The nature of the strepogenin molecule cannot be established before it is isolated in pure condition. However, certain aspects of its structure are apparent now. Evidence has been presented[4] which would indicate that strepogenin is an amphoteric substance. The failure of chloramine T to inactivate the factor, taken in connection with its lability to acid or alkali, and its occurrence in proteins, make it seem probable that it may be a peptide, rather than an α-amino acid. Its solubility behavior and its reaction toward various chemical reagents are likewise in line with the working hypothesis of the peptide nature for the compound.

Whether strepogenin is or is not a peptide, it nevertheless has a bearing on the structure of proteins. The relatively high content of the factor in very highly purified proteins would seem to argue against its presence as a chance contaminant. Likewise, the fact that pure proteins isolated from pancreas were far richer sources than was pancreas itself[7] points to the conclusion that strepogenin is a part of the protein molecule. Furthermore, slow liberation of the factor during tryptic digestion suggests that this compound is an integral part of the protein rather than an impurity. It would be possible for a protein to contain all the amino acids and yet not possess strepogenin activity, for the amino acids might not be linked together in the proper combination. This is one explanation for the absence of strepogenin from egg white and for the rather widely differing strepogenin contents of proteins of similar amino acid composition.

(7) In this connection, it was of interest to assay autolysates of pancreas, for it was found that a major portion of the strepogenin of this organ could be accounted for in the insulin and proteolytic enzymes normally found therein.

Summary

Tryptic digests of many highly purified proteins were found to be rich sources of the bacterial growth factor strepogenin. Crystalline insulin, crystalline trypsinogen, crystalline trypsin, crystalline chymotrypsins and chymotrypsinogen, crystalline ribonuclease, crystalline tobacco mosaic virus, certain crystalline proteins of yeast, hemoglobin, and casein were excellent or good sources. Dialyzed egg white, salmine, and gelatin were very poor sources. Comparative values for the strepogenin contents of tryptic digests of these proteins were determined. The rate of liberation of strepogenin activity by trypsin from different proteins varied, but the release was maximal for all in twenty hours or less under the conditions studied. Two independent procedures for the preparation of rather active concentrates of the growth factor from casein digest were described. Implications of the findings for the study of protein structure, and for the nutritional value of proteins to bacteria, were indicated briefly.

NEW YORK 12, N. Y. RECEIVED JUNE 25, 1945

[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

Gliotoxin. VI. The Nature of the Sulfur Linkages. Conversion to Desthiogliotoxin[1,2]

BY JAMES D. DUTCHER,[3] JOHN R. JOHNSON AND WILLIAM F. BRUCE[4]

The early chemical investigations of gliotoxin ($C_{13}H_{14}N_2O_4S_2$) revealed that this substance is extremely sensitive to alkalies and is altered rapidly even by mild alkaline reagents such as sodium bicarbonate, sodium sulfite and sodium sulfide. These reagents evidently acted upon labile sulfur linkages in gliotoxin but elucidation of the chemical transformations involved has been hampered by the difficulty of isolating well-defined crystalline intermediate degradation products. One of the structural units of the gliotoxin molecule is probably an amino acid related to cysteine, and the complex character of the action of alkalies on cysteine and cystine is well known.[5]

The optical rotation and the antibiotic activity of gliotoxin are changed profoundly under the influence of alkaline reagents. Weindling[6] observed that the inhibitory action against rhizoctonia was lost rapidly even on standing in a buffered solution at *p*H 7.5, and we have found that the action of sodium bicarbonate, sodium bisulfite, and pyridine, also destroys the typical antibacterial activity.

In a previous paper[7] it was shown that exhaustive hydrolysis of gliotoxin with hot aqueous alkalies yields one of the nitrogen atoms as methylamine and the other as indole-2-carboxylic acid;

(1) A preliminary paper was presented at the Cleveland Meeting of the American Chemical Society, April 1944; see Abstracts of Papers, 107th Meeting, pp. 23-24M.

(2) Fifth paper, THIS JOURNAL, **67**, 423 (1945).

(3) Du Pont post doctorate Fellow; present address, Squibb Institute for Medical Research, New Brunswick, N. J.

(4) Present address: Wyeth Institute of Applied Biochemistry, 900 N. Broad Street, Philadelphia 30, Pa.

(5) Clarke and Inouye, *J. Biol. Chem.*, **89**, 399 (1930); **94**, 541 (1932); Zahnd and Clarke, *ibid.*, **102**, 171 (1933); Fruton and Clarke, *ibid.*, **106**, 667 (1934); see also Gortner and Sinclair, *ibid.*, **83**, 681 (1929).

(6) Weindling, *Phytopathology*, **24**, 1153 (1934).

(7) Bruce, Dutcher, Johnson and Miller, THIS JOURNAL, **66**, 614 (1944).

when the alkaline reaction mixture is acidified 40–60% of the sulfur appears as hydrogen sulfide and some as free sulfur. Oxidizing agents such as permanganate or bromine convert the sulfur of gliotoxin to sulfate; reducing agents such as aluminum amalgam, zinc and hydrochloric acid, or hydriodic acid and phosphorus remove both sulfur atoms quantitatively as hydrogen sulfide. On treatment of an alcoholic solution of gliotoxin with mercuric acetate or silver nitrate only one of the two sulfur atoms is removed. The present paper deals with further studies of the sulfur linkages, particularly with the action of alkalies under mild conditions and with the desulfurization by means of aluminum to form desthiogliotoxin. The new experimental data in conjunction with previous work[2,8] permit a tentative formulation of the entire carbon, nitrogen and sulfur skeleton of the gliotoxin molecule.

When an alcoholic solution of gliotoxin is treated with cold aqueous or alcoholic alkali, there develops immediately an intense yellow-orange color which passes through a maximum and fades slowly to a permanent pale yellow. Titration of an aliquot portion after a few minutes' contact shows that approximately two moles of alkali have been consumed per mole of gliotoxin, and one mole of sulfide liberated. The rapid reaction with alkali is followed by a slow reaction that consumes more alkali, so that a total of about 2.7 moles is consumed after nineteen hours at 25°. When gliotoxin is refluxed with methanolic potassium hydroxide solution, approximately 2.5 moles of alkali are consumed in one hour, and there is no evidence of the formation of methylamine during this hydrolysis, in contrast to the formation of methylamine during hydrolysis with hot baryta solution.[9] After two hours' refluxing nearly three moles of alkali are consumed.

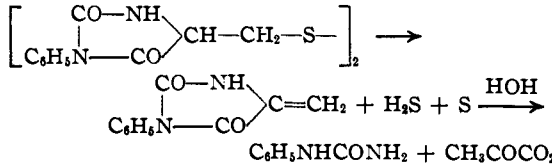
The lability of gliotoxin toward alkali, together with other results to be discussed later, indicates that the sulfur atoms of gliotoxin are present as a disulfide linkage. The characteristic coloration of gliotoxin on treatment with alkali also supports this contention. A general reaction for the alkaline hydrolysis of disulfides is one that leads to the formation of a mercaptide and a sulfenate.[10,11]

$$R{-}S{-}S{-}R + 2KOH \longrightarrow R{-}SK + R{-}S{-}OK + H_2O$$

Investigations of Backer indicate that the formation of the sulfenate is responsible for the characteristic intense yellow coloration, and we observed that the presence of reducing agents such as formaldehyde or thioglycollic acid prevented the development of any coloration when gliotoxin was treated with alkali. Although the above equation may represent the initial hydrolytic reaction, the process usually becomes much more complex owing to oxidation and disproportionation reactions of the unstable sulfenates. The character of the reaction varies widely according to the experimental conditions and the nature of the organic residues; for example, the hydrolysis of benzyl disulfide is accompanied by the formation of some benzoic acid.[10]

