[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

THIO-AMIDES. VI. A PRELIMINARY STUDY OF SOME AMINO ACID DERIVATIVES CONTAINING SULFUR IN THIO-AMIDE COMBINATION[1]

BY ELIZABETH S. GATEWOOD[2] AND TREAT B. JOHNSON

RECEIVED JULY 19, 1926 PUBLISHED NOVEMBER 5, 1926

The importance of obtaining new chemical data that will enable us to acquire a better knowledge of the nature of sulfur linkages in proteins has been emphasized in the earlier papers of this series. While much has been contributed by previous workers to our knowledge of the characteristic reactions of the thio-amide grouping in various types of organic combinations, it is apparently true that no report of a study of thio-amide derivatives of amino acids or polypeptides has been made outside of this Laboratory. In other words, no contribution to our knowledge of thiopolypeptide chemistry has been made since the discontinuation of our study of this problem in 1912, and the question as to whether the thio-amide group, —CS.NH—, functions in protein structure has not been answered.

Harris[3] concludes from the results of his researches on the denaturation of proteins that an undiscovered sulfur linkage apparently exists in ovalbumin, casein and fibrin, and that the production of a grouping that reacts with sodium nitroprusside is an essential feature of the denaturation of these proteins. This investigator has suggested that the well-known nitroprusside reaction, attributed by Arnold to cysteine, may be due to the presence in the natural protein of a thiopolypeptide or similar sulfur grouping which by hydrolysis, or keto-enol transformation, would give rise to an active SH grouping in the resulting protein molecule. For example, egg white is quite non-reactive towards the nitroprusside reagent, but on coagulation by heat or by the action of acetic acid it becomes vividly reactive. If one may judge from the results published by this investigator, it follows that it is very probable that the chemical change accompanying coagulation in some sulfur proteins actually involves the liberation of a reactive grouping of the sulfhydrate type —SH. Harris is the first investigator, so far as we are aware, to suggest the possibility of this nitroprusside reaction being attributable in some cases to a thiopolypeptide linkage.

[1] I. (a) Johnson and Burnham, *J. Biol. Chem.*, **9**, 331 (1911). II. (b) Johnson, *ibid.*, **9**, 439 (1911). III. (c) Johnson and Burnham, *ibid.*, **9**, 449 (1911). IV. (d) Johnson and Burnham, *Am. Chem. J.*, **47**, 232 (1912). V. (e) Johnson, "Synthesis of Autoxidizable Compounds of Biochemical Interest Containing Sulfur in Thiopolypeptide Combination," Organic Chemistry Symposium, Rochester, New York, Dec. 29–31 (1925). Compare Supniewski, *J. Pharmacol.*, **27**, 317 (1926).

[2] Metz Organic Chemistry Research Fellow, 1925–1926.

[3] Harris, *Proc. Roy. Soc.*, **94**, 426 (1923).

$$—CH_2.CS.NH.CH_2— \qquad —CH_2.\underset{\displaystyle SH}{\underset{|}{C}} = N.CH_2—$$

Keto-enol tautomerization

The interest in the chemistry of organic sulfur compounds has been greatly increased by discoveries of workers in the biochemical field. As a result, the biological importance of this element has become increasingly evident in the last few years. Glutathione,[4] an auto-oxidizable constituent of the cell, contains sulfur. It has also been shown that sulfur is an integral part of the insulin molecule and that insulin's physiological activity seems to be dependent upon it.[5] A characteristic sulfur-containing amino acid, which is not identical with cystine, has been isolated from casein, egg albumin[6] and yeast;[7] its structure is not yet known. A thio-sugar of known constitution[8] has also been found in yeast, and according to a recent publication from the laboratory of Levene, vitamin B contains about 4% of sulfur.[9] Very recently, new sulfur compounds of unknown structure have been isolated from blood.[10] Whether any of these naturally occurring substances contains sulfur in thio-amide combination remains to be established by future research. Further evidence that wool contains an unknown sulfur linkage as well as a cystine grouping has been brought forward by Trotman, Trotman and Sutton.[11] Up to the present time, no sulfur linkage of established structure besides cystine has been proved to exist in the protein molecule.

This frequent occurrence of sulfur in both plant and animal organisms makes pertinent and desirable a very thorough study of types of sulfur groupings, other than the sulfhydrate, which may be present in these natural substances. Of these, the characteristic aliphatic thio-amide linkages I, —CS.NH.CH_2—, and II, —CH_2.CS.NH—, seem to be very probable, because they correspond, as has been expressed in our earlier papers, to the prevalent oxygen groupings known to occur in protein structure. Several compounds containing these sulfur linkages have now been prepared and submitted to biological examination. The toxicological properties of selected combinations, which have been determined in the laboratory of Dr. Abel,[12] will be reported elsewhere.

