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Enzymic Synthesis of Oxalic Acid in *Oxalis pes-caprae*

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Oxalic acid is formed in white shoots of bulbs of *Oxalis pes-caprae* by the oxidation of glyoxylic acid (Millerd, Morton & Wells, 1963*a*); glyoxylic acid is also reduced to glycollic acid *in vivo*. The incorporation of ^{14}C from [^{14}C]isocitric acid into oxalic acid suggested that *Oxalis* shoots contained isocitrate lyase, which could catalyse the cleavage of isocitric acid, forming glyoxylic acid and succinic acid.

This paper describes studies with extracts from shoots of *Oxalis*. The extracts have been shown to contain isocitrate lyase (L$_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1, formerly known as isocitritase) and other enzymes concerned with the metabolism of C_2 compounds in this plant. A brief account of some of this work has been published (Millerd, Morton & Wells, 1962).

MATERIALS

General chemicals. All inorganic reagents used were of A.R. grade. DL(+)-Alloisocitric acid (trisodium salt, 42% D form), GSH, sodium glyoxylate monohydrate and α-oxoglutaric acid were A.R. grade (Sigma Chemical Co.). CoA, cytochrome *c* (horse-heart type II, 65%), FMN and ATP were also products of Sigma Chemical Co. Semicarbazide–HCl, 2,4-dinitrophenylhydrazine (British Drug Houses Ltd.) and potassium oxalate (Judex Chemical and Pharmaceutical Co.) were A.R. grade. Glycollic acid (recrystallized before use), succinic acid (British Drug Houses Ltd.), cysteine–HCl (E. Merck and Co.) and trichloroacetic acid (May and Baker Ltd.) were L.R. grade.

DL-*Isocitric acid.* This was prepared from the lactone (A.R.; allo-free; Sigma Chemical Co.) as described by Kornberg & Beevers (1957).

Norit SX-2. This charcoal (Harrington Bros. Ltd.) was washed three times with 6N-HCl and subsequently with water until no chloride was detectable in the washings.

2,6-*Dichlorophenol-indophenol.* Approx. 2 g. of dye (British Drug Houses Ltd.) was dissolved in 80 ml. of N-HCl, shaken well with 20 ml. of diethyl ether and filtered through sintered glass. The ether layer was washed with water (2 × 50 ml.) and extracted with 100 ml. of 2% $NaHCO_3$. The blue aqueous layer was separated and 30 g. of NaCl was added to it. The precipitate was collected and washed with 30 ml. of 30% NaCl.

$NADH_2$. This was prepared from NAD (C. F. Boehringer und Soehne) by reduction with ethanol and alcohol dehydrogenase (Rafter & Colowick, 1955). On completion of the reaction, the mixture was placed in a boiling-water bath for 3 min., cooled rapidly in an ice bath and denatured protein was removed by centrifuging. The supernatant containing $NADH_2$ was stored at −15°.

$NADPH_2$. This was prepared from NADP (C. F. Boehringer und Soehne) by reduction with sodium isocitrate and isocitrate dehydrogenase (Evans & Nason, 1953). On completion of the reaction, the pH of the solution was adjusted to 9·0–9·5 with N-NaOH, placed in a boiling-water bath for 3 min., cooled and centrifuged. The supernatant containing $NADPH_2$ was stored at −15°.

Phosphate buffers. All phosphate buffers were prepared from disodium hydrogen orthophosphate (A.R., British Drug Houses Ltd.) and adjusted to the required pH with 2N-HCl.

^{14}C-*Labelled compounds.* Sodium [1-^{14}C]glycollate, specific activity 19·4 μc/mg., and glyoxylic acid monohydrate, specific activity 32·6 μc/mg., were obtained from The Radiochemical Centre, Amersham, Bucks.

Oxalis *extracts.* Bulbs of *O. pes-caprae* were treated as described by Millerd *et al.* (1963*a*) and the white shoots of the germinated bulbs were allowed to grow in the dark for 1–5 months. Shoots, usually 200 g., were then detached from the bulbs. All subsequent steps were carried out at 2°. The shoots were cut into small pieces (approx. 3 cm.) immediately before grinding in a pre-chilled glass mortar in 3 vol. of 0·2M-phosphate, pH 8·3. This extraction was carried out in stages so that approx. 30–35 g. of *Oxalis* shoots were extracted in 100 ml. of phosphate. Under these conditions the pH of the brei was kept just above pH 7·0 but a check was always made that the pH did not fall below this level from excess of oxalic acid released from the shoots. The green–brown brei was screened through