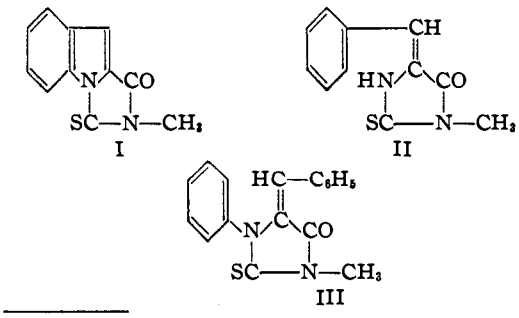
$$5(C_6H_5CH_2{-}S{-})_2 + 12KOH \longrightarrow 9C_6H_5CH_2{-}SK + C_6H_5CO_2K + K_2SO_3 + 7H_2O$$

A typical reaction with cystine derivatives is the elimination of sulfur with a hydrogen from the adjacent carbon; thus, cystine phenylhydantoin reacts with alkali to form the methylene derivative, which undergoes hydrolysis to pyruvic acid and phenylurea.[12]



$$C_6H_5NHCONH_2 + CH_3COCO_2H$$

Several attempts to isolate a well-defined reaction product from the rather mild hydrolysis with alcoholic alkali were unsuccessful. By working with larger amounts of material there was eventually isolated, in yields of 12–15%, a crystalline product, m. p. 188°, which appears to have the molecular formula $C_{11}H_8N_2OS$. The same product could be obtained in somewhat better yields by alkaline hydrolysis of the diacyl derivatives of gliotoxin. This crystalline compound does not seem to be a direct product of hydrolysis but appears to be formed slowly after acidification of the reaction mixture. The C_{11} compound is yellow-orange in color and the ultraviolet absorption spectrum shows three maxima (see Fig. 1). The substance is neutral in reaction and is not hydrolyzed by alkalies; it does not react with acetyl chloride in the presence of pyridine and is indifferent to carbonyl reagents. The available evidence suggests that the C_{11} compound may be a thiohydantoin (I) related to the N-methylamide of indole-2-carboxylic acid. Comparison of the ultraviolet absorption spectrum[13]



(8) Dutcher, Johnson and Bruce, THIS JOURNAL, **66**, 617 (1944).

(9) Marked differences in the rate of deamination of cystine by the action of hot aqueous alkali and alkaline earth hydroxides have been reported by Gortner and Sinclair[4]; deamination is much faster with barium hydroxide than with potassium hydroxide, which in turn acts more rapidly than sodium hydroxide.

(10) Price and Twiss, *J. Chem. Soc.*, **97**, 1175 (1910).

(11) Backer, *Rec. trav. chim.*, **51**, 981 (1932).

(12) Andrews and Andrews, *J. Biol. Chem.*, **102**, 255 (1933).

(13) We are indebted for the absorption spectra to Dr. Nettie Coy of the Squibb Institute for Medical Research, who carried out the spectroscopic work in collaboration with Dr. Dutcher.

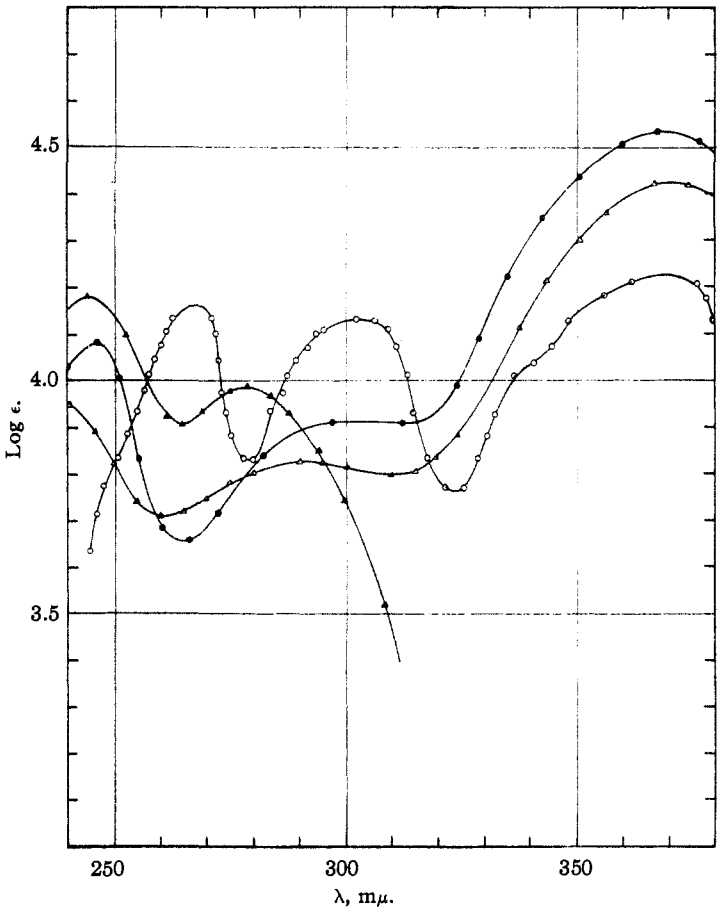


Fig. 1.—Ultraviolet absorption curves: O, compound $C_{11}H_8N_2OS$ from gliotoxin; ●, model thiohydantoin II; △, model thiohydantoin III; ▲. 3-methyl-5-phenyl-2-thiohydantoin. The curve for the methylamide of indole-2-carboxylic acid (not shown) is almost identical with that of indole-2-carboxylic acid shown in Fig. 2. All data are in ethanol solutions.

of the C_{11} compound with the spectra of two model thiohydantoins (II, III) having somewhat similar structural features and with the spectra of several related indole derivatives reveals some similarities. Work is in progress to secure the authentic thiohydantoin of structure I for comparison with the compound from gliotoxin.

The lability of the sulfur atoms of gliotoxin toward alkaline sodium plumbite was investigated by the procedure of Zahnd and Clarke.[5] Under a variety of conditions only 70–78% of the total sulfur was converted to lead sulfide, and no crystalline organic product could be isolated from the reaction mixture after treatment with sodium plumbite. The amount of lead sulfide formed is in close agreement with the findings of Clarke and Inouye,[5] who observed that the disulfide cystine yields 75–80% of its sulfur as lead sulfide. The empirical equation appears to be similar to the reaction of sodium disulfide:

$$4Na_2S_2 + 6PbO + 3H_2O \longrightarrow 6PbS + Na_2S_2O_3 + 6NaOH$$

Perhaps the most convincing evidence for the presence of a disulfide linkage in gliotoxin is afforded by its behavior toward potassium sulfide. It is known that typical organic disulfides are converted by the action of excess potassium sulfide into the potassium salt of the corresponding mercaptan.[14]

$$R—S—S—R + 2K_2S \longrightarrow 2R—SK + K_2S_2$$

When a warm solution of gliotoxin in ethanol is treated with an ethanolic solution of potassium sulfide, a colorless crystalline product begins to separate in a few moments. This substance is optically active, $[\alpha]_D$ *ca.* −60° (in water), and its composition agrees with that of the hydrate of a mercaptide formed by combination of gliotoxin with one mole of potassium sulfide: $C_{13}H_{14}N_2O_4S_3K_2$. Similar deviation from the equation given above has been observed by Goddard and Michaelis[15] and it is likely that the normal reaction does proceed stepwise, with the formation of an intermediate containing the unstable R—S—S—K grouping (a thiosulfenate).

$$R—S—S—R + K_2S \longrightarrow R—S—SK + R—SK$$
$$R—S—SK + K_2S \longrightarrow R—SK + K_2S_2$$

The first step is analogous to the action of alkali to form a sulfenate and is similar to the reaction of certain disulfides with potassium cyanide or sodium sulfite to form an organic thiocyanate or thiosulfate.[16,17]

The potassium mercaptide from gliotoxin was unstable and decomposed gradually even on storage as the dry salt in a vacuum desiccator. An aqueous solution of this product gave a strong nitroprusside reaction and the typical mercaptide precipitates with heavy metals. When the aqueous solution was allowed to stand or was acidified it decomposed with the formation of hydrogen sulfide and an amorphous gum which contained some elemental sulfur. Efforts to stabilize the structure by formation of an S-methyl or S-benzyl derivative led only to products which decomposed readily with the liberation of methyl or benzyl mercaptan. It is significant that the potassium mercaptide on reduction with aluminum gives the same crystalline, optically-active compound, desthiogliotoxin, that is formed from gliotoxin itself by aluminum reduction (see below).

