cule as oxirane oxygen. Details will be published at some later date.

EASTERN REGIONAL RESEARCH LABORATORY[13]
PHILADELPHIA 18, PENNSYLVANIA

(13) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Service, U. S. Department of Agriculture.

## 3-Indolecarboxaldehyde Thiosemicarbazone, a New Antitubercular Compound[1]

BY LOWELL E. WELLER, HAROLD M. SELL AND R. Y. GOTTSHALL

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The inhibition of growth of *Mycobacterium tuberculosis* by many thiosemicarbazones has been reported.[2a,b,3] 3-Indolecarboxaldehyde thiosemicarbazone has been shown to have high bacteriostatic activity *in vitro* and to suppress tuberculosis in mice after injection of virulent tubercle bacilli. Because of these properties the synthesis of this new thiosemicarbazone is described.

**3-Indolecarboxaldehyde Thiosemicarbazone.**—Thiosemicarbazide 8.2 g. (0.09 mole), dissolved in 100 ml. of warm 30% acetic acid was added to a solution of 12.3 g. (0.085 mole) of 3-indolecarboxaldehyde[4,5] in 200 ml. of methanol and the resulting mixture refluxed for 2 hours. After cooling, the precipitate was collected by filtration, washed with cold water and air-dried. The crude product was purified by recrystallization from methanol to give light yellow crystals of 3-indolecarboxaldehyde thiosemicarbazone, m.p. 230–232° dec.

*Anal.* Calcd. for $C_{10}H_{10}N_4S$: N, 25.67. Found: N, 25.48.

**Biological Properties.**—The following organisms were not inhibited by 100 micrograms of 3-indolecarboxaldehyde thiosemicarbazone: *Diplococcus pneumoniae, Streptococcus hemolyticus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Micrococcus pyogenes* var. *aureus, Klebsiella pneumoniae, Proteus vulgaris, Shigella dysenteriae, Corynebacterium diphtheriae, Aerobacter aerogenes, Mycobacterium phlei, M. smegmatis* and *Mycobacterium* 607.

*M. tuberculosis* var. *hominis*, strains $H37R_v$, $H37R_a$ and Ts1760 were inhibited by 6.2, 0.8 and 3.2 micrograms of the chemical, respectively.

When injected intraperitoneally as a suspension, 50 mg. was lethal for 20-g. white mice while 25 mg. was tolerated. Four-and-one-half mg. injected daily on seven successive days was tolerated and this dose was used for a preliminary protection test. For this test, 1.0 mg. of *M. tuberculosis*, $H37R_v$ was injected intravenously into eight mice and the following day, 4.5 mg. of the 3-indolecarboxaldehyde was injected subcutaneously. Daily injections of the same dose of chemical were made for 17 days. Nineteen days after the last injection, seven of the eight treated mice were still alive while of the 10 control mice infected at the same time, but which did not receive the chemical, only three survived.

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DEPARTMENT OF AGRICULTURAL CHEMISTRY
MICHIGAN STATE COLLEGE, EAST LANSING, AND
MICHIGAN DEPARTMENT OF HEALTH
LANSING, MICHIGAN

(1) Journal article No. 1515, Michigan Agricultural Experiment Station.

(2) (a) G. Domagk, R. Behnisch, F. Mietzsch and H. Schmidt, *Naturwissenschaften*, **33**, 315 (1946); (b) G. Domagk, *Schweiz. Z. Path. u. Bakt.*, **12**, 575 (1949).

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## The Keto Acids of the Tulip (*Tulipa gesneriana* with Special Reference to the Keto Analog of γ-Methyleneglutamic Acid

BY G. H. N. TOWERS[1] AND F. C. STEWARD

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Our interest in the keto acids of plants, because of their importance in the understanding of nitrogen metabolism, has prompted the development of a general method for their identification and quantitative determination. This method, which will be published in due course (Towers, Thompson and Steward) depends upon the following procedures.

a. The plant material is killed and the keto compounds fixed with an alcoholic solution of either 2,4-dinitrophenylhydrazine or 1,1-diphenylhydrazine.

b. The keto-acid hydrazones are separated from the amino acids and neutral carbonyl compounds by extraction into ethyl acetate and subsequent extraction of the ethyl acetate solution with dilute sodium carbonate.

c. Specific 2,4-dinitrophenylhydrazones are recognized by chromatography on paper.

d. The hydrazones are converted to the corresponding amino compounds by catalytic hydrogenolysis.

e. The amino compounds so produced are recognized by two-directional chromatography on paper and treatment of the chromatograms with ninhydrin. Thus the keto-acids may be recognized by drawing upon the extensive information, which is now available, on the chromatography of the amino compounds on paper and by utilizing the sensitivity of the ninhydrin method for their detection.

