ACTA CHEMICA SCANDINAVICA 7 (1953) 38–44

α-Keto-Acids in Green Plants

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The occurrence of α-keto-acids in plants, with particular reference to the biological N_2-fixation, was studied in this laboratory in the 1930's. Virtanen and Laine [1] believed that they had found oxaloacetic acid in pea plants by the hydrazine method of Szent-Györgyi and Straub [2]. Since this method gives unreliable results with green parts of plants it was modified later [3]. Virtanen *et al.*[4] were also able to detect oxaloacetic acid in young pea plants by determining the CO_2 produced by the action of aniline. Since oxaloacetic acid seemed to disappear rapidly from green plants on crushing them in dilute sulphuric acid, thus giving variable results, Virtanen and Arhimo [3] modified the method by adding sodium hydroxide to the plants on crushing in order to raise the pH to about 12—13. The addition of alkali stops respiration immediately and consequently, the decomposition of oxaloacetic acid is prevented more effectively than by the use of acid. Oxaloacetic acid could regularly be found by this method both in pea and clover leaves. Virtanen *et al.*[5, 6] studied in detail this method and the effect of light and other factors on the oxaloacetic acid content of different plants (*Pisum*, *Faba*, *Trifolium pratense*, *Phleum*, *Hordeum* and *Avena*). It was noticed that oxaloacetic acid disappeared in the dark, and it was not detected in maturing plants after flowering. In young pea plants 30—50 μg oxaloacetic acid were found per 1 g fresh weight, in red clover about double this amount.

Other methods were also used for estimation of keto-acids in green plants. Virtanen *et al.*[7] determined these acids in crushed plant material by forming from them 2,4-dinitro phenylhydrazones, which were then reduced with sodium amalgam to the respective amino acids. In this manner pyruvic, oxaloacetic, and α-keto glutaric acids were demonstrated in growing pea and red clover (*e.g.* in red clover 12 μg pyruvic acid, 18 μg oxaloacetic acid and 42 μg α-keto glutaric acid per 1 g fresh material). The method was, however, laborious and

unreliable, the results varying in the control experiments from 40 to 80 % of the theoretical values. Moreover, ascorbic acid and dehydroascorbic acid interfered with the determination of oxaloacetic acid [6].

Ketoglutaric acid has also been determined [6] in plants by oxidizing the 2,4-dinitro phenylhydrazones with potassium permanganate according to Krebs [8] and by determining the succinic acid formed from ketoglutaric acid.

Thus we considered that by using different methods we had been able to prove the occurrence of α-keto-acids, essential to the carbohydrate metabolism, in green plants.

Later, James and James [9] have observed that barley roots poisoned with formaldehyde or certain aromatic sulphonic acids give reactions for pyruvic acid not shown by normal plants. Bennett [10] observed that nitroprusside and other tests indicate that pyruvic acid may accumulate in the Ebenezer onion. Morgan [11] estimated pyruvic acid in the juice of onion as 2,4-dinitro phenylhydrazone. He found 0.1034 g pyruvic acid per 100 ml juice. Since no acid was found in the intact onion, he concluded that pyruvic acid was formed from a precursor or precursors while the onion was crushed. Wilson *et al.*[12] were unable to find oxaloacetic acid in pea plants and thus could not confirm the observations made in this laboratory. It is, however, true that these determinations were made on the expressed plant sap from which the proteins were precipitated with tungstate in a sulphuric acid solution, this treatment, according to the above, preventing ineffectively the decomposition of oxaloacetic acid.

In order to check our previous findings of the occurrence of keto-acids in green plants we carried out a number of determinations with pea using a paper chromatographic method for separation and identification of 2,4-dinitro phenylhydrazones of keto-acids.