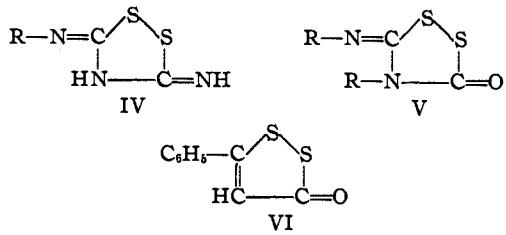
(14) Otto and Rössing, *Ber.*, **19**, 3129 (1886).
(15) Goddard and Michaelis, *J. Biol. Chem.*, **106**, 605 (1934).
(16) Mauthner, *Z. physiol. Chem.*, **78**, 28 (1912); Bodansky, *J. Pharmacol.*, **37**, 463 (1929).
(17) Clarke, *J. Biol. Chem.*, **97**, 235 (1932); Schöberl and Ludwig, *Ber.*, **70**, 1432 (1937).

The reaction of gliotoxin with potassium thioglycollate furnished further evidence for a disulfide linking. In this process the thioglycollate is oxidized to the disulfide and gliotoxin reduced to the sulfhydryl form

$$C_{13}H_{14}N_2O_4S_2 + 2HS{-}CH_2CO_2K \longrightarrow C_{13}H_{14}N_2O_4(SH)_2 + (-S{-}CH_2CO_2K)_2$$

The reduced form of gliotoxin is extremely unstable and splits out hydrogen sulfide very readily. It could not be re-oxidized to gliotoxin nor could crystalline derivatives be obtained. An alcoholic solution of the sulfhydryl form when reduced with amalgamated aluminum, gave the same product (desthiogliotoxin) obtained from the potassium mercaptide and from gliotoxin.

The new experimental evidence indicates clearly the presence of a labile disulfide linkage in gliotoxin. As the molecular formula $C_{13}H_{14}N_2O_4S_2$ and the known degradation products preclude the formulation of a symmetrical disulfide of the cystine type, it is necessary to postulate an unsymmetrical cyclic disulfide structure. Although this type of sulfur linkage does not appear to have been found previously in any naturally-occurring compound, examples of such structures have been described in the literature. Among these are derivatives of 1,2,4-dithiazoledione (IV and V)[18,19] and a 1,2-dithiolone (VI)[20]:



The thiuret type (IV) is cleaved readily by cold alkali to give a product retaining one of the sulfur atoms, whereas 5-phenyl-1,2-dithiolone (VI) yields acetophenone.

A new and significant degradation product of gliotoxin was obtained by reduction in neutral alcoholic solution with amalgamated aluminum at room temperature. The sulfur atoms were eliminated quantitatively as hydrogen sulfide and a colorless crystalline compound, m. p. 243–244°, was isolated from the reaction mixture in 30% yield. This product is optically active, $[\alpha]_D$ −130° (in ethanol), and has the molecular formula $C_{13}H_{16}N_2O_4$. It appears to be formed by replacing the two sulfur atoms by two hydrogen atoms and is therefore designated as desthiogliotoxin. The ultraviolet absorption spectrum of this compound is remarkably similar to that of gliotoxin itself, indicating that no profound structural change has occurred in the desulfurization: both have a maximum of about the same intensity at 270 mμ, and a minimum at 245 mμ (Fig. 2). Their spectra are quite different from that of the previously described hydriodic acid reduction product $C_{13}H_{12}N_2O_2$ (VII)[2,8] and of indole-2-carboxylic acid.

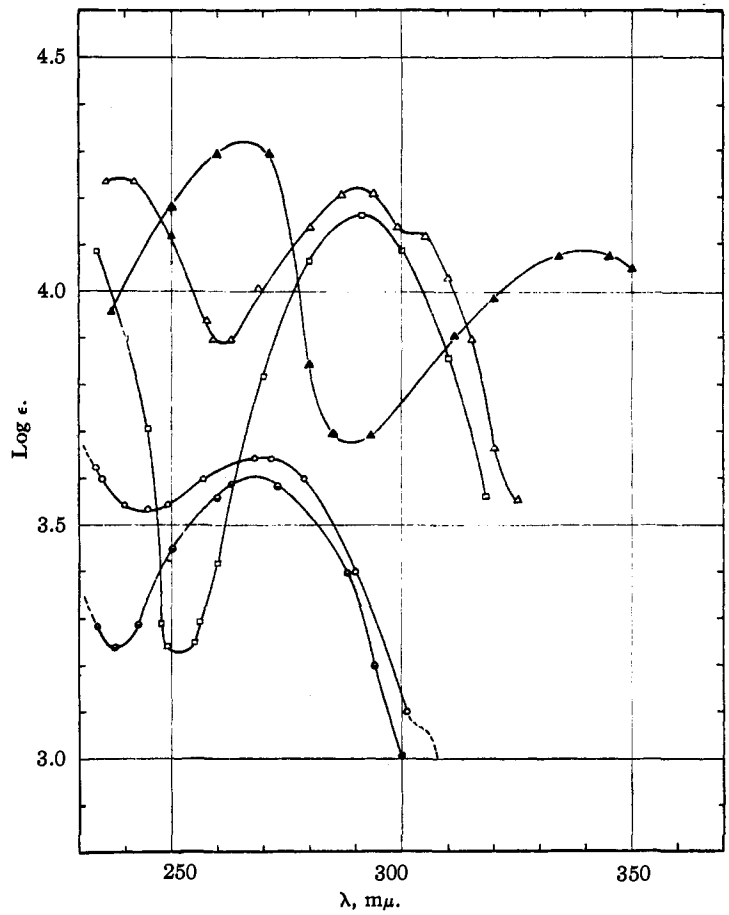


Fig. 2.—Ultraviolet absorption curves: O, gliotoxin; ⊖, desthiogliotoxin; △, hydriodic reduction product, VII; ▲, 2-methyl-α-pyrazindole-1,3,4(2)-trione (selenium degradation product)[2]; □, indole-2-carboxylic acid. All data are in ethanol solutions.

$$C_{13}H_{14}N_2O_4S_2 + 6H \longrightarrow \underset{\text{Desthiogliotoxin}}{C_{13}H_{16}N_2O_4} + 2H_2S$$

Desthiogliotoxin is moderately soluble in water and its aqueous solution decolorizes permanganate and bromine water. It reduces Tollens reagent on gentle warming but is indifferent to carbonyl reagents. It appeared to react with various acid chlorides in the presence of pyridine but no crys-

(18) Fromm, *Ann.*, **375**, 32, 42 (1893).

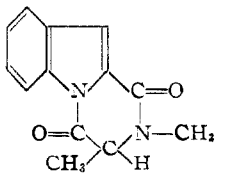
(19) Freund and others, *ibid.*, **385**, 166, 184, 196 (1895); Hantzsch and Wolvekamp, *ibid.*, **331**, 277 (1904).

(20) Baumann and Fromm, *Ber.*, **30**, 110 (1897).

