Estimation of α-Keto Acids in Plant Tissue: a Critical Study of Various Methods of Extraction as Applied to Strawberry Leaves, Washed Potato Slices and Peas

By F. A. ISHERWOOD
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

AND C. A. NIAVIS
Botany School, University of Cambridge

(*Received* 29 *February* 1956)

The accurate estimation of the α-keto acids present in plant tissues is important because these acids occupy a central position in the metabolism of the plant and thus may be vital to our understanding of the physiological conditions which govern the storage of many fruits and vegetables.

The main uncertainty in the estimation is the stability of the α-keto acids and related compounds in contact with the tissue during the procedure used for killing. Preliminary results on strawberry leaves showed that different amounts of α-keto acids were obtained, according to the procedure. This observation focused attention on the peculiarly difficult nature of the estimation of substances, such as α-keto acids, which are intermediates in the metabolic pathways linking fats, carbohydrates and amino acids. Only small amounts are present under normal physiological conditions and they are probably closely associated with multi-enzyme systems, which are confined for the most part in small subcellular units such as mitochondria and microsomes. It is difficult to guess at the importance that this juxtaposition of enzymes and possible substrates will have in the complex reactions that occur during the killing of the tissue by heat treatment. One fact, however, is clear: the enzymes and substrates must be effectively separated if the amounts of α-keto acids in the final extracts are to be representative of those in the tissue at the moment of killing.

In the present study it has been shown that the problem of extracting the α-keto acids unchanged from the plant tissue depended, not only on killing the tissues so that the very active enzymes present should neither produce nor destroy the acids, but also on choosing a medium which did not cause similar reactions chemically. An important question, which arose early in this study, was whether the α-keto acids were bound to other compounds in the tissue and liberated by the action of the extracting medium.

The extraction procedure developed in the present work is to freeze the tissue in a mixture of solid carbon dioxide and methanol (−70°) and then to disintegrate the frozen material in a high-speed blender in cold (−2°) 0·6M metaphosphoric acid (HPO_3). With strawberry leaves, which are very thin, the preliminary freezing can be omitted without apparent detriment.

The principle of this procedure is that it is best to 'arrest' the activity of the enzymes by cooling to a low temperature and then inactivate by chemical treatment while they are still in this arrested state. Any form of heat inactivation is open to the criticism that the enzymes are excited to great activity during the brief warming-up process before inactivation. Cooling can be made much more rapid than any feasible heating process; in addition, the activity of the enzymes is progressively arrested as the temperature falls, whereas the reverse is true of the heating process. This principle, which is of general application, can be applied to the estimation of other labile intermediates in plant tissues, e.g. phosphoric esters. The actual method of chemical inactivation will depend on the nature of the intermediates.

EXPERIMENTAL

Plant material

Freshly picked strawberry and pea leaves, soaked pea cotyledons, fresh garden peas and washed potato slices were used.

For strawberry and pea leaves representative samples of the leaves were taken for most of the experiments, but where the differences expected were small the leaves were split along the midrib, one half being used as control.

The washed potato slices and pea cotyledons were prepared as follows: The potato slices were cut on a large-scale stainless-steel microtome (potato variety King Edward VII; disks diameter 3 cm., thickness 2–8 mm. cut transversely to stem–heel axis of tuber) and were washed in running tap-water 3–4 hr. to remove all the soluble material from the cut cells on the surface. Alternate slices were collected and

treated separately as a control. Just before analysis the slices were dried between filter paper. The dried peas (variety Onward) were split, the testa and plumule carefully removed with a sharp razor and the halves from each pea kept separate. The cotyledons were placed on three layers of Whatman no. 1 filter paper in two covered Petri dishes (12 cm. diameter). Each cotyledon was placed on its outside surface and the filter paper soaked in water so that it was just damp. An extra 5 ml. of water was added to each and the dishes were allowed to stand at room temperature for 18 hr. Two more additions of 5 ml. of water were made to each Petri dish during the next 6 hr. At the end of the period the samples were weighed, after excision of any plumules that had not been removed with the testa. The cotyledons were then washed thoroughly with tap water followed by distilled water. Prepared in this way, the cotyledons should represent strictly comparable samples. They contained very little ethanol, which meant that fermentation had not commenced to any extent. In a typical experiment 10 g. of the dry cotyledons weighed 23·5 g. after taking up water. The figure varied from 19·5 to 25 g. in various experiments, but in any one experiment the control sample was within 0·5 g. of the other.

Killing and extraction of plant tissue

Typical examples of the three basic procedures examined are as follows:

Cold acid extraction. The strawberry leaves (5–10 g.) were plunged into 60 ml. of a mixture of solid CO_2 and methanol (−70°) in a wide-mouthed Dewar vessel and left for a few minutes. Leaving the leaves for periods up to 30 min. did not have any effect on the final results so long as the temperature never rose above −70°.

The frozen leaves were immediately added to 50 ml. of 0·6 M-HPO_3 in the glass container of a high-speed blender (Measuring and Scientific Equipment Ltd.). The temperature of the solution was about −2° and the bowl of the blender was surrounded by a freezing mixture of finely crushed ice and methanol. The leaves were disintegrated at 14000 r.p.m. for 10 min. The mush (temperature 0–2°) was centrifuged and the residue treated in the blender with 50 ml. of ice-cold 0·6 M-HPO_3. The residue after centrifuging was washed once more with 50 ml. of 0·6 M-HPO_3. The extracts were combined.