The first thiopolypeptide combination that we prepared in this Labora-

[4] Hopkins, *Biochem. J.*, **15**, 286 (1921).

[5] Abel and Geiling, *J. Pharmacol.*, **25**, 423 (1925).

[6] Mueller, *J. Biol. Chem.*, **56**, 157 (1923).

[7] Odake, *Biochem. Z.*, **161**, 446 (1925).

[8] Susuki, Odake and Mori, *ibid.*, **154**, 278 (1924).

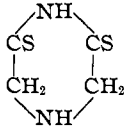
[9] Levene and van der Hoeven, *Science*, **62**, 594 (1925).

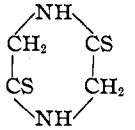
[10] Hunter and Eagles, *J. Biol. Chem.*, **65**, 623 (1925). Benedict, Newton and Behre, *ibid.*, **67**, 267 (1926).

[11] Trotman, Trotman and Sutton, *J. Soc. Chem. Ind.*, **44**, 1115 (1925).

[12] Johns Hopkins University.

tory was 2,5-dithiopiperazine,[1e] IV. It became necessary for us to obtain this compound again for biological research and consequently we have repeated the work of Johnson and Burnham. By introducing some changes in experimental technique we have been able to obtain a much purer product than was originally described, and of required standard for an accurate toxicological examination. Furthermore it was pertinent to confirm the original conclusions regarding its structure. Because of the results of new work now in progress on the action of hydrogen sulfide on other nitriles of amino acids and on nitriles of imino acids, it is possible that the dithiopiperazine described by Johnson and Burnham is to be represented structurally as a 2,6-dithiopiperazine III rather than as a 2,5-dithiopiperazine IV. Formula III would not represent the structure of a cyclic


III


IV

thiopolypeptide derivative of an amino acid. We have now confirmed our original conclusions that Formula IV represents the correct constitution of this substance. On hydrolysis with alkalies or acids it is converted into glycine with evolution of hydrogen sulfide. Ammonia is not a product of the reaction. So far as the writers are aware, the cycle III has not been synthesized. On hydrolysis it would be converted into iminodiacetic acid, $HN(CH_2COOH)_2$, with evolution of ammonia and hydrogen sulfide.

The esters of four acyl derivatives of ethyl amino-acetate have been incorporated in our research. These have been digested with phosphorus pentasulfide in benzene solution to determine, if possible, how the various acyl groups effect the direct replacement of oxygen by sulfur in the —$CONHCH_2$— grouping. The compounds investigated were ethyl hippurate, ethyl acetylamino-acetate, ethyl phenylacetylamino-acetate and ethyl glycylglycate, $H_2N.CH_2CONHCH_2COOC_2H_5$.

Of these four esters, ethyl hippurate was the only one from which we were able to prepare the corresponding sulfur derivative by interaction with phosphorus pentasulfide. It reacted smoothly according to the following scheme.

$$\underset{\text{V}}{C_6H_5CONH.CH_2COOC_2H_5} + P_2S_5 \longrightarrow \underset{\text{VI}}{C_6H_5CSNHCH_2COOC_2H_5}$$

$$\underset{\text{VII}}{C_6H_5CSNHCH_2COOH}$$

By careful saponification of this ester VI we were able to prepare thionhippuric acid VII, which is the first thionacyl amino acid to be described in the chemical literature. Both this sulfur acid VII, and its ester VI were obtained in a crystalline condition. Ethyl acetylamino-acetate

apparently reacts with phosphorus pentasulfide, judging from the prompt development of a yellow color, analogous to the hippuric ester reaction, but from the resulting deep yellow oil no solid product could be isolated. The oil decomposed on distillation and also when attempts were made to saponify it. Ethyl phenylacetylamino-acetate and ethyl glycylglycate did not react with phosphorus pentasulfide. Phenylaceturic acid was the only product obtained when the reaction product of its ester and pentasulfide was subjected to hydrolysis. The glycylglycate was recovered unaltered in all attempts to replace its oxygen with sulfur.