two layers of cheese-cloth and centrifuged at 36 000*g* for 10 min. at 2° in a Vacu-Fuge model VA-2 refrigerated centrifuge (Lourdes Instrument Corp., N.Y., U.S.A.). The supernatant was discarded and the grey–green precipitate homogenized for 15 sec. in 40 ml. of cold acetone at −15° in a glass–Teflon (Potter–Elvehjem-type) homogenizer pre-chilled to −15°. The suspension was centrifuged at 2000*g* for 5 min., and the yellow supernatant was discarded. The grey pellet was homogenized twice in 40 ml. of acetone at −15° and the clear supernatants were discarded. The final pellet was spread over a large area of the glass centrifuge tube, which was placed in a vacuum desiccator containing P_2O_5 and paraffin shavings. Excess of acetone was removed *in vacuo*. The yield of fine white powder obtained was approx. 1 mg./g. fresh wt. of shoots. The powder was extracted immediately before use with 0·1 M-phosphate, pH 7–8, depending on the required pH of the reaction being studied. In general, approx. 200 mg. of powder was extracted at 2° with 8–10 ml. of phosphate buffer by slow stirring for 15 min. The precipitate was removed by centrifuging at 2 000*g* for 5 min., and the clear supernatant containing approx. 1 mg. of protein/ml. was used for enzymic studies. It was found that addition of FMN (50 μM) to the 0·2 M-phosphate buffer, pH 8·3, used in the initial extraction of *Oxalis* shoots, and to the 0·1 M-phosphate buffer used in the extraction of the acetone-dried powder, increased the activity of these extracts in oxidizing glyoxylic acid and glycollic acid.

METHODS

Spectrophotometry. Extinction was measured either in an Optica CF-4 spectrophotometer or a Beckman DK-2 recording spectrophotometer. Wherever the assay of an enzymic system involved a decrease in *E* at a given wavelength, the Beckman spectrophotometer was preferred so that the coenzyme or dye used in the assay could be added to both the control and assay cuvettes. All spectrophotometric assays were carried out at 25° in silica cells of 1 cm. light-path.

Protein estimation. The method of Warburg & Christian (1941) was used.

Assay of isocitrate-lyase activity. (*a*) Semicarbazone assay. Keto acid formation from DL-isocitric acid was measured spectrophotometrically by following the increase in $E_{252\,m\mu}$ due to the formation of semicarbazone (Olsen, 1959). Extinctions of both the control and assay cells were recorded at 252 mμ (Fig. 1). Initial increases in $E_{252\,m\mu}$ were recorded in the control cell and in the assay cell for approx. 5 min. Thereafter $E_{252\,m\mu}$ remained constant in the control cell but increased linearly in the assay cell. In a typical assay the increase in *E* due to keto acid formation from isocitric acid was 0·084/mg. of protein/min.

The increase in $E_{252\,m\mu}$ in the above assay could be due to the formation of α-oxoglutaric acid catalysed by isocitrate dehydrogenase. Therefore the *Oxalis* extract was treated with 5 mg. of Norit SX-2/ml. to remove nicotinamide nucleotide coenzymes. The mixture was stirred for 10 min. at 2° and charcoal removed by filtration. The clear supernatant was assayed for keto acid formation from isocitric acid by the semicarbazide method. The increase in *E* due to semicarbazone formation was 0·055/mg. of protein/min.

(*b*) 2,4-Dinitrophenylhydrazone assay. Keto acid formation from isocitric acid catalysed by *Oxalis* extracts was estimated by the method of Friedman (1955). The reaction was started by the addition of substrate and incubation was carried out at 30°. At 0, 60 and 120 min., 2 ml. portions were removed into 2 ml. of 10% trichloroacetic acid. The denatured protein was removed by centrifuging and 3 ml. portions of the supernatant were added to 1·0 ml. of 0·1% 2,4-dinitrophenylhydrazine in 2 N-HCl. A reference incubation mixture contained trichloroacetic acid and 0·1 M-phosphate, pH 7·5. As glyoxylic acid combines with cysteine (particularly in the absence of a trapping agent) and this complex is not hydrolysed by 2 N-HCl at room temperature, the portions to be assayed were autoclaved at 15 lb./in.^{2} for 15 min. (Rao & Ramakrishnan, 1962). Subsequent procedures for the isolation of 2,4-dinitrophenylhydrazones were as described by Friedman (1955).

Extinctions (1 cm.) of the isolated 2,4-dinitrophenylhydrazones (read against the reference) were recorded at 435 mμ and showed a linear increase through the series 0, 60 and 120 min. The zero-time incubation showed a slight value for $E_{435\,m\mu}$. The spectra of the 2,4-dinitrophenylhydrazones obtained from the incubation mixture are presented in Fig. 2 (A–C).