In treating pea seeds in this way a persistent emulsion formed during the disintegration, which could be broken by adding ether before centrifuging.

Killing in boiling methanol. Strawberry leaves (5–10 g.) were plunged into 60 ml. of boiling methanol in a 200 ml. beaker and kept there for 10 sec. The methanol was decanted and concentrated *in vacuo*. This extract was added to the combined aqueous extracts of the killed leaves obtained later. The killed leaves were added to 60 ml. of warm (50°) 0·2 M-NaH_2PO_4 (pH 4·6) and then disintegrated for 7 min. The residue was re-extracted with 50 ml. of 0·2 M-NaH_2PO_4 and finally with 60 ml. of 0·6 M-HPO_3. The combined extracts (including that from the methanol killing) were centrifuged from any precipitated protein.

Where the leaves were killed and disintegrated in one operation, the final aqueous methanolic or ethanolic extract was neutralized by the addition of dilute aqueous NaOH and concentrated *in vacuo* (final pH of aqueous concentrate about 6·0). The precipitate that sometimes formed was not removed because it adsorbed variable amounts of α-keto acids.

Cold alkali extraction. This was essentially the same as the method described by Virtanen, Miettinen & Kunttu (1953), except that 0·6 M-HPO_3 was used instead of trichloroacetic acid for acidification of the alkaline extract. Trichloroacetic acid is extracted from aqueous solution by organic solvents and interferes with the subsequent chromatography of the 2:4-dinitrophenylhydrazones.

Preparation of 2:4-dinitrophenylhydrazones

To the combined extracts (150–200 ml.) at room temperature was added immediately 20 ml. of 1% (w/v) 2:4-dinitrophenylhydrazine in 5 N-H_2SO_4 and the mixture allowed to stand for 45 min. The hydrazones were extracted by shaking with ether (0·4 vol. repeated four times). The combined ethereal extracts were shaken with a slight excess of saturated $NaHCO_3$ (40 ml.) so that the mixture was alkaline (pH 8·4). (In earlier experiments 10% Na_2CO_3 was used and, though this caused no appreciable destruction of the hydrazones, it did extract much larger quantities of the phenols present because the mixture was usually more alkaline.) Careful tests showed that the use of $NaHCO_3$ for the extraction did not cause any formation of 1-hydroxy-6-nitro-1:2:3-benzotriazole from the 2:4-dinitrophenylhydrazine (cf. Towers & Mortimer, 1954). The aqueous phase was separated and the ethereal layer again shaken with aqueous $NaHCO_3$ (15 ml.) to remove the last traces of hydrazones. Traces of colloidal material sometimes interfered with the clean separation of the phases, and in such cases it was inadvisable to shake vigorously or else a persistent emulsion was formed. Alternatively the mixture could be centrifuged. The combined aqueous phases were acidified to pH 2·0 with 3 N-H_2SO_4 and then extracted (0·5 vol. repeated three times) with $CHCl_3$ containing 15% (v/v) of ether. The combined $CHCl_3$–ether extracts were evaporated under reduced pressure to dryness. The residue was dissolved in absolute ethanol to give a final concentration of hydrazones suitable for the chromatographic separation by the method of Isherwood & Cruickshank (1954).

Chromatography

Three α-keto acids were estimated—pyruvic, α-oxoglutaric and oxaloacetic acids—though the 2:4-dinitrophenylhydrazones of many other keto acids were present on the paper chromatograms.

The solvent systems used were as follows: pyruvic acid, benzene–*tert.*-pentanol–ethanol–water (50:30:10:20 by vol. respectively, water-poor phase used as solvent on paper dipped in 0·2 M-Na borate buffer, pH 8·2, and dried); α-oxoglutaric acid, *tert.*-pentanol–ethanol–water (50:10:40 by vol. respectively on paper dipped in 0·2 M-Na phosphate buffer, pH 6·3, and dried); oxaloacetic acid, *tert.*-pentanol–propanol–NH_3 soln. (sp.gr. 0·880) (65:5:30 by vol. respectively).

RESULTS

Preliminary experiments

A large number of experiments were carried out in which strawberry leaves were disintegrated in different extraction media. Among the media tried were HPO_3 and H_3PO_4 (concentrations 0·6–2·5 M, temperatures 20–70°), $HClO_4$ (0·5 M, 70°), 0·2 M sodium phosphate buffers (pH 2–10·5 at 70°), 70%

aqueous ethanol (at 78°) and N sodium hydroxide plus 0·1 M stannous chloride (to prevent the phenols' oxidizing to give dark-coloured pigments). The results showed that the amount of each of the α-keto acids extracted from the plant tissue varied with the conditions used (200–300 % differences were not uncommon) and that several factors probably contributed. Based on these preliminary experiments, a series of more detailed experiments were carried out to elucidate the reasons for the variations, and these experiments are described below. They are grouped under four main headings according to the main killing and extraction method under investigation. There is some overlapping in detail, but this is understandable where experiments are repeated many times with slight changes in experimental technique as a result of experience with other methods. The four main types were as follows: (1) maceration in acid at different temperatures; (2) maceration in alkali; (3) use of boiling alcohols to inactivate the enzymes; (4) maceration in ice-cold HPO_3.

