S 30. *Gliotoxin. Part I. Synthesis of 2-Thio-3-methylindolo-1′ : 2′-1 : 5-hydantoin and its Identification as a Degradation Product of Gliotoxin.*

By J. A. Elvidge and F. S. Spring.

The yellow compound $C_{11}H_8ON_2S$ isolated from gliotoxin after treatment with alcoholic alkali is shown by synthesis to be *2-thio-3-methylindolo-1′ : 2′-1 : 5-hydantoin* (III). A mechanism for the formation of this compound from gliotoxin is suggested and its bearing upon the location of the disulphide grouping in gliotoxin is discussed.

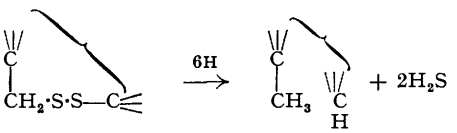
Gliotoxin was isolated by Weindling and Emerson (*Phytopath.*, 1936, **26**, 1068; see also Weindling, *ibid.*, 1932, **22**, 837; 1934, **24**, 1153; 1937, **27**, 1175; 1941, **31**, 991) from culture filtrates of a mould considered to be *Gliocladium fimbriatum*. Subsequently gliotoxin has been recognised as a metabolic product of strains of *Trichoderma viride* (Brian, *Nature*, 1944, **154**, 667; Brian and Hemming, *Ann. Appl. Biol.*, 1945, **32**, 214), *Aspergillus fumigatus* (Waksman and Geiger, *J. Bact.*, 1944, **47**, 391; Menzel, Wintersteiner, and Hoogerheide, *J. Biol. Chem.*, 1944, **152**, 419; Glister and Williams, *Nature*, 1944, **153**, 651), *Penicillia* (Mull, Townley, and Scholz, *J. Amer. Chem Soc.*, 1945, **67**, 1626; Brian, *Trans. Brit. Mycol. Soc.*, 1946, **29**, 211), and an unidentified *Aspergillus* (Stanley, *Australian J. Sci.*, 1944, **6**, 151; Stanley and Mills, *Australian J. Exp. Biol. Med. Sci.*, 1946, **24**, 133). Gliotoxin has marked fungistatic properties having an activity as great as that of mercuric chloride against certain plant pathogens (Brian and Hemming, *loc. cit.*). Johnson, Bruce, and Dutcher (*J. Amer. Chem. Soc.*, 1943, **65**, 2005) found that whereas both still- and submerged-culture methods for the production of gliotoxin were unsatisfactory, shake-cultures produced yields of 50 mg. per l. of culture filtrate in a few days. More recently Brian and Hemming (*loc. cit.*) have described a less rapid but, in some important respects, more convenient process which, in essentials, resembles the quick fermentation method for the manufacture of vinegar.

The chemical constitution of gliotoxin has been the subject of a series of investigations by Johnson and his collaborators (*J. Amer. Chem. Soc.*, 1943, **65**, 2005; 1944, **66**, 614, 617, 619;

1945, **67**, 423, 1736; 1947, **69**, 2364) as a result of which the preferred structure (I) has been proposed. The presence of a disulphide grouping and of a pyrazinoindole nucleus in gliotoxin appear to be established with reasonable certainty. The points of attachment of the disulphide grouping to the pyrazinoindole nucleus on the other hand require support and confirmation.

We have used the method of Brian and Hemming (*loc. cit.*) for the production of gliotoxin with minor modifications described in the experimental section. In the later stages of the growth of the mould, formation of coloured products was observed, and from the mother-liquors obtained from the isolation of gliotoxin we have separated, in very small amounts, two crystalline pigments, m. p.s 225—227° and 116—118° (decomp.). The latter compound differs from fumigatin (m. p. 116°) in that it is insoluble in aqueous sodium hydrogen carbonate.