The recent recognition of γ-methyleneglutamine and of γ-methyleneglutamic acid as constituents of the peanut plant[2] and the proof that these substances are identical with nitrogenous compounds earlier recognized in the tulip plant[3,4] gives rise to interesting possibilities.

Therefore, in making the first extensive examination of the ketoacids of the tulip plant, special attention was paid to the recognition and identification of the keto analog of γ-methyleneglutamic acid. Work has been done on the keto compounds present in the tissue of the resting tulip bulb (in which the γ-methyleneglutamine was first recognized) and also of the green foliage leaves in which it is present in larger amount.

The 2,4-dinitrophenylhydrazones from the tulip bulb yielded on hydrogenolysis the following amino compounds: glycine, alanine, aspartic acid, glutamic acid and valine. In addition to these known amino acids, hydrogenolysis also yielded amino compounds whose identity still remains unknown. Therefore, one could infer that the following keto compounds, corresponding to this list of amino acids, are present as constituents of the tulip plant: glyoxylic acid, pyruvic acid, oxaloacetic acid, α-ketoglutaric acid and unknown keto compounds.

(1) Lalor Foundation, Pre-doctoral Fellow at Cornell University.

(2) J. Done and L. Fowden, *Biochem. J.*, **49**, Proc. XX (1951).

(3) F. C. Steward and J. F. Thompson, *Ann. Rev. Plant Physiol.*, **2**, 233 (1950).

(4) R. Zacharias, J. K. Pollard and F. C. Steward, THIS JOURNAL, **76**, 1961 (1954).

However, the most conspicuous constituent of the chromatograms resulting from hydrogenolysis and treatment with ninhydrin, was a spot that clearly occurred in the position of γ-methylglutamic acid. This position was established with certainty by the use of authentic material furnished to us through the courtesy of Dr. Fowden, of University College, London, England. The natural and the synthetic materials were co-chromatographed in three different solvent systems and could not be resolved from one another. Therefore, α-keto-γ-methyleneglutaric acid is to be regarded as a naturally occurring compound in the tulip leaf.

To confirm the identity of the keto compound which gave γ-methylglutamic acid on hydrogenolysis, the 2,4-dinitrophenylhydrazones of 3 kg. of tulip leaves were separated by chromatographing them successively on columns of cellulose, first with 5:1 butanol:ethanol, equilibrated with 3% ammonium hydroxide, followed, on the second column, by the use of isoamyl alcohol saturated with 10% ammonium hydroxide. In the first of these operations 11 bands were obtained and collected in 80 fractions by means of a fraction collector, and, of these fractions, those numbered 26–56, were consolidated to yield 1.57 g. of a dark oil. This oil was chromatographed on the second column and a slow-moving, conspicuous orange band was removed after extrusion of the column which contained 10 well separated colored bands. The contents of this fraction were eluted with ethanol and concentrated to a yellow oil from which a crystalline product could not be obtained.

1.3 g. of this oil (4/5 of the total) was chromatographed on Fisher activated alumina (80–200 mesh) using ethyl acetate, followed by ethanol which was gradually diluted to water and finally 1% sodium carbonate, as eluants. A substance A crystallized from many of the fractions collected; on recrystallization from methanol 400 mg. of a creamy white, chromatographically pure, substance, which sublimed at 195° and melted at 244°, was obtained. Substance A is insoluble in water, soluble in ethyl acetate and in 1% sodium carbonate to give a brown solution. On analysis substance A gave: C, 45.2%, H, 2.59%, N, 16.0%, O (by difference), 36.2%, and a molecular weight determination by mixed melting point with camphor (the method of Rast) yielded a figure of 328.

On hydrogenolysis, substance A did not yield γ-methylglutamic acid directly but an unknown ninhydrin reactive compound which readily yielded γ-methylglutamic acid on acid hydrolysis. Although Substance A is *not* a typical 2,4-dinitrophenylhydrazone and its constitution is not yet definitely known the important thing is that it yields γ-methylglutamic acid on hydrogenolysis and hydrolysis.