Maceration in acid at different temperatures: yield of pyruvic and α-oxoglutaric acids from strawberry leaves

Experiments were made for the most part with 0·6 M-HPO_3 as extraction medium; at the lowest temperature of extraction, −10°, a solution containing 4 N-HCl and 0·6 M-HPO_3 was used in place of the 0·6 M-HPO_3 in order to prevent the mixture freezing. All conditions except that of temperature were carefully standardized. The figures for the temperature range represent the initial and final temperature of the mixture in the blender. Since the individual experiments were not carried out on samples of leaves at exactly the same physiological state, the results of each experiment have been calculated as a percentage of the amount of α-keto acids found in a comparable sample of leaves analysed by a method arbitrarily assumed as a standard one for the purpose. In the present experiments, the standard method has been to kill the leaves by plunging them into boiling methanol and then extracting them with warm 0·2 M-NaH_2PO_4 (pH 4·6). Comparative results for the various experiments are given in Table 1.

An examination of the results in Table 1 indicates that the yield of both pyruvic and α-oxoglutaric acids is increased if the maceration is made at a higher temperature. The reason for the increase is not the same for both acids, for if an extract made at a low temperature (0–18°) is subsequently heated to 100°, the yield of pyruvic acid increases markedly but that of α-oxoglutaric acid remains stationary. The extra yield of pyruvic acid would appear to be due to the acid hydrolysis of a labile compound in the extract. A clue as to the origin of the extra α-oxoglutaric acid was provided by an experiment made later, based on the fact that if strawberry leaves were plunged for a few seconds into boiling methanol the enzymes were inactivated but very little of the soluble constituents were leached out. It was found that if such killed leaves were macerated with 0·6 M-HPO_3 at different temperatures as described above for fresh leaves, only the yield of pyruvic acid was affected by temperature. The extra α-oxoglutaric obtained with fresh leaves would therefore appear to be due to an enzyme reaction during the killing process.

The recovery of pyruvic and α-oxoglutaric acids added to the extraction media was not less than 90 %.

Labile compound hydrolysed in hot acid to give pyruvic acid. An investigation of the nature of the compound giving rise to pyruvic acid in hot acid suggested that it was possibly phosphoenolpyruvic acid. This acid is known (Lohmann & Meyerhof, 1934) to be readily hydrolysed to pyruvic acid (in N-HCl at 100° about half the acid is split in 10 min.). More specific evidence that this acid might be responsible is given by the experiment described in Table 2.

In this experiment strawberry leaves were extracted with a hot (70°) 0·2 M sodium citrate buffer (pH 5·0) containing mercuric acetate. The

Table 1. *Effect of the temperature of extraction on the yield of α-keto acids from strawberry leaves*

Conditions		Yield of α-keto acids (%)*	
Killing and extraction medium	Temp. range	Pyruvic	Oxoglutaric
0·6 M-HPO_3 + 4 N-HCl	−10–+13°	50	100
0·6 M-HPO_3	0–18	65	94
0·6 M-HPO_3	70–50	120	220
			120
0·6 M-HPO_3	100–70	150	160
		140	120
0·6 M-HPO_3	0–18°	—	—
Extract heated at 100° for 45 min.		380	94

* Compared against extraction with 0·2 M-NaH_2PO_4 (pH 4·6) after killing in boiling methanol.

control experiment contained no mercuric acetate. Mercuric acetate is known to catalyse specifically the breakdown of phosphoenolpyruvic acid, and the results indicate that this acid may be present. The addition of the mercuric acetate increased the yield of pyruvic acid from 140 to 310% (compared with the results of a standard extraction, cf. Table 1) but did not affect the yield of α-oxoglutaric acid.

Maceration in alkali: yield of oxaloacetic acid from strawberry and pea leaves

The results of a series of experiments in which strawberry and pea leaves were extracted with N-NaOH under different conditions are given in Table 3. The results of each experiment are expressed as a percentage of the amounts of the α-keto acids found in a comparable sample of leaves which were extracted with 0·6M-HPO_3 (cf. Table 1).

The results in Table 3 (Expts. 2 and 6) show that maceration of fresh leaves in alkali in the presence of air produces a much larger yield of oxaloacetic acid than does disintegration in 0·6M-HPO_3 (1900 and 200% respectively), but that if air is replaced by nitrogen (Expts. 1 and 5) the yield is practically the same. The extra oxaloacetic acid is formed only if oxygen is present during the disintegration of the leaves. The reaction producing oxaloacetic acid does not depend on the presence of intact enzymes in the leaf, for strawberry leaves which had been killed by plunging into boiling methanol for a short time gave as large a yield of oxaloacetic acid as fresh leaves (1910 and 1900% respectively, Expts. 2 and 3).

The nature of the oxidation reaction which produces oxaloacetic acid is uncertain, but it is known that quinones in alkaline solution can convert amino and hydroxy acids into α-keto acids. The alkaline extract of strawberry leaves is rich in phenols, and in the presence of air will contain the corresponding quinones. Even disintegration in 0·6M-HPO_3 will extract enough phenols for the reaction to occur if the extract is subsequently made alkaline and aerated (Expt. 4). More direct confirmation of this idea is provided by the model experiments given in Table 4. Catechol, and the phenols extracted with ethyl acetate from strawberry leaves disintegrated in 0·6M-HPO_3, were added to hydroxy and amino acids in N-NaOH and treated as described above for strawberry leaves.