Our examination of gliotoxin, $C_{13}H_{14}O_4N_2S_2$, has in the first place been limited to a study of the mode of attachment of the disulphide grouping to the nucleus. Reduction of gliotoxin with aluminium and water gives a sulphur-free compound $C_{13}H_{16}O_4N_2$, dethiogliotoxin, represented by Dutcher, Johnson, and Bruce (*J. Amer. Chem. Soc.*, 1945, **67**, 1736) as a simple derivative of gliotoxin (II). Estimation of *C*-methyl in gliotoxin and dethiogliotoxin pointed to the appearance of one *C*-methyl group in dethiogliotoxin during the change gliotoxin ⟶ dethiogliotoxin, and accordingly one point of attachment of the disulphide grouping in gliotoxin appeared to be at C_{3a}. In view of their great significance in the elucidation of the structure of gliotoxin, these experiments have been repeated and extended. Estimation of *C*-methyl in gliotoxin and dethiogliotoxin confirmed the presence of one *C*-methyl group in the latter and the absence of a *C*-methyl in the former. Additional support for this conclusion was obtained from a comparative examination of esters of gliotoxin and dethiogliotoxin. Dutcher, Johnson, and Bruce (*loc. cit.*) observed that dethiogliotoxin appeared to react with various acid chlorides in the presence of pyridine, but no crystalline acyl derivatives could be isolated. We find that dethiogliotoxin can be acetylated smoothly by reaction with acetic anhydride to give a crystalline ***diacetyl*** derivative which is a normal *O*-acetyl derivative since it is reconverted into dethiogliotoxin by treatment with methanolic ammonia. Comparative estimation of *C*-methyl in gliotoxin dibenzoate (gliotoxin diacetate has not been obtained crystalline) and dethiogliotoxin diacetate again indicated that one *C*-methyl group is developed in passing from gliotoxin to dethiogliotoxin, and that this change can be represented as



C-Methyl determinations.

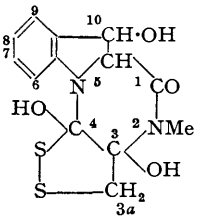
	Found, %.	Calc., %.	No. of *C*-Me groups.
Gliotoxin	0·9	4·6 } for 1 *C*-Me	0·2
Dethiogliotoxin	4·35	5·7 } for 1 *C*-Me	0·76
Gliotoxin dibenzoate	1·0	2·8 } for 1 *C*-Me	0·36*
Dethiogliotoxin diacetate	11·3	12·9 for 3 *C*-Me	2·63

* High value possibly due to volatility of benzoic acid.

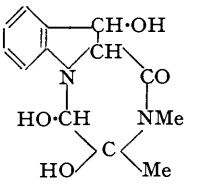
The isolation of a diacyl derivative of dethiogliotoxin is a valuable indication that dethiogliotoxin is a simple derivative of gliotoxin, which also forms diacyl derivatives.

Treatment of gliotoxin or its diacyl derivatives with methanolic potassium hydroxide gives in low yield a compound $C_{11}H_8ON_2S$ which Dutcher, Johnson, and Bruce (*J. Amer. Chem. Soc.*, 1945, **67**, 1736) suggested might be a thiohydantoin (III). An examination of this compound is of some importance, since its identification may be of value in locating the position of the disulphide group in gliotoxin. For this reason it appeared desirable to synthesise the thiohydantion (III). An attempt to cause indole-2-carboxymethylamide to react with dimethyl trithiocarbonate proved unpromising. An alternative approach in which it was hoped to obtain the intermediate (IV) by a Fischer indole synthesis from the phenylhydrazone (V) was abandoned since methyl pyruvate and pyruvic acid both reacted with 2-phenyl-4-methylthiosemicarbazide to yield the *triazine* (VI). Finally it was found that methyl indole-2-carboxylate reacts smoothly with methyl *iso*thiocyanate in a sealed tube at 180°, to give a product, $C_{11}H_8ON_2S$, which proved to be identical with the degradation product from gliotoxin. Confirmation of identity was provided by a comparison of the ultra-violet absorption spectra of

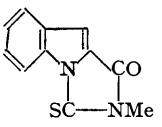
both compounds (see Fig.). In view of the reactivity of the 3-position in the indole nucleus, it is possible to formulate the compound $C_{11}H_8ON_2S$ obtained from methyl indole-2-carboxylate and methyl *iso*thiocyanate as either (III) or (VII). Although the thiohydantoin structure (III) would appear to be the more probable, additional evidence was sought to enable us to differentiate between these two structures. Indole-2-carboxylic acid when refluxed with acetic anhydride is converted into 1-*acetylindole*-2-*carboxylic acid*, whereas the compound $C_{11}H_8ON_2S$ is unaffected by similar treatment, a behaviour which points to the structure (III). Conclusive evidence for structure (III) was obtained as follows : *Methyl* 3-*methylindole*-2-*carboxylate* (VIII),



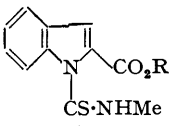
(I.)