Fom an ether extract of the remaining 1/5 of the yellow oil referred to above 9.5 mg. of a substance B, which had a m.p. of 268–270° decomposition, was obtained. This substance was chromatographically very similar to, but not identical with, the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. It yielded directly on hydrogenolysis a compound which was chromatographically identical in three different solvent systems with γ-methylglutamic acid. Substance B is therefore considered to be the 2,4-dinitrophenylhydrazone of a keto-dicarboxylic acid (α-keto-γ-methyleneglutaric acid).

Therefore, by the use of 2,4-dinitrophenylhydrazine, two compounds (A and B) have been obtained from tulip which on hydrogenolysis ultimately yielded γ-methylglutamic acid. The white substance A and the yellow substance B are interrelated because A may be converted to B by treatment with alkali followed by acidification with acetic acid. This is consistent with the idea that the substance B is the free 2,4-dinitrophenylhydrazone of a keto-dicarboxylic acid, the other (A) being a modification of this probably arising from the treatment with alumina and with methanol. Therefore, α-keto-γ-methyleneglutaric acid may be regarded as a naturally occurring compound in the tulip leaf. Although the possibility exists that the keto analog of γ-methylglutamic acid itself occurs in the tulip plant this is unlikely, inasmuch as γ-methylglutamic acid is not known as a natural constituent of the tulip plant.

While proof is lacking it is evident that the γ-methylene-glutamic acid could exist in tautomeric form in which the double bond would be located between the β and γ carbons. There is as yet no direct evidence for this except the curious point mentioned above that there are two compounds with 2,4-dinitrophenylhydrazine which give rise to γ-methylglutamic acid on hydrogenolysis. The possibilities of isomerism and cyclization are, however, too numerous for this alone to be considered as evidence of the two forms in question.

To obtain evidence of the presence in the plant of the keto analog of γ-methyleneglutamine, 1,1-diphenylhydrazones from tulip leaves were prepared in neutral or weakly acid solution. This was done to avoid the hydrolysis of the keto analog of the amide which would be liable to occur in the formation of the 2,4-dinitrophenylhydrazones in the more acid solution. The hydrazones were extracted with ethyl acetate, the extract washed with water and the product from half of this extract was hydrogenated in 80% ethanol, chromatographed and tested for amino acids. The product from the remaining half was chromatographed without being hydrogenated.

The most interesting constituents on the chromatograms resulting from hydrogenolysis of the diphenylhydrazones of tulip leaves were two substances that occurred in excess of any other spots in the areas on blanks using the non-hydrogenated hydrazones. One of these substances was chromatographically identical with γ-methylglutamic acid and this occurred in quantity. The other substance occurred in the region occupied by γ-methylglutamine, the position of which was established by comparison with a sample prepared by reduction of authentic γ-methyleneglutamine as described in a previous note.[4] These results again furnish evidence that the keto analog of γ-methyleneglutamic acid exists free in the tulip but they suggest that the keto analog of γ-methyleneglutamine may also be a constituent and metabolite of the tulip plant.

From this work it is clear that the list of metabolically important keto acids of plants requires to be greatly extended. Hitherto, pyruvic acid, oxaloacetic acid and $\alpha$-ketoglutaric acid have been most prominent in views on the role of keto acids in plant metabolism. These views should now take into account other keto acids. In particular, and with special reference to the tulip plant, they should recognize that the keto analogs of glycine and of the new $\gamma$-methyleneglutamic acid occur and even embrace the possibility that the keto analog of the amide $\gamma$-methyleneglutamine may also occur free.

Grateful acknowledgment is made that this work forms part of investigations that are supported by the Grasselli Grant to the New York State College of Agriculture at Cornell University for work being directed by F. C. S..