The results show that the α-keto acids formed represent about 5–8% of those theoretically possible if the oxidation went to completion. In the strawberry leaf the effective agents may be trihydroxyphenols or leucoanthocyanins which are not soluble in aqueous acids.

Table 2. *Effect of extraction with a hot citrate buffer containing mercuric acetate on the yield of pyruvic acid and α-oxoglutaric acid from strawberry leaves*

Conditions		Yield of α-keto acids (%)*	
Killing and extraction medium	Temp. range	Pyruvic	α-Oxoglutaric
0·2M-citrate buffer (pH 5·0)†	70–50°	140	121
0·2M-citrate buffer (pH 5·0) + 0·06M mercuric acetate†	70–50	310	118

* Compared against extraction with 0·2M-NaH_2PO_4 (pH 4·6) after killing in boiling methanol (cf. Table 1).
† Comparable half-leaves were used for the two parts of each experiment.

Table 3. *Yield of α-keto acids from strawberry and pea leaves with aqueous alkali as extraction medium*

The figures in parentheses are recoveries of added α-keto acids.

Expt.	Extraction conditions	Yield of α-keto acids (%)* Pyruvic	α-Oxoglutaric	Oxaloacetic
1	N-NaOH (pH 13·0); strawberry leaves; nitrogen gas present throughout (i.e. absence of oxygen)	112 (65)	95 (93·5)	102 (81)
2	N-NaOH (pH 13·0); strawberry leaves; air present	—†	29 (36)	1900 (83)
3	N-NaOH (pH 13·0); strawberry leaves killed by plunging into boiling methanol; air present	—	—	1910
4	Strawberry leaves extracted with 0·6M-HPO_3; extract made alkaline (pH 13·0); air present	—	101	205
5	N-NaOH (pH 13·0); pea leaves; nitrogen gas present throughout	—	60	75
6	N-NaOH (pH 13·0); pea leaves; air present	—	55	200

* Compared against extraction with 0·6M-HPO_3.
† Dark-coloured pigments obscured the pyruvic hydrazones on the paper chromatogram.

The stability of the α-keto acids in the alkaline extract from strawberry leaves varied widely. Figures for the recovery of the three α-keto acids added to a typical experiment are given in Table 3 above. Oxaloacetic acid (83%) is much more stable than the other two (less than 40%) and any increase in amount is therefore more obvious.

Use of boiling alcohols to inactivate the enzymes in strawberry leaves

α-Keto acids extracted with aqueous ethanol. The results of two experiments in which strawberry leaves were killed and disintegrated in one operation in boiling ethanol are given in Table 5. The results have been expressed in the same way as those in Table 1. To make sure that all the α-keto acids had been extracted, the residue was finally treated with a warm aqueous buffer and the α-keto acids were estimated in this extract.

The figures in Table 5 show that although extraction with boiling 70% (v/v) ethanol removes practically all the α-keto acids, very little being left in the insoluble residue, extraction with 90% (v/v) ethanol removes only a part. With pyruvic acid about 20% remains in the insoluble residue, whereas with α-oxoglutaric acid the figure is 64%. α-Keto acids added during the distintegration of the leaves in boiling 90% (v/v) ethanol incurred similar losses, so that the difficulty would appear to be in the method of extraction rather than in the presence of bound forms of the α-keto acids. The figures in Table 5 for the recovery of added α-keto acids show that even in the absence of the insoluble residue an appreciable amount of the α-keto acid is lost (40 and 12% for pyruvic and α-oxoglutaric acids respectively). Part of this loss has been traced to the critical nature of the neutralization of the ethanolic extract before concentration under reduced pressure. A precipitate forms which tends to absorb part of the α-keto acids. In addition, there is a suspicion that some destruction takes place during the evaporation of the ethanolic extracts catalysed by compounds in the extracts. The interaction of these various effects probably explains why we have found that, in practice, extraction with either 70 or 90% (v/v) ethanol gives inconsistent duplicates.

Table 4. *Yield of α-keto acids produced by quinone-catalysed oxidation reactions with aqueous alkali as extraction medium*

Conditions	Yield of α-keto acids (mg.) α-Oxoglutaric	Oxaloacetic
Catechol (20 mg.), glutamic acid (1 mg.) and aspartic acid (1·5 mg.) in water (40 ml.), made alkaline (pH 13·0) and aerated for 10 min.	0·07	0·074
Catechol (20 mg.), *iso*citric acid (1·5 mg.) and malic acid (2·0 mg.) in water (40 ml.), made alkaline (pH 13) and aerated for 10 min.	0·094	0·075
Strawberry-leaf phenols,* *iso*citric acid (1·5 mg.) and malic acid (2·0 mg.) in water (40 ml.), made alkaline (pH 13·0) and aerated for 10 min.	0·099	0·065

* Phenols extracted with ethyl acetate from strawberry leaves (20 g.) after disintegration in 0·6M-HPO_3.

Table 5. *Yield of α-keto acids from strawberry leaves with hot aqueous ethanol as the extraction medium*

Figures in brackets are recoveries of α-keto acids added during disintegration of the leaves *a* and to the final ethanolic extract *b*.

