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 α-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 γ-methyleneglutamic acid occur and even embrace the possibility that the keto analog of the amide γ-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..

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γ-Methyleneglutamine and γ-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 γ-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 γ-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 γ-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 γ-methyleneglutamine and Unknown No. 4 was γ-methyleneglutamic acid. Through the coöperation of Dr. Fowden, samples became available of γ-methyleneglutamine as isolated by Done and Fowden[3] from the peanut plant and of γ-methyleneglutamic acid from the same source as well as synthetic γ-methylglutamic acid.[4]

To enable the necessary comparisons to be carried out, 12 mg. of the γ-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 γ-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.

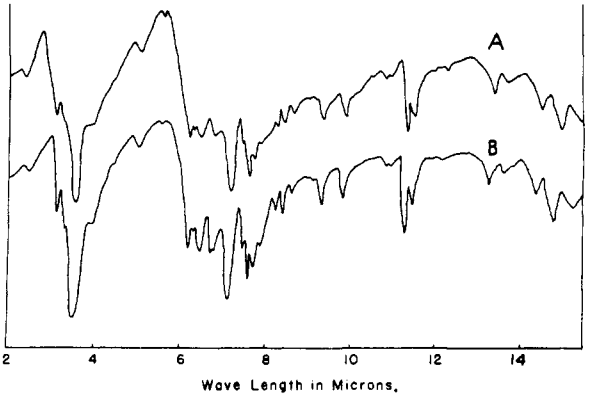

Fig. 1.—Infrared absorption curve in a Nujol mull of: A, γ-methyleneglutamine isolated from the tulip; B, γ-methyleneglutamine isolated from peanut by Done and Fowden.[4]

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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(2) R. M. Zacharius, Ph.D. Thesis, Univ. of Rochester, 1953.

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(4) J. Done and L. Fowden, *ibid.*, **51**, 451 (1952).

from 177–181° for all determinations. Gas was evolved on melting but no discoloration was observed. (Done and Fowden reported melting with decomposition in a sealed tube between 174–183°.)

As further proof of these relationships, γ-methyleneglutamine from the tulip was hydrolyzed to the γ-methyleneglutamic acid which in turn was hydrogenated and proved to be chromatographically identical with the synthetic γ-methylglutamic acid as furnished by Dr. Fowden.

Through the interest and coöperation of Dr. Alton Meister of the National Cancer Institute, Bethesda, it was possible to investigate the enzymatic oxidation of the isolated γ-methyleneglutamine. The effect of L-amino acid oxidase of rattle snake venom was followed manometrically by the method of Meister[5] and it was found to be oxidized to the extent of 65% as compared with 85% for L-glutamine and 88% for L-asparagine. The isolated material may tentatively be considered to have the L-configuration.

As a result of the evidence documented above, it is now possible to conclude that the substance recognized and described[1] as Unknown No. 12 in the tulip bulb is identical with the substance described as γ-methyleneglutamine by Done and Fowden.[4] Also the substance described as Unknown No. 4 in the tulip bulb is to be regarded as γ-methyleneglutamic acid. Both of these substances exist free in the tulip plant and their metabolic role will be of great interest.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY, SANTA BARBARA COTTAGE HOSPITAL RESEARCH INSTITUTE]

The Dispersion of Testosterone in Aqueous Bovine Serum Albumin Solution

BY FRITZ BISCHOFF AND ROYCE D. STAUFFER

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By means of dialysis through a semipermeable membrane it is shown that the distribution of testosterone between water and aqueous bovine serum albumin solution follows the classic laws of distribution between two phases, based on the ratio of solubilities in these phases, over a sixfold concentration of albumin, a shift of *p*H from 5.2 to 7.4 and a twelve-fold change in testosterone concentration. At *p*H 5.2 and saturation, the albumin adsorbs a maximum of 3 moles testosterone per mole albumin, at *p*H 7.4, 4 moles. According to the Scatchard concept, there is one class of adsorbing points, the number of points being large. Under the conditions of dialysis the testosterone in the water phase is in a metastable state in which the solubility exceeds that previously reported; albumin thus brings about an increase in the fugacity of testosterone in the water phase. The conditions under which the two solubility equilibria are attained are described and considered according to the Gibbs concept. The extinction coefficient for testosterone, not previously reported in aqueous solution, was ascertained.

Introduction

The marked dispersive action of serum albumin solutions upon testosterone and other steroids[1–3] is undoubtedly a governing factor in the transport of steroids in body fluids and has led to the preparation of solutions suitable for intravenous injection.[4–6] Testosterone appeared idealy suited for a study of the nature of the attraction between albumin and an un-ionizable steroid, since its solubility in water is sufficiently high to assure accuracy in analysis. The orientation by adsorbents of organic ions of various sizes, as well as inorganic ions has been the subject of intensive investigation.[7,8]

The original plan, which was successfully consummated, sought to obtain the equilibrium concentrations of testosterone in water and in an albumin solution separated by a semipermeable membrane. The results were such that the solubility of testosterone in water had to be reinvestigated. In the case of progesterone, solubility depended upon two equilibrium phases in aqueous non-protein solution.[1] An analog was therefore sought in the case of testosterone and found.

Experimental

Equilibrium between Water and Albumin.—Armour crystalline bovine serum albumin and Schering testosterone were used in these experiments. The dialyzing chambers were replicas of those described by Jorgensen.[9] The junction between the two chambers and the dialyzing membrane was sealed with paraffin, which is ideally suited for the purpose as it was shown not to adsorb testosterone from aqueous solutions.

The dialysis was carried on in a cabinet at 37.5° with rocking by a mechanical shaker. Preliminary experiments indicated that equilibrium in dialysis was established in 24 hours. This was confirmed by the experiments in which the results for transfer from albumin to water and water to albumin are in substantial agreement.

The membrane used was a vegetable parchment supplied by Central Scientific Company and was selected because the adsorption of testosterone by it was nil. The blank was appreciably reduced by washing with distilled water and air drying. In testing for albumin leakage across the membrane, it was found that the foam test was more sensitive than chemical tests. A concentration of 1 part albumin in 100,000 parts water is detectable by the foam test. In all the experiments reported, the leakage of albumin, if any, into the water phase did not exceed this concentration. It was necessary to reject a considerable number of experiments because of imperfect parchment.

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