tyrosine is present and consequently tyrosine could not contribute to this fraction. This would appear to conflict with some of our earlier conclusions and it may be possible that some of the nitrogen in the soluble humin fraction of a normal protein hydrolysate is derived from tryptophane. We have already shown that traces of the tryptophane may appear in this fraction.[4] However, the experiments referred to show conclusively that only a small fraction of the tryptophane is converted into "soluble" humin in a 24-hour hydrolysate (0.20 mg. of soluble humin *N* from 100 mg. of tryptophane). In the same experiments no "insoluble humin" *N* was found.

[1] Gortner and Holm, THIS JOURNAL, **39**, 2738 (1917).

[2] *Ibid.*, **39**, 2742 (1917).

[3] Cf. Fig. 1, *ibid.*, **39**, 2736–2745 (1917).

[4] Gortner and Holm, *ibid.*, **42**, 821–827 (1920).

A colorimetric determination upon the soluble humin fraction also shows that it still retains some of the properties of the tryptophane molecule; at least it gives color with the phenol reagent. A separation of the clear filtrate from the soluble humin into a "basic" and "non-basic" fraction by means of phosphotungstic acid indicates, as we have formerly pointed out, that there is no clear-cut separation of tryptophane into the "bases" or into the "filtrate from the bases." The result in case of a Van Slyke analysis will be that, if tryptophane is present, all of the non-amino nitrogen of a filtrate cannot with certainty be calculated as proline nitrogen and, in fact, might with equal certainty be calculated as non-amino tryptophane nitrogen. The bases, on the other hand, will be in error just to the extent of precipitated tryptophane which, as we have already suggested, will appear and be calculated largely as histidine.

The colorimetric determinations indicate that the "bases" and the "filtrate from the bases" contain substances, which, if they are not unchanged tryptophane, are, at any rate, tryptophane which has been only slightly altered. The color produced by solutions of the bases is approximately equal (82% of a tyrosine equivalent) to that given by unaltered tryptophane (85–90% of a tyrosine equivalent), while the substances in the filtrate from the bases gives a somewhat lower color value, indicating a somewhat greater alteration.

We intend in the near future to make a more exact study of some of the factors involved in tryptophane decomposition.

Summary.

We have studied certain changes produced by boiling tryptophane with 20% hydrochloric acid for various lengths of time and have reached the following conclusions.

1. Tryptophane is slowly altered and parts of the molecule are broken down by long acid-hydrolysis.

2. Tryptophane, in the absence of aldehydes or other reactive compounds, contributes but an insignificant fraction of its nitrogen to the "acid-insoluble" humin. A much larger amount of the tryptophane appears in the "soluble humin" after 144 hours' boiling with acid. Since, however, a normal protein hydrolysis rarely requires more than 24 hours' boiling, it appears extremely improbable that the "total" humin of such a hydrolysate is derived from tryptophane without the intervention of some other reactive compound, which we have postulated in our earlier papers to be of the nature of an aldehyde.

3. Tryptophane is relatively easily deaminized by boiling with 20% hydrochloric acid. Probably some of the ammonia of a normal protein hydrolysate is derived from tryptophane instead of being entirely derived from amide groupings.

4. When tryptophane has been boiled with 20% hydrochloric acid the

distribution of the nitrogen is such that errors may be introduced into both the "basic" nitrogen and the "non-basic nitrogen" fractions of a Van Slyke determination.

ST. PAUL, MINN.

[COMMUNICATION FROM THE CHEMICAL LABORATORY OF JOHNS HOPKINS UNIVERSITY.]

A SULFIDE ACID OR THE BUTYL ETHER OF THIOGLYCOLIC ACID.

BY YOSHISUKE UYEDA AND E. EMMET REID.

Received August 30, 1920.

Introduction.

Sulfur, when present in an organic compound in the sulfide condition, $= CH—S—CH =$, may have comparatively slight influence on the chemical and physical properties of the compound while in some cases it gives rise to peculiar properties, as in β,β'-dichloro-ethyl sulfide. In this remarkable compound the presence of the sulfur atom renders the chlorine atoms very reactive.

It seemed of interest to prepare other compounds containing a sulfide grouping along with some other group with a distinctive function. The present investigation is a study of the acid, $CH_3CH_2CH_2CH_2 — S — CH_2COOH$, and some of its derivatives. Thiophene has nearly the same boiling point as benzene but a considerably higher density and the same may be said of ethyl sulfide as compared with hexane. In physical properties the above acid and its esters should resemble caprylic acid, $C_8H_{16}O_2$, and its esters to which they are nearest in molecular weight. This has been found to be true in a general way, though the acid boils at 282.2°, which is 45° higher than caprylic.

The sulfide acid forms salts readily, the sodium salt being very soluble in water while the barium and calcium salts are only moderately so. When mineral acids are added to water soltuions of its salts the acid separates readily as a heavy oil which would easily be mistaken for one of the fatty acids of about its molecular weight, even to the odor. It is very slightly soluble in water and distils without decomposition. The odors of its esters are somewhat like those of the higher aliphatic esters.

The acid is readily prepared by the action of sodium chloro-acetate on the sodium salt of butyl mercaptan in water solution. Its esters and many of its salts are easily obtained by the usual methods. The esters give good results on quantitative saponification as the acid is accurately titrated with phenolphthalein.

The remarkable fact about β,β'-dichloro-ethyl sulfide is that the influence of the sulfur renders the chlorine atoms quite movable.[1] This

[1] Marshall, *J. Am. Med. Assoc.*, **73**, 684 (1919); *C. A.*, **13**, 2929 (1919).