Expt.	Conditions	Yield of α-keto acids (%)* Pyruvic	α-Oxoglutaric
1	Boiling ethanol; final concentration 90% (v/v); two extractions	70 [65*a*] [60*b*]	33 [35*a*] [88*b*]
	Residue from above extracted with 0·2M-NaH_2PO_4 (pH 4·6); two extractions	20	64
	Total	90	97
2	Boiling ethanol; final concentration 70% (v/v); two extractions	82 [75*a*] [77*b*]	87 [85*a*] [85*b*]
	Residue from above extracted with 0·2M-NaH_2PO_4 (pH 4·6); two extractions	2	2
	Total	84	89

* Compared against extraction with 0·2M-NaH_2PO_4 (pH 4·6) after killing in boiling methanol.

α-Keto acids extracted with aqueous media. A development which avoids many of the uncertainties of the method described above is to kill the leaf tissue by a very short treatment with boiling methanol and then to disintegrate and extract the residue with an aqueous solution. Preliminary experiments with this method showed that the recovery of pyruvic and α-oxoglutaric acids added to killed strawberry leaves before disintegration was excellent, and that duplicate estimations of the α-keto acids in strawberry leaves agreed closely. The method was then investigated in more detail, various modifications in both the killing and extraction procedure being tried. The results of the experiments are given in Table 6.

The results of Expt. 1 show that when strawberry leaves are killed in boiling methanol it is immaterial whether the α-keto acids are extracted with cold 0·6M-HPO_3 or warm 0·2M-NaH_2PO_4 (pH 4·6). The figures are almost identical, and it seems that if the killed tissue is not extracted under conditions that cause chemical reactions (hot acid or hot and cold alkali) the yield of the two α-keto acids is the same.

For the other experiments in Table 6 a solution containing 5N-H_2SO_4 and 0·6M-HPO_3 at −10° was used as extractant. This solution was developed for killing and extracting the α-keto acids from fresh leaves, with the object of ensuring by the use of the lowest possible temperature without freezing that the activity of the enzymes in the cells was arrested before the disintegration of the leaves into the acid solution. In practice, the final extract was immediately diluted with two volumes of ice-water, because otherwise some of the labile compound that gives pyruvic acid would have been hydrolysed by the very acid extract when the hydrazones were prepared.

In Expts. 2 and 3, the leaves were frozen for ½ hr. in a mixture of methanol and solid CO_2 (−70°). The original idea in these experiments was to replace the water present in the leaves by methanol while the enzymes and α-keto acids were held in an arrested state by the low temperature. It was supposed that in the virtual absence of water there would be no danger of the subsequent heat treatment with boiling methanol activating the enzymes present. In practice, however, it was found that if the temperature of the methanol–solid CO_2 mixture remained below −70° no water was extracted. The treatment with cold methanol was therefore not successful in testing the original idea, but the results of Expt. 2 in which it was used did show that freezing the tissue before killing in boiling methanol had no effect on the figures for pyruvic and α-oxoglutaric acids. In Expt. 3, both samples of the half leaves were frozen in methanol–solid CO_2 (−70°), but only one sample was killed in boiling methanol before extraction with cold acid. The results show that killing in boiling methanol had a striking effect on the figures for pyruvic and oxaloacetic acids. It appeared that the oxaloacetic acid was largely destroyed to give a corresponding amount of pyruvic acid. Inspection of the figures for Expt. 3 (Table 6) indicates that when oxaloacetic acid is negligible (boiling-methanol killing) the pyruvic acid is increased by approximately the equivalent amount (nearly 920 μg. of oxaloacetic acid disappears and 530 μg. of pyruvic acid is formed).

The destruction of oxaloacetic acid is clearly a serious objection to any method of killing in which hot methanol is used (ethanol and propanol give similar results). In cold HPO_3 the α-keto acids are stable, and the use of this reagent for killing and extracting several different plant tissues was investigated in detail.

Table 6. *Comparison of various combinations of methanolic and aqueous extraction methods as applied to strawberry leaves**

Expt.	Preliminary treatment	Extraction conditions	Yield of α-keto acids (μg./100 g.) Pyruvic	α-Oxoglutaric	Oxaloacetic
1	Plunged into boiling methanol	Ice-cold 0·6M-HPO_3	1000	17 500	<20
	Plunged into boiling methanol	Warm 0·2M-$NaHPO_4$ (pH 4·6)	1089	17 500	<20
2	Plunged into boiling methanol	5N-H_2SO_4–0·6M-HPO_3 at −10°	1060	30 300	<20
	Frozen in methanol–solid CO_2 at −70° (left 30 min.), then plunged into boiling methanol	5N-H_2SO_4–0·6M-HPO_3 at −10°	950	28 400	<20
3	Frozen in methanol–solid CO_2 at −70° (left 30 min.), then plunged into boiling methanol	5N-H_2SO_4–0·6M-HPO_3 at −10°	1600	30 400	<20
	Frozen in methanol–solid CO_2 at −70° (left 30 min.)	5N-H_2SO_4–0·6M-HPO_3 at −10°	1070	29 800	920

* Half-leaves were used for each part of the experiments.

Maceration in cold 0·6 M-metaphosphoric acid

The results of a series of experiments in which strawberry leaves, washed potato slices, soaked pea cotyledons and fresh garden peas were used as the plant material are given in Table 7.