Spectra of the 2,4-dinitrophenylhydrazones of α-oxoglutaric acid and of glyoxylic acid. α-Oxoglutaric acid (0·3 μmole) and 0·3 μmole of glyoxylic acid, and a mixture of the two, were incubated for 120 min. exactly as for the enzymic reaction. Phosphate (0·1 M, pH 7·5) was substituted for *Oxalis* extract and the appropriate keto acid for isocitric acid. The reference mixture contained no keto acid. The spectra of these 2,4-dinitrophenylhydrazones are shown in Fig. 2 (D–F).

Comparison of the spectra of enzymically formed 2,4-dinitrophenylhydrazones (Fig. 2A–C) and those of the

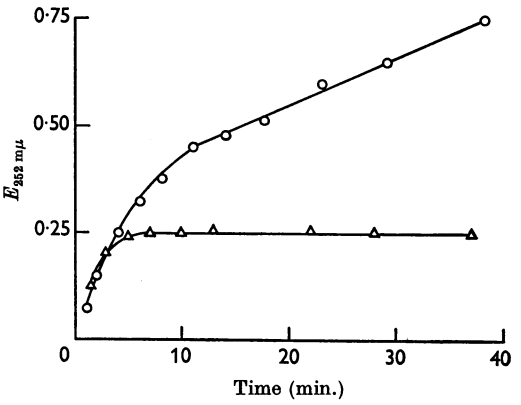


Fig. 1. Formation of keto acid from DL-isocitric acid catalysed by *Oxalis* extract. The complete system (○) contained, in 3 ml.: 200 μmoles of sodium phosphate, pH 6·0, 10 μmoles of $MgSO_4$, 5 μmoles of GSH, 40 μmoles of semicarbazide, 0·25 ml. of *Oxalis* extract, pH 7·0, and 5 μmoles of DL-isocitric acid. All reactants were adjusted to pH 6·0 immediately before use. Keto acid formation was measured in an Optica CF-4 spectrophotometer as the rate of change in $E_{252\,m\mu}$ consequent upon the formation of the semicarbazone (Olsen, 1959). Extinctions (1 cm.) were recorded against a reference cuvette containing all reactants except semicarbazide. From the control cuvette (△) DL-isocitric acid was omitted.

reference keto acids (Fig. 2D–F) shows that glyoxylic acid has been formed from isocitric acid. The spectra of the 2,4-dinitrophenylhydrazones obtained at zero-time (enzymic reaction, curve C) and with α-oxoglutaric acid (reference mixture, curve F) are almost identical with a broad extinction maximum at approx. 420 mμ. It was apparent that the *Oxalis* extract contained some endogenous α-oxoglutaric acid, which would account for the observed initial increase in $E_{252\,m\mu}$ in the semicarbazone assay in the absence of isocitric acid (Fig. 1). The presence of α-oxoglutaric acid was separately demonstrated by paper chromatography. Although the glyoxylic acid derivative by itself has an extinction maximum at 450 mμ under the conditions used (Fig. 2E), the presence of the 2,4-dinitrophenylhydrazone of α-oxoglutaric acid resulted in an extinction maximum at 440 mμ (Fig. 2D). The 2,4-dinitrophenylhydrazone derivatives prepared from the keto acids formed enzymically (after 60 and 120 min.) have maxima at approx. 440 mμ (Fig. 2A, B).