Experimental Part

2,5-Dithiopiperazine IV.—Twenty g. of finely powdered amino-acetonitrile sulfate[13] was added to 50 cc of concd. aqueous ammonia in 100 cc. of ethyl alcohol. Ammonium sulfate separated at once. The mixture was well cooled by ice and salt and hydrogen sulfide was passed in for four hours. The ammonium sulfate was then filtered off and the clear, light yellow filtrate was left in a vacuum desiccator overnight. The dithiopiperazine separated as an almost colorless, crystalline powder, which was collected and washed with water, alcohol and ether. The yield varied from 2.5 to 3.0 g. It was purified by dissolving small quantities in cold, very dilute alkali and precipitating *at once* with dil. hydrochloric acid. The cyclic thio-amide was obtained in this manner as a colorless powder. The solution of its sodium salt is colorless but turns yellow if allowed to stand even a short time, and then the precipitated dithiopiperazine always has a greenish-gray color, doubtless due to slight decomposition by the alkali. When the product is subjected to hydrolysis, hydrogen sulfide and glycine are the products of the reaction; ammonia is not evolved. The sulfur in this compound is very labile; it can be removed easily by boiling with either dil. sodium carbonate solution or dil. acids, and even by boiling water.

Anal. Calcd. for $C_4H_6N_2S_2$: C, 32.84; H, 4.14; N, 19.16. Found: C, 33.5, 33.4; H, 3.89, 4.08; N, 19.12, 19.19.

After filtering off the dithiopiperazine, the ammoniacal filtrate was neutralized with dil. acetic acid and saturated with sodium chloride. A light brown powder (1.4 g.) separated slowly. It was insoluble in water, alcohol and ether, but highly soluble in very dilute alkali and acid. It did not melt below 280° though it turned black gradually. The compound was quite unstable, especially when exposed to light, and rapidly turned dark brown. As it could not be obtained in a pure condition, it was not analyzed.

Action of Phosphorus Pentasulfide on Esters of Acyl Amino Acids

Ethyl Thionhippurate, $C_6H_5CSNHCH_2COOC_2H_5$.—Hippuric acid was esterified in the usual manner and the ester separated and purified according to the directions of Conrad.[14] From 30 g. of the acid we obtained 20 g. of the pure ester melting at 60°. Twenty g. of the hippurate, vacuum-dried, was dissolved in 200 cc. of dry benzene and the solution was refluxed with 4.5 g. of powdered pentasulfide for two hours. Within five minutes the solution became deep yellow. Most of the benzene was distilled in a vacuum and the remainder allowed to evaporate spontaneously. Sixteen g. of a deep yellow oil remained which solidified on cooling. It was repeatedly extracted with petroleum ether. The ester separated from the latter solvent in long, silky, pale yellow needles. After several extractions, a little unchanged ethyl hippurate was extracted each time

[13] Klages, *Ber.*, **36**, 1511 (1903).

[14] Conrad, *J. prakt. Chem.*, **15**, 246 (1877).

with the thio compound. The ester crystallized in short, colorless needles and the thionhippurate was easily separated from them mechanically. The unchanged ester and the oily residue were refluxed again with phosphorus pentasulfide in benzene solution. The total yield of pure ethyl thionhippurate was 8.0 g.; m. p., 38–40°. It is very soluble in alcohol, ether and benzene, and insoluble in cold water though somewhat soluble in hot water. It cannot be distilled without decomposition even under reduced pressure.

Anal. Calcd. for $C_{11}H_{13}O_2NS$: N, 6.28. Found: 6.36, 6.31.

Thionhippuric Acid, $C_6H_5CSNH.CH_2COOH$.—Two g. of the ethyl ester described above was dissolved in a solution of 1 g. of potassium hydroxide in 20 cc. of ethyl alcohol and the solution allowed to stand at room temperature for four days. The alcohol was then evaporated in a vacuum desiccator and the yellow oil remaining was dissolved in a little water, cooled with ice and acidified with dil. hydrochloric acid. The thionhippuric acid separated in the form of pale yellow crystals, weighing 1.55 g. The acid was recrystallized from hot water; m. p., 148–150°. This acid is stable in cold alkali but loses its sulfur when boiled with acid or alkali solutions.

Anal. Calcd. for $C_9H_9O_2NS$: N, 7.18. Found: 7.16, 7.22.

Action of Phosphorus Pentasulfide on $CH_3CONHCH_2COOC_2H_5$.—This ester was obtained directly from ethyl amino-acetate hydrochloride according to the method of Radenhausen.[15] Nine g. of the pure ester was dissolved in 100 cc. of dry benzene and refluxed with 3 g. of phosphorus pentasulfide for two hours. The solution became yellow in a very short time. After the benzene had been evaporated, 8 g. of a deep yellow oil remained. This did not crystallize and could not be distilled without decomposition. It was very soluble in alcohol, ether, benzene and chloroform and very difficultly soluble in petroleum ether, from which it separated as a yellow oil again even at very low temperatures.