The results of Expt. 1 show that freezing strawberry leaves in methanol–solid CO_2 had no significant effect on the yields of the α-keto acids when ice-cold 0·6 M-HPO_3 was used in the blender. Freezing the tissue arrests the activity of the enzymes, and in ideal circumstances they remain in this arrested state until the cells have been broken open by the action of the blender and the contents of the cells are in intimate contact with the HPO_3. If the enzymes are influencing the results obtained when ice-cold HPO_3 is used, then freezing should show a significant effect. Since this does not occur, it follows that with strawberry leaves the enzymes present must be inactivated very rapidly.

Strawberry leaves, however, are thin and open in structure, and reagents such as cold HPO_3 penetrate rapidly into the tissue. Consequently the period before the enzymes are inactivated is short and similar results are obtained by the various methods of killing. With thicker and more compact tissues this is unlikely to be the case, and the other experiments in Table 7 have therefore been made on thicker tissues such as washed potato slices, soaked pea cotyledons and fresh garden peas.

The results of experiments on soaked pea cotyledons (2 and 4) show that freezing the pea cotyledons at −70° before disintegration in ice-cold 0·6 M-HPO_3 causes a small difference whose direction and size appear to vary with the physiological state of the tissue (i.e. amount of water absorbed and age of the peas). In the corresponding experiments (8 and 9) with potato slices, the difference is very small and is not affected by the thickness of the tissue.

Expt. 3 illustrates the effect of allowing the unfrozen pea cotyledons to remain in contact with the HPO_3 for a short time before disintegration. The figures for pyruvic and α-oxoglutaric acids are low compared with those obtained without this delay, and it appears that the reagent has stimulated destruction of the α-keto acids. The importance of this observation is that a similar process may occur during the disintegration of the unfrozen pea cotyledons even if the disintegration is carried out as rapidly as possible. Considerable amounts of cellular tissue were still present even after 1–2 min. in the high-speed blender used in these experiments. Model experiments showed that after 1 min. 28 %, and after 2 min. 1 %, would not pass a 10-mesh sieve. Even after 5 min. 68 %, and after 10 min. 32 %, failed to pass a 60-mesh sieve.

In Expts. 5 and 6 a check has been made of the amount of enzyme action which occurs during disintegration of frozen peas in ice-cold HPO_3. The results were compared with those obtained by disintegrating the frozen pea cotyledons in a solution of 0·6 M-HPO_3 in aqueous methanol (1:2·5, v/v) at −70°. This solution does not freeze, and the extraction can be carried out at a temperature which virtually precludes enzyme action. The disintegration is effective and the mixture does not froth as with aqueous HPO_3. The results show a small difference in Expt. 5 and none in Expt. 6. The difference in Expt. 5 may be the result of the rise in temperature of the aqueous HPO_3 from −1° to +5° during the disintegration, which allows some enzyme action analogous to that mentioned above in connexion with Expt. 3 to occur in thawed particles.

Expt. 7 indicates that heat inactivation of thick tissue such as peas may give results different from those obtained by killing at low temperature in 0·6 M-HPO_3. The figure for pyruvic acid was five times and that for α-oxoglutaric acid almost twice that obtained by the cold-acid method.

DISCUSSION

The results described above for the hot-acid, cold-alkali and boiling-methanol or ethanol extraction methods have shown clearly that in each method chemical reactions leading either to the formation or destruction of α-keto acids occur; only cold HPO_3 is free from chemical reactions affecting the amount of α-keto acid present. Information about the influence of enzymic reactions in the plant material during the killing procedure is, however, less conclusive, though in extreme cases the effect can be demonstrated. Disintegration of strawberry leaves in hot HPO_3 gave a much higher yield of α-oxoglutaric acid than disintegration in cold HPO_3 (220 and 94 % respectively, Table 1), and the extra α-oxoglutaric acid was not formed if the leaves were first killed by plunging into boiling methanol for a short time (cf. Table 6). With thicker tissues such as peas the enzymic effect was noticed in Expt. 3, Table 7. The pea cotyledons, left in contact with cold HPO_3 for 5 min. before disintegration commenced, gave a much lower yield of α-keto acids even though the HPO_3 cannot have penetrated far into the cotyledon.

In the recommended method the tissue was first frozen in a mixture of solid CO_2 and methanol to arrest the action of the enzymes and then disintegrated at a low temperature in aqueous or aqueous–methanolic 0·6 M-HPO_3. This method is certainly free from the chemical reactions which complicate the other methods examined, but the results obtained may still be affected by reactions in the tissue due to the killing treatment. Since no absolute method is available, it is not easy to detect

Table 7. *Comparison of various cold-acid-extraction methods as applied to strawberry leaves, pea cotyledons, fresh peas and potato slices*