BOTANY DEPARTMENT
CORNELL UNIVERSITY
ITHACA, NEW YORK

## $\gamma$-Methyleneglutamine and $\gamma$-Methyleneglutamic Acid in the Tulip (*Tulipa gesneriana*)

BY R. M. ZACHARIUS, J. K. POLLARD AND F. C. STEWARD

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As part of a survey of the soluble nitrogenous compounds of plants, extracts of the tulip bulb were examined by paper chromatographic methods. In the map as published by Steward and Thompson[1] two unidentified substances were recognized and designated Unknowns No. 4 and 12. One of these was an acid-unstable substance that reacted in a characteristic manner with ninhydrin to give a chocolate brown color on the paper; the other, No. 4, an obviously acidic substance, also reacted brown with ninhydrin on paper, although on long standing this spot turned purple. The presence of free ammonia in the acid hydrolysate of Unknown No. 12 gave rise to the idea that this substance was an amide.[2]

With the publication of the evidence leading Done and Fowden[3] to regard $\gamma$-methyleneglutamine as a constituent of the peanut plant, it was an obvious suggestion that this substance might be identical with Unknown No. 12 from the tulip bulb.

To establish the identity of unknown No. 12 as it occurs in the tulip bulb with that of $\gamma$-methyleneglutamine as it occurs in the peanut plant, small quantities of these two substances were isolated from the respective plants by the use of paper chromatographic methods. This was done on a number of sheets of chromatographic paper by the use, successively, of phenol–$H_2O$, and collidine–lutidine–$H_2O$, but applying the chromatographic procedures not to a single spot of the extract but to a broad band applied to one edge of the paper. In this way by successive operations chromatographically pure products corresponding to $\gamma$-methyleneglutamine from the peanut and Unknown No. 12 from the tulip were obtained. This was also done for the corresponding free acid. It was possible to show by one directional co-chromatography in phenol–$H_2O$, collidine–lutidine–$H_2O$ and butanol–acetic acid–$H_2O$, that these two compounds as isolated from the tulip and the peanut plant, respectively, were identical.[2]

At this point there was a strong presumption that Unknown No. 12 in the tulip was $\gamma$-methyleneglutamine and Unknown No. 4 was $\gamma$-methyleneglutamic acid. Through the coöperation of Dr. Fowden, samples became available of $\gamma$-methyleneglutamine as isolated by Done and Fowden[3] from the peanut plant and of $\gamma$-methyleneglutamic acid from the same source as well as synthetic $\gamma$-methylglutamic acid.[4]

To enable the necessary comparisons to be carried out, 12 mg. of the $\gamma$-methyleneglutamine was isolated from the tulip bulb. This was carried out broadly as follows: An aqueous extract of the bulb was treated with mercuric nitrate, the mercury precipitate centrifuged and retained, and the mercury removed by hydrogen sulfide after suspension in 40% ethanol. The mercuric sulfide was washed and discarded and the supernatant liquid was evaporated to a small volume and applied to a powdered cellulose (200 g.) column for chromatography with butanol saturated with water, using an automatic fraction collector. All fractions containing chromatographically pure Unknown No. 12 were combined, evaporated to a small volume and absolute ethanol was added until crystals formed. The $\gamma$-methyleneglutamine as isolated in this manner was recrystallized from ethanol–$H_2O$ and used for the following tests.

1. By co-chromatography it was established that the material as isolated by Fowden from the peanut plant and the material as isolated above from the tulip bulb were chromatographically identical.

2. The infrared absorption spectra of both of these materials were obtained and the curves, as shown at Fig. 1, were identical.

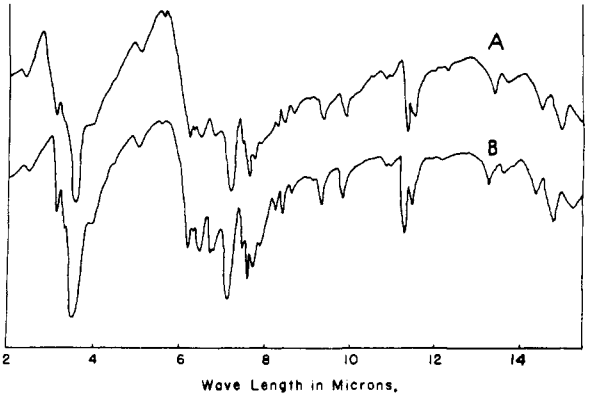


Fig. 1.—Infrared absorption curve in a Nujol mull of: A, $\gamma$-methyleneglutamine isolated from the tulip; B, $\gamma$-methyleneglutamine isolated from peanut by Done and Fowden.[4]

3. Such other physical properties as are known (solubility, crystalline form and melting point) are consistent with the identity of the material isolated from tulip and the material from the peanut. Melting points determined between glass cover slips on a Fisher melting point block showed identical behavior for the tulip amide, the peanut amide and mixtures of both. Melting occurred

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