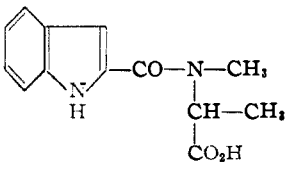
talline acyl derivative could be isolated. Attempts to prepare a crystalline acyl derivative of desthiogliotoxin by aluminum reduction of acyl derivatives of gliotoxin were also unsuccessful. Desthiogliotoxin showed a positive iodoform reaction and gave 0.93 mole of acetic acid in the Kuhn–Roth determination of C—CH_3 groups.[21] As gliotoxin itself gives a negligible C—CH_3 test, this result implies that one end of the disulfide linkage was attached to a methylene group as the system C—CH_2—S—S—, which was converted to C—CH_3.

Desthiogliotoxin is neutral in reaction but dissolves readily in strong aqueous hydrochloric acid. It reacts rapidly with hot methanolic potassium hydroxide and a sparingly soluble crystalline material separates directly from the hot solution. This substance is an accessory product of the reaction and is formed in relatively small amount (60 mg. from 350 mg. of desthiogliotoxin). On recrystallization from glacial acetic acid it forms fine, colorless needles that do not melt up to 300°, but it is not a potassium salt for it leaves no ash on ignition. Carbon and hydrogen analyses indicate an empirical formula $C_{13}H_{12}N_2O_2$, corresponding to the removal of two molecules of water from desthiogliotoxin. Further study will be necessary to establish its molecular formula and constitution.

The alkaline hydrolysis of desthiogliotoxin gave as the principal product (200 mg. from 350 mg. of desthiogliotoxin), a nitrogenous organic acid which crystallized in colorless prisms melting at 186–188°. This acid proved to be identical with the acid $C_{13}H_{14}N_2O_3$, m. p. 187°, previously obtained by mild alkaline hydrolysis of the hydriodic acid reduction product of gliotoxin (VII).[7] This acid has been shown to be *dl*-N-2-indolecarbonyl-N-methylalanine (VIII), and its formation under these circumstances suggests that desthiogliotoxin may be converted by alkali first into compound VII, or its tautomeric form XI, which is subsequently hydrolyzed. This view leads to the



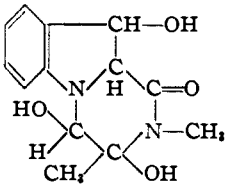
VII



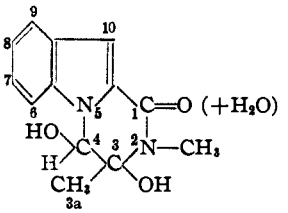
VIII

conclusion that alcoholic alkali acts upon desthiogliotoxin to produce two isomeric compounds of the formula $C_{13}H_{12}N_2O_2$, formed by two different modes of splitting off water; one of these products is stable to alkali (see below, formula XII) and the other is hydrolyzed to the C_{13} amino acid (VIII).

All of the available evidence is compatible with the hypothesis that desthiogliotoxin possesses the same ring system as the hydriodic acid reduction product (VII), and on this basis it can be formulated tentatively as the trihydroxy derivative of a dihydro-α-pyrazindolone (IX). An alternative formulation as the hydrate of a dihydroxypyrazindolone (X) appears less likely,



IX

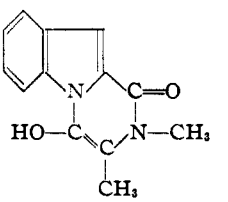


X

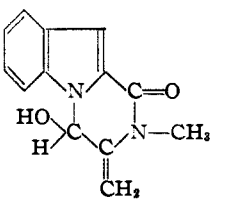
since desthiogliotoxin does not suffer dehydration on prolonged heating in vacuum and does not show an absorption band at 290 mμ, which is present in various indole derivatives having a carbonyl group attached in the alpha position. Further, none of the pyrazindolones hitherto examined has formed a hydrate.

A distinctive feature of formula IX is the presence of a 3-hydroxy-2,3-dihydroindole (β-hydroxyindoline) structure, which must be postulated to undergo dehydration readily to the corresponding indole. This assumption is justified by the behavior of β-hydroxyindoline itself, which is converted easily into indole by acids or bases.[22] The loss of water would undoubtedly be facilitated by the carbonyl group attached at the alpha position of the indoline ring, and this process would convert IX into the pyrazindolone system shown in X. The synthesis of model compounds related to 3-hydroxyindoline-2-carboxylic acid is now in progress for the purpose of examining their chemical behavior and absorption spectra.

Formulas IX and X are similar with reference to the hydroxyl groups at positions 3 and 4 of the pyrazindolone system. This structure could undergo elimination of water by loss of hydroxyl from position 3 with hydrogen from either position 4 or 3a. The former mode of elimination is the more likely and would give the enol form (XI) of the hydriodic acid reduction product; this would lead eventually to the C_{13} amino acid (VIII), which is actually the principal product observed. The alternative elimination of hydrogen from the side-chain methyl group to give the isomeric compound XII would be less likely. The latter would be much more resistant to alkaline



XI

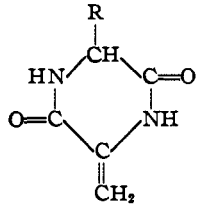


XII

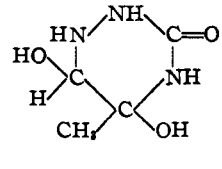
(21) Kuhn and Roth, *Ber.*, **66**, 1274 (1933)

(22) German Patents 516,675 and 518,515 (1928); *Frdl.*, **17**, 642, 649 (1930).

hydrolysis; this structure, or a transformation product from it, may represent the insoluble accessory product formed along with the C_{13} amino acid. Structure XII bears some formal resemblance to the sparingly soluble methylene compounds (XIII) which are obtained by the



XIII

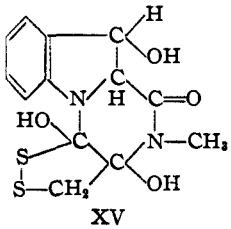


XIV

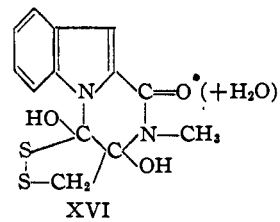
action of alkalies on diketopiperazines having present a serine or cystine residue.[23]

The dihydroxypyrazine system of IX and X may be regarded as an addition product of methylglyoxal with the methylamide of an α-amino acid, and for this reason one might expect such structures to be rather unstable toward hydrolytic agents. However, methylglyoxal bisulfite reacts with semicarbazide[24] to form a compound of very similar constitution (XIV), which is quite stable to alkalies and dissolves in cold mineral acids to form stable salts.

If desthiogliotoxin is formulated as IX or X, it is evident that the two hydrogen atoms introduced during desulfurization were added at positions 3a and 4, and gliotoxin could then be represented by the corresponding disulfide structures XV and XVI (preferably the former). These

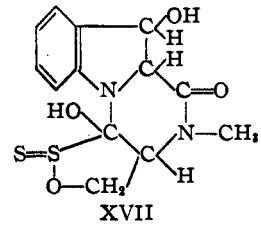


XV

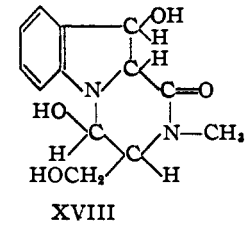


XVI

structures are of a rather novel type and in the present state of our knowledge must be presented with reservations, particularly with reference to the disposition of the oxygen atoms. There is the possibility that an oxygen atom is attached to one of the sulfur atoms in a thiosulfinate linkage,[25] —S—S— (O) or —O—S— (S), and structures such as XVII have been considered. On reduction with aluminum a compound of this structure might eliminate the sulfur quantitatively as hydrogen sulfide and give a desthio compound (XVIII) containing all of the original oxygen atoms. This desthio compound does not contain a C—CH_3 group but it could conceivably undergo dehydration to a derivative of the enol form of methylglyoxal (such as XII), which would yield acetic acid in the Kuhn–Roth determination and give a positive iodoform reaction.