EXPERIMENTAL

Being labile compounds α-keto-acids are not suitable to the paper chromatographic technique. Their phenylhydrazones are more stable and therefore more suitable for the purpose. The first to determine keto-acids in preparations of animal origin by paper chromatography as 2,4-dinitro phenylhydrazones were Cavallini *et al.*[13]. Since the detection of keto-acids in plants requires pretreatment of the material for stabilization of these acids, particularly oxaloacetic acid, and special measures for liberation of the 2,4-dinitro phenylhydrazone fraction from impurities disturbing the chromatography, a suitable method had to be devised. Development of the chromatograms also required more elucidation, in order to enable separation of the three α-keto-acids in question. After the affecting factors had been proved experimentally the following method was worked out and used in the determination of keto-acids in plant material.

Treatment of the plant material: 10 g of plant material were crushed in a mortar with 10 ml of 2 *N* NaOH which brought the pH to about 13. The crushed material was squeezed

through linen, the mortar and the precipitate were washed with 200 ml of 8 % trichloroacetic acid and the washings added to the extract whereby its pH fell to about 1. The solution was centrifuged and the supernatant liquid added to 10 ml of 1 % 2,4-dinitro phenylhydrazine reagent, mixed well and allowed to stand at room temperature for about 1 h. Crushing and centrifugation were regularly performed within 15 min. Since the plants taken from nature could not be treated as rapidly as the plants taken from the greenhouse of the laboratory, attempts were made to cool the plants rapidly by placing them in a glass bowl in a mixture of CO_2-ice and alcohol. The keto-acids then remained undecomposed for a longer time.

Reagents: 8 % solution of trichloroacetic acid was regularly made just before use from a 50 % basal solution which was kept in an icebox and renewed weekly. 1 % solution of 2,4-dinitro phenylhydrazine was prepared by dissolving 1 g in 5 *N* H_2SO_4. This, also, was kept in an icebox and renewed weekly.

Extraction: The solution containing 2,4-dinitro phenylhydrazones was shaken with ethylacetate until no further colour could be extracted. The combined extracts were then shaken with small amounts of 10 % Na_2CO_3 until the new soda phases remained colourless. 2,4-dinitro phenylhydrazones of keto-acids were brought to the soda solution. To this was added 2 *N* H_2SO_4 until the reaction was acid. The yellow colour was again extracted into ethylacetate and dried with anhydrous Na_2SO_4 overnight, after which the salt was separated by filtering and washing with a small amount of ehylacetate. The combined ethylacetate solutions were then evaporated *in vacuo* to about 1 ml.

Removal of fatty acids and other impurities: The paper chromatographic analysis of the ethylacetate solutions proved to be impossible because the spots of the dinitro phenylhydrazine derivatives were badly deformed due to impurities, principally fatty acids, present in the solution. These were removed as follows: Equal amounts of "bentonite"* and "Celite 545"** were mixed and of this mixture an adsorption column of 1 cm diam. and approximately 3 cm length was formed. Ether was allowed to pass through the column at the rate of 4 drops per minute, after which the ethylacetate extract was added to the column. Fatty acids *etc.* were then eluted with approximately 30 ml of ether. The adsorbent, which contained the adsorbed 2,4-dinitro phenylhydrazones of keto-acids, was then pushed out from the chromatographic tube into a centrifuge tube, where the dinitro phenylhydrazone derivatives were displaced from the adsorbent by three extractions with 10 ml of 10 % soda solution, the extract being clarified each time by centrifugation.

The combined extracts were made acid with 2 *N* H_2SO_4 and the 2,4-dinitro phenylhydrazones were extracted into ethylacetate which was then dried and concentrated *in vacuo* to 1 ml. 0.1 ml of this solution, added in small fractions to form a sharp concentrated starting spot, usually contained enough material for one chromatogram.

Paper chromatographic analysis: Both partition chromatographic separations with many organic solvents and adsorption chromatographic separations with different buffers in water solution were studied. Although separation by both principles was possible the latter method proved to be superior in our work. All buffers above pH 7.0 gave a distinct separation, the glycine-NaOH buffer of Sörensen, with pH 8.4 being the most suitable. This buffer was, therefore, used as a 0.1 molar solution whereby the three keto-acids in question were already well separated in one-dimensional runs (Fig. 1). Best results

* "Wyoming's" bentonite.