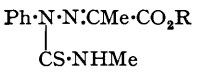
(II.)



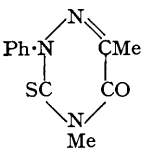
(III.)



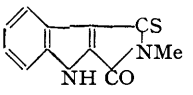
(IV.)



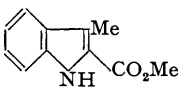
(V.)



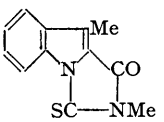
(VI.)



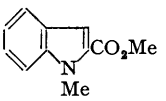
(VII.)



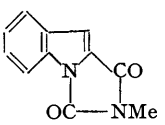
(VIII.)



(IX.)



(X.)

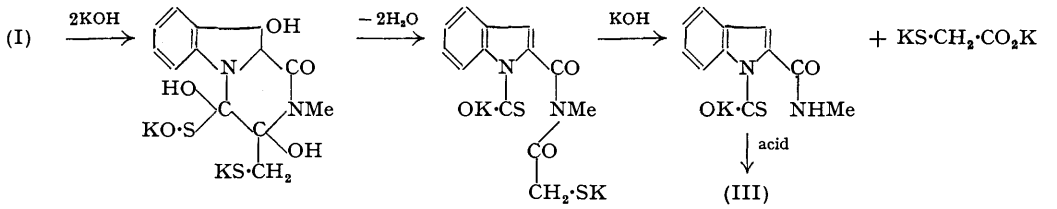


(XI.)

prepared by treatment of the phenylhydrazone of β-ketobutyric acid with methanolic sulphuric acid, was found to react with methyl *iso*thiocyanate to yield a compound $C_{12}H_{10}ON_2S$, which can only have the thiohydantoin structure (IX). The ultra-violet absorption spectrum of this *thiohydantoin* is very similar to that of the compound $C_{11}H_8ON_2S$ (see Fig.). On the other hand methyl 1-methylindole-2-carboxylate (X) failed to react with methyl *iso*thiocyanate under even more drastic conditions than those employed in the synthesis of the compound $C_{11}H_8ON_2S$ and of the thiohydantoin (IX), thus showing that the 3-position in the indole nucleus is not involved in this type of reaction. We conclude therefore that the compound $C_{11}H_8ON_2S$ from gliotoxin is 2-*thio*-3-*methylindolo*-1′ : 2′-1 : 5-*hydantoin* (III). As expected, oxidation of the thiohydantoin (III) with hydrogen peroxide gives the corresponding *hydantoin* (XI).

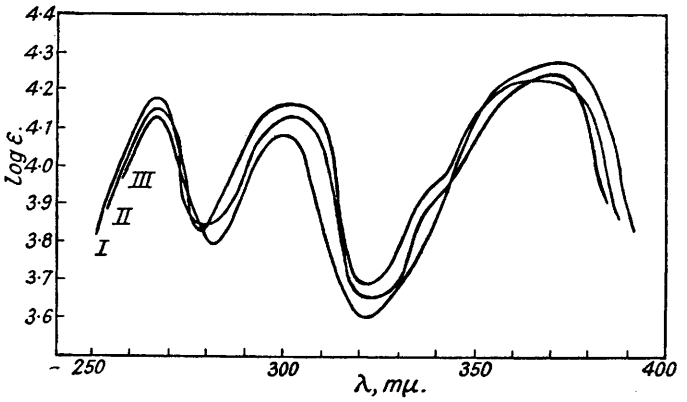
The establishment of the structure of the sulphur-containing degradation product of gliotoxin affords additional support for the view that the second position of attachment of the disulphide

grouping in gliotoxin is at C_4. The formation of the thiohydantoin from gliotoxin, involving a contraction of a six- to a five-membered ring, may be represented as follows :



In accord with this scheme it is observed experimentally that three equivalents of alkali are consumed per mole of gliotoxin. Furthermore, the scheme is in harmony with the observation of Dutcher, Johnson, and Bruce (*loc. cit.*) that the thiohydantoin is not a primary product of the degradation of gliotoxin with alkali, but is formed after the reaction mixture has been acidified; this observation has been confirmed by us.

FIG. 1.



Absorption spectra in ethanol: I. *2-Thio-3-methylindolo-1′:2′-1:5-hydantoin.* II. *Compound* $C_{11}H_8ON_2S$ *from gliotoxin.* III. *2-Thio-3:3′-dimethylindolo-1′:2′-1:5-hydantoin.*

EXPERIMENTAL.