Action of Phosphorus Pentasulfide on $C_6H_5CH_2CONHCH_2COOC_2H_5$.—The nitrile of phenylaceturic acid was prepared from amino-acetonitrile and phenylacetyl chloride according to the method of Klages and Haack.[16] This nitrile was then converted into the ester by digestion with alcohol acidified with hydrochloric acid. Four g. of the ester was refluxed in benzene with 1.5 g. of phosphorus pentasulfide for one hour. The solution remaining colorless, more phosphorus pentasulfide was added and the solution refluxed for another hour. A pale yellow oil remained after the solvent had been evaporated. It did not solidify and on treatment with alcoholic potassium hydroxide was converted into phenaceturic acid.

Action of Phosphorus Pentasulfide on $H_2NCH_2CONHCH_2COOC_2H_5$.—This ester was prepared according to Fischer's directions.[17] No reaction took place after refluxing the ester in benzene with phosphorus pentasulfide for several hours.

Summary

1. 2,5-Dithiopiperazine is the chief product of the reaction between amino-acetonitrile and hydrogen sulfide, as originally shown by Johnson and Burnham.

2. Phosphorus pentasulfide interacts with ethyl hippurate with replacement of oxygen by sulfur in the amide grouping.

3. Ethyl thionhippurate and the free thionhippuric acid were isolated

[15] Radenhausen, *J. prakt. Chem.*, **52**, 437 (1895).

[16] Klages and Haack, *Ber.*, **36**, 1646 (1903).

[17] Fischer and Fourneau, *Ber.*, **34**, 2870 (1901).

in a crystalline condition. This is the first acylthionamino acid to be described in the chemical literature. Acetylthionamino-acetate or its corresponding acid could not be separated.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION FROM THE HAVEMEYER CHEMICAL LABORATORY OF NEW YORK UNIVERSITY]

STUDIES ON UNSATURATED 1,4-DIKETONES. I. SYNTHESIS AND STRUCTURE OF 1,2-DI(2,4,6-TRIMETHYLBENZOYL)-ETHENOL

BY ROBERT E. LUTZ

RECEIVED JULY 20, 1926 PUBLISHED NOVEMBER 5, 1926

An interesting substance assumed to be an hydroxy-dibenzoyl-ethylene, $C_6H_5COC(OH)=CHCOC_6H_5$ (dibenzoyl-ethenol), a tautomer of the hypothetical dibenzoyl-ethanone, $C_6H_5COCOCH_2COC_6H_5$, was described in a previous paper[1] dealing with certain derivatives of dibenzoyl-ethylene; it was prepared by the action of sodium acetate both on dibenzoyl-dibromoethane and on dibenzoyl-acetylene, which was considered to be an intermediate in the reaction. It was extremely difficult to purify the substance and the analyses were unsatisfactory; although a copper salt was prepared, no other crystalline derivatives were obtained from it and therefore the structure was not clearly established. Experiments were undertaken in an attempt to obtain more evidence concerning the structure of this substance and to prepare some derivative possessing somewhat modified properties, which could be more easily studied and in which the system, $ArCOC(OH)=CHCOAr$, could definitely be shown to be present. It has been possible to prepare the dimesityl derivative, di(2,4,6-trimethylbenzoyl)-ethenol, $(CH_3)_3C_6H_2COC(OH)=CHCOC_6H_2(CH_3)_3$, which has been successfully studied. This paper deals primarily with the synthesis and structure of this compound and its ketonic modification, and with the general reaction,

$$ArCOCHBrCHBrCOAr \xrightarrow{CH_3COONa} ArCOC(OH)=CHCOAr \qquad (1)$$

A preliminary study was made of derivatives of di(bromobenzoyl)-ethylene,[2] $BrC_6H_4COCH=CHCOC_6H_4Br$, and a yellow substance was obtained which was difficult to purify but which gave a characteristic copper derivative and yielded satisfactory analyses. The substance was undoubtedly di(bromobenzoyl)-ethenol, $BrC_6H_4COC(OH)=CHCOC_6H_4Br$.

When the mesityl group was substituted for phenyl the desired derivatives proved to be more crystalline in character, more easily purified

[1] Conant and Lutz, THIS JOURNAL, **47**, 881 (1925).

[2] The Friedel and Crafts reaction with aluminum chloride, fumaryl chloride and aryl hydrocarbons has made easily accessible many derivatives of dibenzoyl-ethylene. Conant and Lutz, THIS JOURNAL, **45**, 1303 (1923). See also Ref. 1.