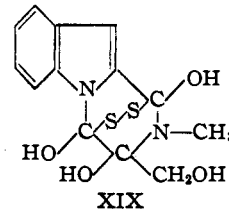


XVII

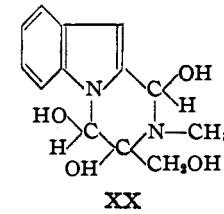


XVIII

In a preliminary report[1] we proposed for gliotoxin a structure (XIX) having the same carbon and nitrogen framework as the present formulas but with the disulfide linkage placed across the 1- and 4-positions of the pyrazine system. This would lead to a structure such as XX for desthiogliotoxin, having —CHOH— groups at the 1- and 4-positions. These variants have not been excluded definitely but now appear less probable than other formulas. The several structures advanced for gliotoxin and desthiogliotoxin will serve as a guide for further experimental work to secure new structural evidence.



XIX



XX

The antibiotic activity of gliotoxin is probably closely associated with the unusual type of sulfur linkage that is present, since even the mildest alkaline reagents destroy the characteristic activity against fungi and bacteria. The water-soluble mercaptide obtained by treating gliotoxin with potassium sulfide appears to be inactive also, although its structure must differ only slightly from gliotoxin; however, the mercaptide is quite unstable and it is uncertain whether the experiments indicate lack of activity of the mercaptide itself or of its products of decomposition. Cavallito and Bailey[26] have reported that gliotoxin is inactivated by cysteine but their observation is at variance with our finding that there is no decrease in activity of gliotoxin toward *Staphylococcus aureus* in the presence of cysteine in a buffered solution at *p*H 6.8.[27] The inactivation observed by Cavallito and Bailey probably resulted from their use of sodium bicarbonate in preparing the test solutions and represents merely

(23) Bergmann and collaborators, *Ann.*, **445**, 17 (1925); **448**, 32 (1926); **458**, 40 (1927); *Z. physiol. Chem.*, **140**, 128 (1924); **152**, 189 (1926).

(24) Ekeley and O'Kelley, THIS JOURNAL, **50**, 2731 (1928).

(25) Cavallito, Buck, Suter and Bailey (*ibid.*, **66**, 1952 (1944); **67**, 1032 (1945)) have postulated structures of this type in formulas proposed for the antibacterial principle of garlic (*Allium sativum*).

(26) Cavallito and Bailey, *Science*, **100**, 390 (1944).

(27) We are indebted to Miss C. McKee of the Squibb Institute for Medical Research, for the antibacterial tests of gliotoxin and various liotoxin derivatives.

the inactivation by alkali rather than by reaction with a sulfhydryl group. This explanation is supported by our observation that there is no chemical reaction between gliotoxin and thioglycollic acid until alkali is added to the mixture. It is of interest to note also that none of the crystalline degradation products obtained from gliotoxin has shown any appreciable bacteriostatic activity against several test organisms.

We wish to thank E. I. du Pont de Nemours and Company and the Cornell Research Foundation for generous support of this work.

Experimental

Mild Alkaline Hydrolysis of Gliotoxin.—Fifty milligrams of gliotoxin was dissolved in 10 ml. of warm methanol, and 10 ml. of warm water was added, followed by 5.0 ml. of 0.1 *N* sodium hydroxide solution. The nearly colorless solution turned deep yellow-orange on the addition of alkali; the color faded somewhat upon standing. A 5-ml. aliquot portion was removed at once and titrated with standard hydrochloric acid using phenolphthalein as indicator. The solution was kept at room temperature and the consumption of alkali was determined at hourly intervals by removal of aliquot portions. The quantity of sulfide liberated was determined in the same aliquot portions by adding to the neutralized solutions 1 ml. of 10% sulfuric acid and 10 ml. of 0.025 *N* iodine solution; after standing for ten minutes the excess iodine was titrated with standard thiosulfate solution.

The results, which are tabulated below, indicate that gliotoxin reacts practically instantly with two moles of alkali and one mole of titrable sulfide is liberated. On standing a further, slow reaction takes place which consumes a third mole of alkali but does not affect the iodine titer.

TABLE I

ACTION OF ALKALI ON GLIOTOXIN (AT 25°)

Time elapsed	Moles of NaOH consumed	Moles of I_2 required
10 min.	1.96	0.98
1 hr.	2.14	1.02
2 hr.	2.25	1.0
7 hr.	2.47	1.1
19 hr.	2.72	1.0

When 50 mg. of gliotoxin in methanol was refluxed under nitrogen with methanolic potassium hydroxide, at the end of one hour 2.5 moles of alkali were consumed per mole of gliotoxin; after two hours' refluxing, the consumption of alkali was 2.9 moles per mole.

The effect of alkaline reagents on the rotatory power of gliotoxin is quite remarkable. When a solution of 16 mg. of gliotoxin, $[\alpha]_D$ −300°, in 20 ml. of ethanol was treated with 5 ml. of 0.1 *N* sodium hydroxide and the pale yellow solution read immediately in a polarimeter the specific rotation was +125°. After several hours the rotation had fallen slightly, to +110°. An aqueous solution of sodium sulfite, buffered at *p*H 7.5–8.0, caused the rotation of gliotoxin to shift slowly to +125° over a period of twenty-four hours. Aqueous solutions of sodium sulfide or sodium cyanide (unbuffered) shifted the rotation to −65°. There can be little doubt that the pronounced effect of these reagents on the optical activity of gliotoxin results from transformations of a labile disulfide system.

Action of Alkaline Plumbite Solution on Gliotoxin.—Two 10-milligram samples of gliotoxin were dissolved in 5-ml. portions of methanol and treated at room temperature with 5- and 10-ml. portions, respectively, of a sodium plumbite solution (prepared by mixing 50 ml. of 25% sodium hydroxide solution and 10 ml. of 10% lead acetate solution). The solutions immediately turned orange, became turbid and soon precipitated black lead sulfide. After standing for twenty-four hours, the precipitate was filtered off, washed and dried; from each sample 11.5 mg. of lead sulfide was obtained. This corresponds to 78.5% of the sulfur present in gliotoxin and is similar to the behavior of typical disulfides, as Zahnd and Clarke[5] have shown that only about 75% of the labile sulfur is precipitated as lead sulfide (the remainder being converted to soluble thiosulfate).

With two similar test portions of gliotoxin, 100 mg. of stannous chloride was added to one sample before adding the plumbite solution, and to the other only the plumbite solution was added. Both of these solutions were refluxed for two hours, and the lead sulfide which formed was filtered off and converted to barium sulfate by the method of Zahnd and Clarke. The weights of barium sulfate obtained were 10.2 and 10.6 mg., respectively, which correspond to 71 and 73% of the sulfur present in the gliotoxin.

Twenty milligrams of gliotoxin was refluxed with 10 ml. of 25% sodium hydroxide and 10 ml. of 10% lead acetate solution under nitrogen, and the vapors were trapped in standard acid solution. Within three hours the liberation of volatile base (methylamine) had ended and in the distillate an amount of acid equivalent to 1 mole of base per mole of gliotoxin had been neutralized. The supernatant alkaline solution was decanted from the precipitate of lead sulfide in the reaction flask and the lead sulfide decomposed with strong hydrochloric acid. The hydrogen sulfide evolved was trapped in hypobromite solution, and from this solution there was obtained an amount of barium sulfate equivalent to 70% of the sulfur in gliotoxin.

These experiments show that with alkaline plumbite, under a variety of experimental conditions, only 70–78% of the sulfur of gliotoxin is converted to lead sulfide.