Gliotoxin.—Trichoderma viride (strain 211) was grown on blotting-paper supports in glazed earthenware pots (32 cm. × 24 cm. diameter) into which the medium (Weindling, pH 3·5) slowly dripped, and through which sterile air was blown continuously. Early blockage of the system (during the third or fourth week) was prevented by raising the corrugated paper supports 3 cm. above the base of the pots by means of glass-rod stools. Inoculation was with spore suspensions prepared from 8-day cultures on Czapek-Dox agar (pH, 6·5), the rate of flow of the medium was 8—10 l. per week, and the temperature was kept at 18—20°. The culture solution was extracted with chloroform (3 × 0·1 vol.), the extract was filtered, washed with a little water, and evaporated under slightly reduced pressure, and the residue was crystallised from methanol, from which gliotoxin separates (charcoal) as colourless silky needles or laths, m. p. 190° (decomp.) with some softening below this temperature; it has an instantaneous decomposition point at 220° (Found : C, 48·0; H, 4·3; N, 8·4; S, 18·9. Calc. for $C_{13}H_{14}O_4N_2S_2$: C, 47·85; H, 4·3; N, 8·6; S, 19·6%).

Run no.	Time, days.	Vol. of culture solution, l.	Yield of gliotoxin, g.
1 (One production unit)	42	56	3·92
2 (Two units)	43	112	8·62
3 (,, ,,)	41	97	8·22

Gliotoxin dibenzoate separates from chloroform–methanol as parallellogrammic plates, m. p. 202° (Found : C, 60·85; H, 4·5; N, 5·4; S, 12·0. Calc. for $C_{27}H_{22}O_6N_2S_2$: C, 60·7; H, 4·1; N, 5·2; S, 12·0%); Bruce, Dutcher, Johnson, and Miller (*J. Amer. Chem. Soc.*, 1944, **66**, 616) give m. p. 192—193° (decomp.).

The mother liquors from the crystallisation of gliotoxin were combined and evaporated under reduced pressure. The dark red viscous oil was kept with methanol, and the solid separating was fractionated from methanol to yield gliotoxin as needles, m. p. 186—190° (decomp.), a pigment which forms pale salmon-pink plates, m. p. 225—227°, and a second pigment which separates as maroon-coloured plates, m. p. 116—118° (decomp.). The last compound dissolves in aqueous sodium hydroxide to give a deep red solution, but is insoluble in sodium hydrogen carbonate or hydrochloric acid solutions.

Dethiogliotoxin.—A solution of gliotoxin (2·0 g.) in ethanol (600 c.c.) containing water (40 c.c.) was stirred for 8 hours with amalgamated aluminium foil (20 g., 1 cm. squares). The hot mixture was filtered, the alumina was washed with boiling ethanol (2 × 200 c.c.), and the combined filtrates were evaporated under reduced pressure. Crystallisation of the residue from *n*-propanol gave dethiogliotoxin (497 mg.), m. p. 245—249° (decomp.), which on recrystallisation was obtained as stout prisms, m. p. 248—249° (decomp.) (Found: C, 59·0; H, 6·0; N, 10·8. Calc. for $C_{13}H_{16}O_4N_2$: C, 59·1; H, 6·1; N, 10·6%); Dutcher, Johnson, and Bruce (*J. Amer. Chem. Soc.*, 1945, **67**, 1743) give m. p. 243—244°.

Dethiogliotoxin Diacetate.—Dethiogliotoxin (280 mg.) was refluxed with acetic anhydride (30 c.c.) for 1 hour. The solution was evaporated (reduced pressure), and the residue warmed with methanol. On removal of the solvent under reduced pressure at room temperature, crystallisation occurred. *Dethiogliotoxin diacetate* (161 mg.) separated from di*iso*butyl ketone in shiny hexagonal plates, m. p. 175° (Found: C, 58·2; H, 5·6; N, 7·9, 8·2. $C_{17}H_{20}O_6N_2$ requires C, 58·6; H, 5·75; N, 8·0%).

A solution of the diacetate (4 mg.) in methanol (1 c.c.) was treated with aqueous ammonia (*d* 0·88; 5 drops), and after 16 hours the solution was concentrated under reduced pressure. Dethiogliotoxin separated as characteristic stout prisms, m. p. 238—242° (decomp.), undepressed in m. p. when mixed with a pure specimen.