Expt.	Plant material	Killing and extraction conditions	α-Keto acids μg./100 g. fresh wt. as taken for analysis: Pyruvic	α-Oxoglutaric	Oxaloacetic
1	Strawberry* leaves	(*a*) Disintegrated in 0·6M-HPO_3 at 0°	870	26 400	650
		(*b*) Frozen in methanol–solid CO_2, −70°; disintegrated in 0·6M-HPO_3 at 0°	870	28 400	650
2	Pea cotyledons (old stock 1953) 10 g. (dry) swollen to 21 g.	(*a*) Frozen in methanol–solid CO_2, −70°, for 5 min.; disintegrated in 0·6M-HPO_3 at 0°	338	3 000	122
		(*b*) Disintegrated in 0·6M-HPO_3; temperature rose from −2° to 0° during blending	310	2 740	99
3	Pea cotyledons (old stock 1953), 10 g. (dry) swollen to 25 g.	(*a*) Frozen in methanol–solid CO_2, −70°, for 30 min.; disintegrated in 0·6M-HPO_3 at 0°	784	2 720	No observation
		(*b*) Pea cotyledons added to 0·6M-HPO_3 caused temperature to rise to +5°; mixture cooled for 5 min. till temperature was 0°, then blended	404	800	No observation
4	Pea cotyledons (new stock 1955) 10 g. (dry) swollen to 16·2 g.	(*a*) As 1 (*a*)	432	1 490	No observation
		(*b*) As 1 (*b*)	518	1 385	No observation
5	Pea cotyledons (new stock 1955) 10 g. (dry) swollen to 17·5 g.	(*a*) Frozen in methanol–solid CO_2, −70°, for 30 min.; disintegrated in solution of methanol (2·5 vol.) and 1·2M-HPO_3 (1 vol.) at −70°; glass cup surrounded by methanol–solid CO_2 freezing mixture; final temperature after 15 min. disintegration, −36°	188	795	74
		(*b*) Frozen in methanol–solid CO_2, −70°, for 30 min.; disintegration for 10 min. in 0·6M-HPO_3, temperature −1° to +5°	161	960	86
6	Pea cotyledons (new stock 1955) 10 g. (dry) swollen to 21 g.	(*a*) As 4 (*a*); final temperature after 15 min. disintegration, −65°	258	1 780	110
		(*b*) As 4 (*b*); final temperature after 15 min. disintegration, −1°	252	1 760	115
7	Fresh garden peas purchased from market	(*a*) As 1 (*a*)	280	1 290	No observation
		(*b*) Plunged into boiling methanol for 5 min.; disintegrated in 0·6M-HPO_3 at 0°	1 460	2 200	No observation
8	Washed potato slices 2 mm. thick	(*a*) As 1 (*a*)	266	200	106
		(*b*) As 1 (*b*)	280	200	92
9	Washed potato slices 4 mm. thick	(*a*) As 1 (*a*)	160	186	No observation
		(*b*) As 1 (*b*)	200	182	No observation

* Comparable half-leaves were used for the two parts of the experiment.

the presence of such reactions. The possibility that such reactions, especially enzymic ones, are present, is considered below. An attempt is made to assess the magnitude of these reactions by an analysis of the experimental results, particular attention being given to factors affecting the rate of killing.

One of the most important factors is the effect of temperature on the tissue and the enzymes in the cells. In the recommended method the rate of chilling to the point at which enzyme activity virtually ceases is much higher than the corresponding rate of heating in a typical heat-inactivation method. Some comparative figures for pea cotyledons are given in Table 8.

The figures have been calculated by the methods of Carslaw & Jaeger (1947) and Olson & Schultz (1942). The cotyledons were assumed to be flat plates 0·5 cm. thick.

Comparison of the two sets of figures in Table 8 shows that in the freezing treatment the bulk of the tissue would be below −50° in 3 sec. and at a temperature which largely precluded enzyme reactions, whereas treatment with hot ethanol would raise the temperature comparatively slowly (average temperature 62° after 20 sec.) through the region in which inactivation and maximum stimulation of the enzymes might occur. The effect of enzymic reaction rates in the freezing and hot-ethanol methods will become particularly important when the cell organization begins to break down and allows the uncontrolled association of enzymes and substrates.

In the recommended method the temperature at which disorganization occurs is about −5° when the tissue freezes. At this temperature the enzyme reaction will proceed at about one-eighth of the corresponding rate at room temperature (assuming that a 10° drop in temperature changes the rate by a factor of 2). In practice the formation of ice will possibly restrict the association of enzymes and substrates and still further decrease the rate. In addition there may be a sharp break in the relationship between temperature and reaction velocity near −5°, with very high activation energies characterizing the system below −5° (Sizer, 1943).

In the other method mentioned above, the treatment with boiling ethanol may not inactivate the enzymes until the temperature reaches 50–60° (Sizer, 1943). Even at these temperatures the process may require a considerable time. A typical protein such as egg albumin requires about 30 min. for half the protein to be denatured at a temperature of 70° (Lewis, 1926), and most of the proteins and enzymes are denatured or inactivated at the same speed within a factor of 10^2 (Stearn, 1949). If we assume arbitrarily that 55° is the temperature at which cell breakdown occurs, then the rate of enzyme reactions will be about eight times that at room temperature.

These rough calculations suggest that if the freed enzymes and substrates are held at the appropriate temperatures in the two methods for about the same time, any consequent difference between the results will reflect a change of 50–100 times in enzymic reaction rates. In practice the enzymic effect is likely to be emphasized, because the time at the critical temperature is much greater in the hot-ethanol method than in the recommended freezing method. These considerations are relevant to the practical problem of the rapid inactivation of the enzymes in plant tissue, as is shown by the results given in Table 7 for fresh garden peas (Expt. 6). The amount of pyruvic acid found after the hot-ethanol killing was 4–5 times that obtained by the recommended freezing method, and the difference is too great to be accounted for by the breakdown of oxaloacetic acid (if all the oxaloacetic acid were broken down it would be equivalent only to 5–8 % of the difference), though other labile compounds which break down to pyruvic acid may be present in the tissue. The amounts of α-oxoglutaric acid found by the two methods show a similar difference. If the calculations apply even roughly to the conditions when plant tissue is disintegrated in 0·6 M-HPO_3 at 0°, the enzymic effect is likely to be small. The cell disorganization produced by the rapid freezing of the cotyledons, as in the recommended freezing method, would appear to have only a small

Table 8. *Temperature calculated at centre of pea cotyledons immersed in (a) freezing mixture of solid carbon dioxide–methanol (−75°) and (b) boiling ethanol (+78°)*

The figures in parentheses are average temperatures of tissue.