Formation of the Compound $C_{11}H_8N_2OS$. (a) From Gliotoxin.—One gram of gliotoxin was refluxed under nitrogen with 25 ml. of 20% methanolic potassium hydroxide for two hours; during this period no methylamine was evolved. Through a side arm 25 ml. of 15% hydrochloric acid was introduced and the solution was refluxed for thirty minutes to drive off the hydrogen sulfide, which was collected in sodium plumbite solution. The lead sulfide which was formed corresponded to about 1 mole of hydrogen sulfide per mole of gliotoxin. The acidified alcoholic solution of the organic products was diluted with a large volume of water and extracted with ether. The ether extract was then washed with 5% sodium carbonate solution, which removed most of the yellow pigment from the ether layer. From the washed and dried ether solution there was obtained a yellow crystalline residue which on recrystallization from aqueous acetone yielded 80 mg. of yellow prismatic rods, m. p. 188°. This is a 12% yield on the basis of the formula $C_{11}H_8N_2OS$.

On acidification of the sodium carbonate wash liquor there was obtained at first a gummy precipitate, which was filtered off; on standing the solution gradually deposited 15–20 mg. of yellow-orange crystals, which were identical with the product obtained directly from the ether solution. From this experiment and subsequent preparations, it appears that formation of the crystalline C_{11} compound takes place rather slowly, following acidification, from the products of alkaline hydrolysis. A somewhat better yield of the same compound was obtained by alkaline hydrolysis of the dibenzoyl or di-*p*-bromobenzoyl derivative of gliotoxin, following the procedure described below.

(b) **From the Di-*p*-bromobenzoyl Derivative of Gliotoxin.**—A solution of 2.55 g. of the di-*p*-bromobenzoyl derivative of gliotoxin in 25 ml. of pyridine was treated with 20 ml. of 2 *N* methanolic potassium hydroxide. The mixture, which turned deep red-brown, was allowed to stand at room temperature for twelve hours and then made up to 100 ml. with water. Titration of an aliquot portion showed the consumption of 4.7 moles of alkali per mole of ester, corresponding to the expected consumption of two moles of alkali by the acyl groups and 2.7 moles by gliotoxin itself. From the titration data the amount of

hydrochloric acid required to neutralize the total solution was calculated and this quantity was then added. No precipitate was formed at once but on standing for forty-eight hours at room temperature a crop of long, red-orange needles was deposited, weighing 150 mg. (17% yield). Recrystallization from alcohol yielded small yellow prisms, m. p. 188°, which showed no depression of the melting point when mixed with the product from the hydrolysis of gliotoxin by alcoholic potassium hydroxide. On slow crystallization from alcohol the substance crystallizes in large red-orange needles.

Anal.[28] Calcd. for $C_{11}H_8N_2OS$: C, 61.11; H, 3.70; N, 12.97; S, 14.82; mol. wt., 216. Calcd. for $C_{10}H_8N_2OS$: C, 58.82; H, 3.92; N, 13.72; S, 15.68; mol. wt., 204. Found: C, 60.46; H, 3.79; N, 13.29; S, 14.81; mol. wt. (Rast), 222.

The molecular formula $C_{11}H_8N_2OS$ fits the analytical data better than any other and is accepted tentatively for the compound, although it is not yet possible to account for the loss of two carbon atoms in passing from gliotoxin ($C_{13}H_{14}N_2O_4S_2$) to this product.

The C_{11} compound is neutral in reaction and has no optical activity. It is insoluble in water and in aqueous alkalies, and does not consume alkali when refluxed for two hours with methanolic potassium hydroxide. It does not give the Ehrlich reaction for indole derivatives. There is no reaction with acetyl chloride in toluene and pyridine as measured quantitatively by the method of Shriner.[29] When an alcoholic solution is treated with zinc and hydrochloric acid or with aluminum amalgam, hydrogen sulfide is liberated immediately.

Action of Potassium Sulfide on Gliotoxin.—One-half gram of gliotoxin was dissolved by warming in 90 cc. of absolute ethanol and to the warm solution (50°) was added 5 ml. of potassium sulfide solution (prepared by saturating 10 ml. of 10% ethanolic potassium hydroxide with hydrogen sulfide and then adding 10 ml. of untreated alkali). The solution developed a transient orange coloration that faded quickly to yellow, and within a few moments a colorless crystalline precipitate began to form. The solution was chilled for an hour and the precipitate collected, washed with absolute alcohol and dried in a vacuum desiccator. The weight was 405 mg., $[\alpha]_D$ *ca.* −60° (in water). Analyses indicated that this substance is the hydrate of a mercaptide formed by addition of one molecule of K_2S to gliotoxin.

Anal. Calcd. for $C_{13}H_{14}N_2O_4S_2K_2 + 4H_2O$: C, 30.7; H, 4.33; N, 5.50; S, 18.8; K, 15.3; H_2O, 14.2. Found: C, 30.98; H, 4.20; N, 5.50; S, 17.47; K, 15.6; H_2O (loss at 100° in vacuum), 14.0.

The fresh aqueous solution was alkaline to litmus and gave an intense nitroprusside reaction; it gave a white precipitate with mercuric chloride, a yellow orange precipitate with lead acetate, and a yellow precipitate with copper sulfate. On standing, the aqueous solution became turbid and gradually an amorphous precipitate settled out. Acidification of the fresh aqueous solution liberated some hydrogen sulfide and produced an amorphous, colorless precipitate which could not be crystallized.

When the fresh aqueous solution of this mercaptide was treated with aluminum amalgam, hydrogen sulfide was rapidly eliminated and there was formed a sulfur-free compound, $C_{13}H_{16}N_2O_4$, identical with that obtained by the action of aluminum amalgam on gliotoxin itself (see desthiogliotoxin).

It was not found possible to re-oxidize the mercaptide to the disulfide state, nor could a crystalline derivative be prepared by alkylation with methyl iodide or benzyl chloride.

Action of Thioglycollic Acid on Gliotoxin.—One gram of gliotoxin (3.1 millimoles) was dissolved in 70 ml. of hot ethanol and after cooling to 50° an ethanolic solution of 1.1 g. (12 millimoles) of thioglycollic acid was added. As no reaction was apparent in the acidic solution, 10 ml. of 2 *N* methanolic potassium hydroxide was then added. The solution developed a pale yellow coloration and soon deposited a crystalline precipitate. After cooling and standing for an hour the precipitate was collected, washed with cold ethanol and dried. The salt-like product weighed 1.0 g. and its potassium content agreed with that of potassium dithioglycollate (K = 30.3%). This salt dissolved readily in water, and the aqueous solution after acidification and extraction with ether gave an organic acid. The latter crystallized from benzene as clusters of prisms, melting at 106–107° (dithioglycollic acid melts at 107–108°).

On treatment with aluminum amalgam, the filtrate from the alkaline reaction mixture gave the same sulfur-free compound (desthiogliotoxin) obtained from the potassium mercaptide and from gliotoxin.

Reduction of Gliotoxin by Aluminum: Desthiogliotoxin.—A solution of 1 g. of gliotoxin in 150 ml. of absolute ethanol was treated with 20 g. of amalgamated aluminum turnings. After the addition of 5 ml. of water a gentle reaction occurred and hydrogen sulfide was slowly liberated. Nitrogen was bubbled slowly through the solution, and after twenty-four hours no more hydrogen sulfide could be detected in the escaping gases. The aluminum hydroxide and unreacted aluminum were filtered off and washed thoroughly with hot ethanol. The clear, slightly greenish filtrate was concentrated in vacuum. When the volume had been reduced to a few milliliters a crystalline precipitate began to separate out; distillation was stopped and the solution allowed to chill for twelve hours. The crop of colorless crystals was separated from the sirupy mother liquor by filtration and washing with a little cold alcohol. The yield was 250 mg. including a small second crop obtained by further concentration of the mother liquors. The crude material could be recrystallized from hot water in which it was fairly soluble. It separated as dense, colorless prisms melting at 243–244°; $[\alpha]^{23}D$ −240°, c = 0.4 in glacial acetic acid; $[\alpha]_D$ −130°, c = 0.2 in ethanol. Qualitative test for sulfur was negative; the substance showed no loss of weight on drying in a vacuum at 147° for four hours.