1-*Acetylindole*-2-*carboxylic Acid.*—*o*-Nitrophenylpyruvic acid (Carlo, *J. Amer. Chem. Soc.*, 1944, **66**, 1420) was reductively cyclised to indole-2-carboxylic acid, m. p. 204° (Kermack, Perkin, and Robinson, *J.*, 1921, 1625). The acid (0·2 g.) was refluxed with excess of acetic anhydride for 1·5 hours, and the solution evaporated (reduced pressure). The residue was crystallised from acetone–water (charcoal) to yield 1-*acetylindole*-2-*carboxylic acid* as leaflets, m. p. 168°, which dissolve with effervescence in aqueous sodium hydrogen carbonate (Found: C, 65·3; H, 4·6; N, 7·0. $C_{11}H_9O_3N$ requires C, 65·0; H, 4·4; N, 6·9%).

2-*Thio*-3-*methylindolo*-1′ : 2′-1 : 5-*hydantoin* (III).—(*a*) Methyl *iso*thiocyanate was prepared by shaking a cold mixture of methylamine hydrochloride (6·7 g.), carbon disulphide (6·0 c.c.), and sodium hydroxide (8 g.) in water (50 c.c.) until homogeneous solution was obtained (*ca.* 40 minutes). A hot solution of lead acetate (50 g.) in water (100 c.c.) was added, and the mixture was at once steam distilled. The product was dried between paper and over calcium chloride (yield 3·4 g., m. p. 34—35°) (cf. Delépine, *Compt. rend.*, 1907, **144**, 1126).

Methyl indole-2-carboxylate (0·8 g.) and methyl *iso*thiocyanate (0·4 g.) were heated in a sealed tube at 180° for 3 hours. Crystallisation of the reaction product first from methanol and then from acetone–water gave 2-*thio*-3-*methylindolo*-1′ : 2′-1 : 5-*hydantoin* as yellow needles (0·6 g.), m. p. 189° (Found: C, 61·6; H, 3·8; N, 12·7; S, 14·2. $C_{11}H_8ON_2S$ requires C, 61·1; H, 3·7; N, 13·0; S, 14·8%).

The thiohydantoin was recovered unchanged after refluxing with acetic anhydride for 1·25 hours.

An attempt to prepare the thiohydantoin by heating indole-2-carboxylic acid (0·8 g.) and methyl *iso*thiocyanate (0·4 g.) in a sealed tube at 160—170° for 3 hours gave a product which crystallised slowly from ethanol–water. It was sublimed at 160—170°/1 mm., and recrystallised from ethanol–water to yield indole-2-carboxymethylamide as laths, m. p. 224° undepressed when mixed with an authentic specimen (Found: N, 16·4. Calc. for $C_{10}H_{10}ON_2$: N, 16·1%).

(*b*) A solution of gliotoxin (321 mg.) in dry pyridine (5 c.c.) was treated with methanolic potassium hydroxide (4·0 c.c., 1·972N) and kept at room temperature for 23 hours. Water (20 c.c.) was added, and the solution neutralised with hydrochloric acid (5·6 c.c., 0·892N), using phenolphthalein externally (alkali consumed, 2·9 equivs.). The solution was diluted with water (85 c.c.). After 48 hours it was evaporated to dryness under reduced pressure, and the residue extracted with boiling acetone. Evaporation of the extract gave a brown-yellow solid (30 mg.), m. p. 179—183°, which was sublimed in high vacuum and recrystallised from acetone–water to give 2-thio-3-methylindolo-1′ : 2′-1 : 5-hydantoin as yellow needles, m. p. 189° either alone or when mixed with the specimen prepared by method (*a*).

3-*Methylindolo*-1′ : 2′-1 : 5-*hydantoin* (XI).—A hot solution of 2-thio-3-methylindolo-1′ : 2′-1 : 5-hydantoin (0·3 g.) in ethanol (150 c.c.) was treated with hydrogen peroxide (1 c.c., 30%) and then with methanolic potassium hydroxide (2·5 c.c., 2N). Potassium sulphate separated; after 1 hour the mixture was evaporated to dryness (reduced pressure), and the solid washed with dilute hydrochloric acid and with water. Crystallisation from ethanol (charcoal) gave 3-*methylindolo*-1′ : 2′-1 : 5-*hydantoin* (0·25 g.) as needles, m. p. 182° (Found: C, 66·1; H, 4·0; N, 13·8. $C_{11}H_8O_2N_2$ requires C, 66·0; H, 4·0; N, 14·0%). Light absorption in ethanol: Maxima at 2350 A., ε = 13,280, 2570 A., ε = 10510, and 3100 A., ε = 14200.