** Johns Manville Co., Box 60, New York 16, N.Y., U.S.A.

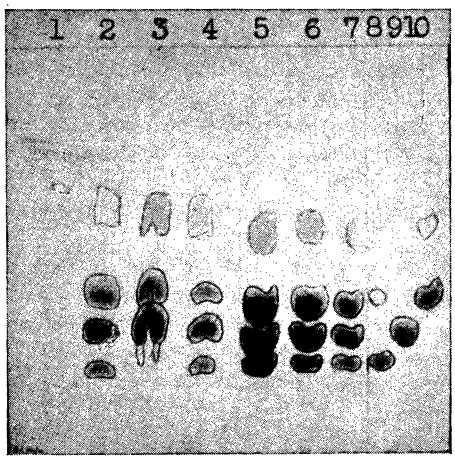

Fig. 1. Paper chromatographic analysis of an experiment with pea 20—21.VI.1952. Paper adsorption chromatogram by 0.1 M *Sörensen glycine-NaOH-buffer, pH 8.4. Whatman No. 1 paper, at 22° C for 8 h.*

1. *Plants harvested in the night, no sun for 9 h, NaOH added.*
2. *Plants harvested in the afternoon, kept at −10° C for 1 ½ h after which NaOH added.*
3. *Plants harvested in the afternoon, H_2SO_4 added.*
4. *Plants harvested in the afternoon, NaOH added.*
5. *Control, pyruvic, α-ketoglutaric, and oxaloacetic acids, 80 μg of each.*
6. *Control, as 5 but 50 μg of each acid.*
7. *Control, as 5 but 30 μg of each acid.*
8. *Control, oxaloacetic acid, 30 μg.*
9. *Control, α-ketoglutaric acid, 30 μg.*
10. *Control, pyruvic acid, 30 μg.*

were obtained in an atmosphere containing phenol vapours and traces of HCN. R_F-values: oxaloacetic acid 0.78, ketoglutaric acid 0.69, and pyruvic acid 0.45 (the faster and more distinct of the two spots produced by pyruvic acid). The "satellite" spots of the dinitro phenylhydrazone derivatives, probably due to isomeric forms, were all more strongly adsorbed having R_F-values below 0.45.

In the semiquantitative determinations the procedure was as follows: The 2,4-dinitro phenylhydrazone spots were cut off and the paper strips extracted in a beaker with 3 × 1 ml of 10 % Na_2CO_3. 3 ml of 2 *N* NaOH were added to the filtered solution. The red colour formed was measured photometrically by using the 525 mμ filter and the solution obtained by the same treatment from a strip of paper cut from the same sheet outside the spots was used as a standard. The "satellite" spots were thus left out from the determinations but usually they did not represent more than about 10 % of the total material.

As an example of the determination of keto-acids in plants by the above method we present the following:

20.6.1952. Samples were taken in the afternoon from pea plants growing in the greenhouse of the laboratory. The plants had been sown on May 21 and were not yet in flower. The weather was sunny on the day of harvest and had been so on the day before. The aim of the experiment was to elucidate not only the presence and quantities of ketoacids in plants but also the effect of alkali and acid treatment on the preservation of keto-acids. Further, the effect of low temperature on the preservation of keto-acids was examined.

Next morning before sunrise new samples were taken from the same lot of plants and crushed with alkali. This experiment was performed to control the earlier findings about the rapid disappearance of oxaloacetic acid in the dark when assimilation does not take place.