Anal. Calcd. for $C_{13}H_{16}N_2O_4$: C, 59.07; H, 6.11; N, 10.59; mol. wt., 264. Found: C, 58.81, 58.43; H, 6.0, 6.0; N, 10.7, 10.8; mol. wt. (Rast), 257.

This sulfur-free product from gliotoxin is designated as desthiogliotoxin. It is slightly soluble in cold methanol or ethanol, but dissolves readily on warming; it is soluble also in warm water, acetone, acetic acid or pyridine. The aqueous solution is neutral in reaction and the compound is not soluble in cold dil. hydrochloric acid or potassium hydroxide solution. It dissolves in concentrated hydrochloric acid but gives no precipitate with chloroplatinic acid or picric acid. When desthiogliotoxin was treated with methanolic potassium hydroxide solution at room temperature there was no apparent reaction and titration showed that no alkali had been consumed after twenty-four hours contact; however, desthiogliotoxin reacts rapidly with hot alcoholic alkali to give two crystalline products (see below).

By the acetylation method of Shriner[29] desthiogliotoxin gave good values for one hydroxyl group; attempts to prepare a crystalline acyl derivative were unsuccessful, although the compound appeared to react with various acid chlorides in the presence of pyridine. Reduction of the dibenzoyl derivative of gliotoxin by amalgamated aluminum in dioxane also failed to give a crystalline acyl derivative of desthiogliotoxin.

An aqueous solution of desthiogliotoxin readily decolorizes potassium permanganate solution and bromine water, and reduces Tollens reagent on gentle warming. However, it does not give a color with Schiff aldehyde reagent and is completely indifferent to the usual carbonyl reagents. It is not oxidized by periodic acid.

The addition of a solution of iodine in potassium iodide

(28) The Kuhn-Roth C-methyl determinations and a large number of the microanalyses and Rast molecular weight determinations reported in this paper were carried out by Mr. J. F. Alicino of the Squibb Institute for Medical Research.

(29) Shriner, "Quantitative Analysis of Organic Compounds," Edwards Brothers, Inc., Ann Arbor, Michigan, 1938.

to a dilute aqueous alkaline solution of desthiogliotoxin caused at first the development of an intense violet-brown color; further addition of iodine solution discharged this color and there was formed slowly a crystalline precipitate, which was identified as iodoform by its melting point and crystalline form. The other products of the reaction formed a dark oil from which no crystalline material could be separated. Desthiogliotoxin reacts rapidly with 3 moles of sodium hypochlorite solution and there is a slow continuous consumption of more hypochlorite until as much as 16 moles have reacted per mole of gliotoxin. Acidification of the solution after reaction with 3 moles of hypochlorite gave a minute quantity of a crystalline acid, m. p. 265–270°, which has not been identified.

By the Kuhn-Roth[21] method for the determination of C—CH_3 groups, desthiogliotoxin gave 0.93 mole of acetic acid, showing the presence of one methyl group linked to carbon. In the Kuhn-Roth determination gliotoxin itself gave a value of only 0.12 mole acetic acid, an amount too small to have structural significance in view of the limitations of the method. The hydriodic acid reduction product from gliotoxin, which is known to contain one C—CH_3 group, gave 0.83 mole of acetic acid.

Action of Alkali on Desthiogliotoxin.—When 350 mg. of desthiogliotoxin was refluxed with 2 *N* methanolic potassium hydroxide the solution quickly became cloudy and a colorless product separated in fine crystals. After an hour of refluxing the solution was cooled and the precipitate collected and washed; it weighed 60 mg. This substance is very sparingly soluble in most organic solvents but can be recrystallized from hot glacial acetic acid, from which it forms fine needles that do not melt up to 300°. The carbon and hydrogen content of this material is compatible with a C_{13} formula, but further data are necessary before a definite molecular formula can be assigned.

Anal. Calcd. for $C_{13}H_{12}N_2O_2$: C, 68.40; H, 5.26. Found: C, 68.25; H, 5.40.

A similar insoluble compound was isolated in very small amounts as an accessory product from the hydriodic acid reduction of gliotoxin. This material formed colorless prisms, m. p. 280° (dec.), and had about the same carbon and hydrogen content: C, 68.80; H, 4.89.

After separation of the insoluble product the alkaline filtrate was acidified with hydrochloric acid and evaporated to dryness in vacuum. The residue was extracted with hot acetone, which on cooling deposited clusters of colorless prisms; weight, 200 mg. After one crystallization from acetone–toluene the substance melted at 182–183°, and recrystallization from chloroform raised the m. p. to 186–188°. The appearance and properties of this acid were identical with those of the acid $C_{13}H_{14}N_2O_3$, m. p. 187°, obtained by hydrolysis of the hydriodic acid reduction product of gliotoxin. A mixed melting point with the known C_{13} acid and preparation of the ethyl ester (m. p. 129°) confirmed the identity of the product as *dl*-N-2-indolecarbonyl-N-methylalanine.

Distillation of Gliotoxin with Zinc Dust.—Quite early in the study of gliotoxin the classical procedure of zinc dust distillation was undertaken but the yield of product by the conventional methods was discouragingly small (a few mg. from 500 mg. of gliotoxin). A modified technique recently developed by Clar[30] gave considerably better yields of a crystalline product but we have not succeeded in establishing its constitution. As our work in this direction has been set aside temporarily it is deemed advisable to record our preliminary results at this time.

A sample of 500 mg. of gliotoxin was intimately mixed with 5 g. of zinc dust, 1 g. of sodium chloride and 5 g. of fused zinc chloride. The powder was placed in an Erlenmeyer flask and heated gradually in a metal bath. At 150° water vapor came off as well as some hydrogen sulfide; at 200° some free sulfur sublimed off. At 240° the dark mix began to foam and it was necessary to stir with a glass rod. The mass was heated to 275–280° and stirred until most of the foaming ceased; at this point the melt was greenish-black with a bluish iridescence, and had a naphthalenic odor. No sublimate appeared on the walls of the flask.

After cooling, the hard glassy mass was warmed with water until all was in suspension. This suspension was extracted with ether which took on an intense bluish fluorescence. After drying and evaporating, a semi-crystalline residue weighing 188 mg. was obtained. The crystalline material was quite insoluble in alcohols and only slightly soluble in hot acetone, from which it was recrystallized; m. p. 258° (darkening at 250°). This material is soluble in concd. sulfuric acid and is precipitated unchanged on addition of water. It is insoluble in aqueous sodium hydroxide even on warming. It forms a picrate immediately upon treatment with picric acid solution. This picrate may be recrystallized from hot toluene; m. p. 193–195°, after darkening at 180°. Similarly it forms a microcrystalline chloroplatinate, which melts at 204°.

Anal. Base: C, 72.38; H, 5.0; N, 11.37. Picrate: C, 51.96; H, 3.37; N, 14.56.

Solutions of the base in ether, alcohol or benzene showed a marked blue fluorescence. The base gave a deep green color in the Ehrlich test, a blue color in the pine splinter test, and a blue-green color in the Keller reaction. The analytical data show that all of the oxygen was not removed by zinc dust distillation (O, by difference: 11.3%). The properties of the product suggest that it is not a simple indole compound derived from one molecule of gliotoxin but is probably a complex structure derived from two or more molecules of gliotoxin.