Methyl 3-*Methylindole*-2-*carboxylate.*—Ethyl ethylacetoacetate (64·5 g., b. p. 93°/20 mm.) was dissolved in a solution of sodium acetate (150 g.) in aqueous ethanol (70%, 1000 c.c.), and treated at 0° with a solution of benzenediazonium chloride prepared at 5° from aniline (45 c.c.), concentrated hydrochloric acid (120 c.c.), water (120 c.c.), and sodium nitrite (37·5 g.) in water (90 c.c.). After 48 hours the dark solution was extracted with ether, the extract was evaporated, and the oil was treated with a solution of potassium hydroxide (21 g.) in ethanol (75 c.c.). The mixture was diluted with water, filtered (filter-aid), treated with charcoal, and acidified with concentrated hydrochloric acid to give β-ketobutyric acid phenylhydrazone (19 g.), which separated from ethanol–water as yellow prisms, m. p. 150—152°.

The phenylhydrazone (6 g.) was refluxed for 3 hours with a mixture of methanol (60 c.c.) and sulphuric acid (6 c.c.). The mixture was concentrated and extracted with ether. The extract was washed with water and evaporated to yield *methyl* 3-*methylindole*-2-*carboxylate* which separates from methanol as colourless prisms, m. p. 148° (Found: C, 69·4; H, 5·9; N, 7·6. $C_{11}H_{11}O_2N$ requires C, 69·8; H, 5·8; N, 7·4%).

2-*Thio*-3 : 3′-*dimethylindolo*-1′ : 2′-1 : 5-*hydantoin* (IX).—Methyl 3-methylindole-2-carboxylate (0·9 g.) and methyl *iso*thiocyanate (0·4 g.) were heated in a sealed tube at 240° for 2·5 hours. Crystallisation of the product from glacial acetic acid gave 2-*thio*-3-: 3′-*dimethylindolo*-1′ : 2′-1 : 5-*hydantoin* as orange-yellow needles (0·5 g.), m. p. 222° (Found: C, 62·2; H, 4·5; N, 11·9. $C_{12}H_{10}ON_2S$ requires C, 62·6; H, 4·35; N, 12·2%).

An attempt to effect the reaction by heating the components at 180° for 3 hours was unsuccessful, methyl 3-methylindole-2-carboxylate (90%) being recovered. Attempts to cause methyl 3-methyl-

indole-2-carboxylate (0·9 g.) (Johnson, Hasbrouck, Dutcher, and Bruce, *J. Amer. Chem. Soc.*, 1945, **67**, 428) and methyl *iso*thiocyanate (0·4 g.) to react by heating the mixture (*a*) at 180° for 3 hours, (*b*) at 240° for 2·5 hours, and (*c*) at 250—260° for 2 hours were all unsuccessful, the unchanged indole derivative being recovered.

5-*Keto*-3-*thio*-2-*phenyl*-4 : 6-*dimethyl*-2 : 3 : 4 : 5-*tetrahydro*-1 : 2 : 4-*triazine* (VI).—A solution of methyl *iso*thiocyanate (1·3 g.) and phenylhydrazine (1·9 g.) in ethanol (12 c.c.) was warmed for several minutes, and evaporated (reduced pressure). Treatment of the residue with ether gave 2-phenyl-4-methylthiosemicarbazide (2 g.), m. p. 89—90° (cf. Beilstein, 4th Ed., **15**, 278). A solution of the thiosemicarbazide (1·7 g.) in acetic acid (4 c.c.) was treated with pyruvic acid (0·8 c.c.), and the mixture warmed for 5 minutes. The solution was cooled and diluted with water, and the solid separating was recrystallised from aqueous ethanol to give the *triazine* (VI) as needles (1·2 g.), m. p. 150° (Found : C, 56·8; H, 4·7; N, 17·4. $C_{11}H_{11}ON_3S$ requires C, 56·7; H, 4·7; N, 18·0%). Light absorption in ethanol : Maxima at 2270 A., $\varepsilon = 17{,}260$ and 2740 A., $\varepsilon = 14{,}300$. The triazine is also obtained by adding methyl pyruvate to a solution of the thiosemicarbazide in cold methanol.

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