Time (sec.)	0	1	2	3	5	7	10	20
(a)*	25	23	9 (−13°)	−3 (−50°)	−27	—	−57	—
(b)†	25	25·5	—	—	31·5	38	46 (57°)	62 (67°)

* Cotyledon assumed to be composed of ice, thermal conductivity 0·005 (average between +20° and −70°) cal. cm.$^{-1}$ sec.$^{-1}$ °C^{-1}.

† Similarly assumed to be composed of water, thermal conductivity 0·002 cal. cm.$^{-1}$ sec.$^{-1}$ °C^{-1}.

effect on the amounts of α-keto acid found. An experiment in which pea cotyledons were chilled to 0°, but not frozen, and then disintegrated in 0·6 M-HPO_4 at −2°, gave results which were within 10% of those obtained by the recommended freezing method. In this experiment, great care had to be taken to prevent the temperature of the mixture in the blender rising above 0–2°. When frozen pea cotyledons are used, the temperature is much easier to control.

Apart from the effect of temperature on the tissue, the other important factor to be considered is the efficiency with which the tissue is disintegrated during the killing process. There must be no possibility of the enzymes in the frozen tissue becoming active through a rise in temperature before the HPO_3 has penetrated into the tissue, because a momentary rise of temperature may occur when the blade of the blender strikes the hard pea cotyledon. To test whether this occurs in the recommended freezing method, the results obtained have been compared with those obtained by blending the frozen pea cotyledons in an aqueous methanolic solution of 0·6 M-HPO_3 at −50°. The assumption was that at −50° the enzymes would be inactive even if the blending process was inefficient and required a relatively long time to disintegrate the tissue completely. Comparison of the results (Expts. 4 and 5, Table 7) shows that there is no difference, and indicates that the blending of the frozen pea cotyledons at 0° as in the recommended method is satisfactory.

The experiments discussed above indicate that freezing the tissue before disintegration in ice-cold HPO_3 does not result in enzymic or chemical changes that seriously affect the estimation of the α-keto acids. Although this may be generally true for other metabolites such as phosphoric esters, it is advisable to re-examine the effect of disintegration at −2°, with and without a previous freezing at low temperature, when the method is applied to other compounds, as the effect of cell disorganization due to freezing may not be the same in all cases. With the materials examined in the present study, freezing ensured that no rise of temperature occurred during the blending procedure.

SUMMARY

1. Various methods of inactivating the enzymes in strawberry leaves, in washed potato slices and in peas have been critially compared as a preliminary to the estimation of the α-keto acids by the chromatographic method of Isherwood & Cruickshank (1954).

2. The results showed that the use of hot acid or strongly alkaline media, or boiling methanol, led to both the formation and destruction of α-keto acids by chemical reactions occurring in the tissue extract during disintegration.

3. Any method of heat inactivation was open to the suspicion that the enzymes were not inactivated sufficiently rapidly to avoid a brief period of heat stimulation, thus causing a significant change in the α-keto acids in the tissue.

4. In the recommended method, the tissue was frozen in a mixture of methanol and solid carbon dioxide and then disintegrated in 0·6 M metaphosphoric acid at −2°. The principle of this procedure, that it is best to arrest the activity of the enzymes by cooling to a low temperature and inactivate them by chemical treatment while they are still in this arrested state, is important in the estimation of other labile intermediates such as phosphoric esters.

As far as one of us is concerned (F.A.I.) the work forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The other author (C.A.N.) thanks the Greek State Scholarship Foundation for a grant and the Agricultural College of Athens for permission to work in Cambridge. We would like to thank Dr J. Barker, F.R.S., for his interest in this work. We would also like to thank Dr H. G. Wager for helpful criticism and permission to quote his unpublished results on fresh garden peas. Mr F. C. Barrett carried out the experimental work on washed potato slices and pea cotyledons.

REFERENCES

Carslaw, H. S. & Jaeger, J. C. (1947). *Conduction of Heat in Solids*, p. 84. Oxford University Press.

Isherwood, F. A. & Cruickshank, D. H. (1954). *Nature, Lond.*, **173**, 121.

Lewis, P. S. (1926). *Biochem. J.* **20**, 965.

Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.

Olson, F. C. W. & Schultz, O. T. (1942). *Industr. Engng Chem. (Industr.)*, **34**, 874.

Sizer, I. W. (1943). *Advanc. Enzymol.* **3**, 35.

Stearn, A. E. (1949). *Advanc. Enzymol.* **9**, 23.

Towers, G. H. N. & Mortimer, D. C. (1954). *Nature, Lond.*, **174**, 1189.

Virtanen, A. I., Miettinen, J. K. & Kunttu, H. (1953). *Acta chem. scand.* **7**, 38.