The experiments thus included the following:

1. 10 pea plants, fresh weight 12 g. The plants were crushed with alkali. (About 20 μg oxaloacetic acid, 40 μg α-ketoglutaric acid, and 20 μg pyruvic acid per 1 g fresh weight were found).
2. 10 pea plants, fresh weight 18 g. The plants were crushed with diluted sulphuric acid. (Oxaloacetic acid was not found.)
3. 10 pea plants, fresh weight 18 g. The plants were cooled to below $-$ 10° C by placing them in a glass bowl and keeping this in a mixture of CO_2-ice and alcohol for about 1 $\frac{1}{2}$ h, fresh CO_2-ice being added to the alcohol solution as required. The plants were then crushed with alkali. The results were practically the same as in experiment 1.
4. 10 pea plants, fresh weight 12 g. The plants which had received no sun for 9 h, were crushed with alkali. No oxaloacetic acid, and very small amounts of α-ketoglutaric acid and pyruvic acid were found.

(In experiments 1,2, and 3 the plants were removed in the afternoon at 16.30—17.00, in experiment 4 in the morning at 3.00.)

DISCUSSION

Fig. 1 illustrates the results of the experiments described above (20.6—21.6). The results show that all the three keto-acids, oxaloacetic acid, α-ketoglutaric acid and pyruvic acid are found in pea plants grown in the light, thus confirming the earlier results from this laboratory. Oxaloacetic acid evidently produces some pyruvic acid in the analysis but, judging from the chromatograms, pyruvic acid is present to some extent in the intact plants and is not formed only from oxaloacetic acid during the treatment and analysis of the plant material. The values found are obviously too low for oxaloacetic acid and too high for pyruvic acid. The two spots given by 2,4-dinitro phenyl-hydrazones of pyruvic acid and oxaloacetic acid, the slower ones being much weaker than the faster, are probably caused by cis-trans-isomeric forms. For this further reason the values recorded in the experimental part are only semiquantitative.

Oxaloacetic acid disappears rapidly from the plants in the dark in accord with the previous results from this laboratory. Under this condition both ketoglutaric acid and pyruvic acid are also decreased in quantity. The accumulation of oxaloacetic acid in the light and rapid disappearance in the dark suggest that the formation of this keto-acid is connected with the CO_2-assimilation.

On crushing the pea plants oxaloacetic acid disappears very rapidly, an indication of great activity of oxaloacetic acid decarboxylase and possibly even of some other oxaloacetic acid-consuming system in the cells. Since oxaloacetic acid is stabilized both in acid and alkaline reaction, it can be expected that its disappearance is prevented when the plant is crushed either with acid or alkali. The results obtained in this work as well as in the previous work of this laboratory indicate that the effect of alkali is far superior to that of acid, the chief reason obviously being that acid does not intrude into the cell so rapidly as does alkali.

When the plants are crushed with dilute sulphuric acid, oxaloacetic acid often disappears entirely as happened in the experiment described above. In two out of three samples of young pea plants, crushed with sulphuric acid, no oxaloacetic acid was found; in the third was found a little, which shows that in the treated plant material oxaloacetic acid may appear sometimes, but not regularly. In all these samples oxaloacetic acid was found when the pea plants were crushed with alkali. Oxaloacetic, α-ketoglutaric, and pyruvic acids were found both in the plants grown in the greenhouse of the laboratory and in nature providing that they were harvested in light. Rapid cooling of plants to below $-10°$ C prevents the disappearance of oxaloacetic acid for at least 2 hours.

Pea seeds germinated in the dark for 7 days contained no oxaloacetic acid but only α-ketoglutaric and pyruvic acids.

SUMMARY

A paper chromatographic method has been worked out for determination of α-keto-acids in green plants in the form of 2,4-dinitro phenylhydrazones. This has corroborated the results obtained in this laboratory in the 1930's by different methods on the presence of oxaloacetic acid, α-ketoglutaric acid and pyruvic acid in green plants. Other keto-acids were not detected in our chromatograms. In accord with the earlier observations it has been possible to confirm the rapid disappearance of oxaloacetic acid in the dark. On crushing the plant material oxaloacetic acid disappears, more readily in acid than in alkaline solution.

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Received November 20, 1952.