The color reactions of this base were similar to those of an unidentified crystalline substance obtained in small quantity by subjecting the hydriodic acid degradation product of gliotoxin to further reduction by means of amalgamated aluminum. This base gave an emerald green color in the Ehrlich test, a blue-violet color in the pine splinter test, and a blue-green color in the Keller reaction.

Preparation of Model Thiohydantoins.—The benzylidene derivatives of the thiohydantoins were obtained by reaction with benzaldehyde in the presence of sodium acetate and glacial acetic acid, according to the general procedure of Johnson and Scott.[31]

5-Benzylidene-3-methyl-2-thiohydantoin (II).—A mixture of 1.8 g. of 3-methyl-2-thiohydantoin,[32] m. p. 160–161°, 1.9 g. of benzaldehyde, 5.3 g. of fused sodium acetate and 14 ml. of glacial acetic acid was heated for four hours at 140–150°. The product was precipitated by pouring the reaction mixture into 100 ml. of cold water. Recrystallization from glacial acetic acid gave 2.5 g. (83% yield) of yellow needles, m. p. 200–201°.

Anal. Calcd. for $C_{11}H_{10}N_2OS$: N, 12.84. Found: N, 13.06.

5-Benzylidene-3-methyl-1-phenyl-2-thiohydantoin (III).—The intermediate 3-methyl-1-phenyl-2-thiohydantoin was prepared in 30% yield by heating 5 g. of N-phenylglycine and 2.1 g. of methyl isothiocyanate at 100° for half an hour and then gradually increasing the temperature of the bath to 150°. Recrystallization from ethanol gave white crystals, m. p. 113–113.5° (Calcd. for $C_{10}H_{10}N_2OS$: N, 13.60; Found, 13.07, 13.20).[33] By the procedure given above 100 mg. of this hydantoin was converted to the benzylidine derivative. After recrystallization from ethanol there was obtained 50 mg. of yellow needles, m. p. 138°.

Anal. Calcd. for $C_{17}H_{14}N_2OS$: N, 9.52. Found: N, 9.41.

Summary

The behavior of gliotoxin toward alkali under mild conditions has been investigated and a colored crystalline compound, $C_{11}H_8N_2OS$, was

(30) Clar, *Ber.*, **72**, 1645 (1939).

(31) Johnson and Scott, THIS JOURNAL, **37**, 1851 (1915).

(32) Marckwald, Neumark and Stelzner, *Ber.*, **24**, 3278 (1891).

(33) We are indebted to Dr. Richard L. Sawyer for the preparation of this hydantoin and of several derivatives of N-(o-formylphenyl)-glycine, which will be described in another report.

isolated after acidification of the alkaline hydrolysate. The reactions of gliotoxin with potassium sulfide, potassium thioglycollate, and alkaline plumbite solution indicate the presence of a disulfide linkage.

Reduction of gliotoxin by amalgamated aluminum under mild conditions eliminated the sulfur atoms quantitatively as hydrogen sulfide and gave a colorless, crystalline, optically-active compound of the formula $C_{13}H_{16}N_2O_4$, which has been designated as desthiogliotoxin. The action of hot alcoholic alkali on desthiogliotoxin yields as the principal product the same C_{13} amino acid previously obtained from the hydriodic reduction product of gliotoxin, namely, N-2-indolecarbonyl-N-methylalanine. There is formed concurrently a small quantity of a crystalline compound corresponding to the empirical formula $C_{13}H_{12}N_2O_2$.

On the basis of the available evidence, provisional structural formulas for desthiogliotoxin and for gliotoxin have been proposed.

ITHACA, NEW YORK RECEIVED JULY 23, 1945

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

A Plan for Distinguishing between Some Five- and Six-membered Ring Ketones

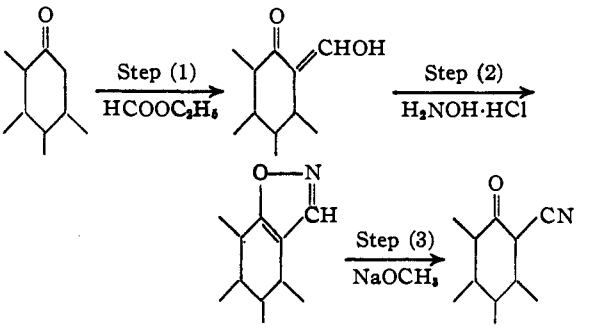
BY WILLIAM S. JOHNSON AND WESLEY E. SHELBERG

The importance of a method for the accurate discrimination between five- and six-membered ring ketones is strikingly illustrated by the classical problem of the structure of the sterols, in which the application of the Blanc rule[1] served as the means of determining ring size.[2] The Blanc rule, however, is not general,[3] and erroneous conclusions resulting from its failure led to an incorrect structure of the sterol nucleus. The position of prominence which this rule, nevertheless, has since occupied in the study of structure apparently is due to the lack of other methods.

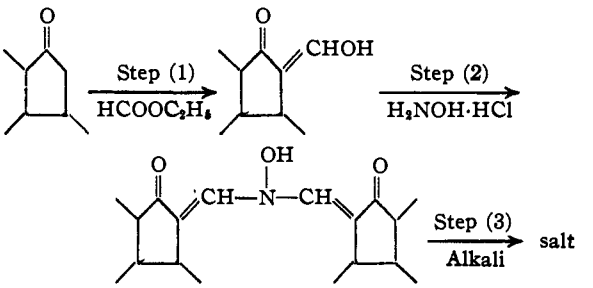
During the course of a study of the reaction of α-hydroxymethylene ketones with hydroxylamine we have observed a striking difference in behavior between cyclohexanone and cyclopentanone derivatives. This difference suggested a possible plan for distinguishing between five- and six-membered ring ketones which are capable of conversion to the hydroxymethylene derivatives, *e. g.*, ketones containing at least one reactive (α) methylene group. The ketones involved in the proof of structure of the steroid nucleus fall into this class and should afford interesting examples for testing the generality of the plan.

The scheme consists of: (1) condensation of the ketone with ethyl formate, (2) treatment of the resulting hydroxymethylene ketone with hydroxylamine hydrochloride in acetic acid, and (3) examination of the condensation products, particularly as to their behavior toward alkali. In the cases which have been studied it has been found that the cyclohexanone derivatives reacted according to the conventional scheme A, giving rise to isoxazoles at step (2). These substances were non-acidic, but were readily cleaved with sodium methoxide (step 3) to the acidic β-ketonitriles. In sharp contrast the cyclopentanone derivatives did not give isoxazoles, but condensed to form di-substituted hydroxylamine derivatives (see scheme B). These substances, unlike the isoxazoles, were colored and weakly acidic. They were conclusively distinguished from the isoxazoles by analysis for nitrogen.

SCHEME A FOR SIX-MEMBERED RINGS



SCHEME B FOR FIVE-MEMBERED RINGS



Cyclohexanone Derivatives

The formation of isoxazoles from open chain α-hydroxymethylene ketones and hydroxylamine is well-known.[4] That the generality of this reaction extends to cyclohexanone derivatives is indicated by previous work. For example, v. Auwers, Bahr and Frese[5] have thoroughly investigated the

(1) Blanc, *Compt. rend.*, **144**, 1356 (1907).

(2) For a review see Fieser, "The Chemistry of Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1937.

(3) *Cf.* the failure of the Blanc rule in the case of ring C; Wieland, *Z. physiol. Chem.*, **108**, 306 (1920).

(4) For early work see Claisen (a) *Ber.*, **36**, 3664 (1903), and (b) *ibid.*, **42**, 59 (1909).

(5) v. Auwers, Bahr and Frese, *Ann.*, **441**, 54 (1925).