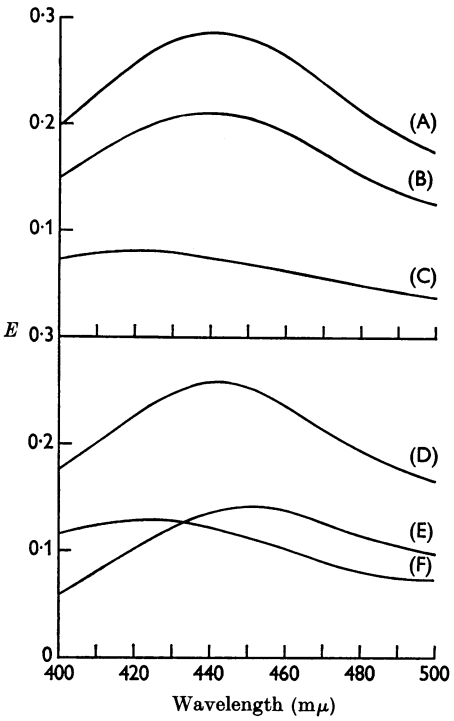


Fig. 2. Spectra of 2,4-dinitrophenylhydrazones of keto acids formed enzymically from DL-isocitric acid, and of 2,4-dinitrophenylhydrazone derivatives of a mixture of glyoxylic acid and α-oxoglutaric acid, of glyoxylic acid and of α-oxoglutaric acid. The complete enzymic system contained, in 8·5 ml.: *Oxalis* extract (5·0 ml. in 0·1 M-sodium phosphate, pH 7·5), 25 μmoles of cysteine, 50 μmoles of $MgSO_4$ and 40 μmoles of DL-isocitric acid. Reactions incubated at 30° were stopped at 0, 60 and 120 min. by removing 2 ml. portions of the incubation mixture into trichloroacetic acid. The 2,4-dinitrophenylhydrazones were isolated from incubation mixtures as described by Friedman (1955) for total keto acids. Spectra (1 cm.) of the keto acid derivatives were read against a reference solution originally containing sodium phosphate, pH 7·5, in a Beckman DK-2 recording spectrophotometer. (A) 120 min. incubation; (B) 60 min. incubation; (C) 0 min. incubation. The formation and isolation of 2,4-dinitrophenylhydrazone derivatives of reference keto acids was carried out under the same conditions as for the enzymic experiment except that sodium phosphate, pH 7·5, substituted for *Oxalis* extract and the appropriate keto acid for isocitric acid. The spectra of the 2,4-dinitrophenylhydrazone derivatives were recorded as described above. (D) A mixture of glyoxylic acid and α-oxoglutaric acid; (E) glyoxylic acid; (F) α-oxoglutaric acid.

RESULTS

Stoicheiometry of the reaction catalysed by isocitrate lyase. The formation of glyoxylic acid from isocitric acid, catalysed by *Oxalis* extracts, was measured by the semicarbazide method. The reaction was stopped after 53 min. by the addition of hydrochloric acid (final concentration 1 N), the reaction mixture was adjusted to pH 7·5 (approx.) with sodium hydroxide and portions were assayed for succinic acid.

Succinic acid formed from the cleavage of isocitric acid was measured by the specific reduction of ferricytochrome *c* in the presence of succinate dehydrogenase (Keilin & Hartree, 1940) essentially as described by Morton (1958). The portions to be assayed for succinic acid contained GSH; this was oxidized by aerating these samples vigorously for approx. 5 min.

Samples (0·25 ml.) from the control (without isocitric acid) and assay (with isocitric acid) mixtures were used to start the reaction catalysed by succinate dehydrogenase. No increase in $E_{550\,m\mu}$ was observed in the cell containing the control sample from the semicarbazone assay. Extinctions in the cell containing the assay sample increased linearly for 10 min., after which the rate of reduction decreased and finally remained constant after 20 min.

The amount of glyoxylic acid formed from isocitric acid in the above experiment was calculated by using $\epsilon_{252\,m\mu}$ $12{\cdot}4 \times 10^3$ (Olsen, 1959). The total glyoxylic acid formed was 0·13 μmole. Allowing for volume changes and by using ϵ (reduced − oxidized) $18{\cdot}5 \times 10^3$ for ferricytochrome *c* (Margoliash & Frohwirt, 1959), the calculated amount of succinic acid formed was 0·12 μmole. These results are in agreement with the stoicheiometry of the reaction catalysed by isocitrate lyase.

The condensation of glyoxylic acid and succinic acid to form isocitric acid, catalysed by isocitrate lyase, was assayed in a coupled reaction with isocitrate dehydrogenase in the presence of NADP. The reaction was followed spectrophotometrically by the increase in $E_{340\,m\mu}$ thus:

$$\underset{\text{acid}}{\text{Glyoxylic}} + \underset{\text{acid}}{\text{succinic}} \xrightarrow[MgCl_2]{\text{isocitrate lyase}} \underset{\text{acid}}{\text{isocitric}} \qquad (1)$$

$$\text{Isocitric acid} + \text{NADP} \xrightarrow[MgCl_2]{\text{isocitrate dehydrogenase}} \alpha\text{-oxoglutaric acid} + NADPH_2 \quad (2)$$

In the presence of isocitric acid, magnesium chloride, *Oxalis* extract and NADP there was a rapid increase in $E_{340\,m\mu}$ (see below). Hence the reversal of isocitrate lyase, resulting in the production of isocitric acid, could be assayed in *Oxalis* extracts without the addition of exogenous isocitrate dehydrogenase.

As shown in Fig. 3, there was no increase in E on the addition of succinic acid, but a slow increase in $E_{340\,m\mu}$ after the subsequent addition of glyoxylic acid to the assay cell. After approx. 2 min. there was a steady decrease in E. On the addition of isocitric acid (approx. 1 μmole) to the assay cell there was a further increase in $E_{340\,m\mu}$ for approx. 2 min., and then a decrease, in a pattern similar to that obtained on addition of succinic acid and glyoxylic acid. Thus although NADP was reduced initially the $NADPH_2$ so formed was either re-oxidized or rapidly destroyed. When the *Oxalis* extract was left at 25° for 3 hr. and the above assay was repeated, there was a slow but steady increase in $E_{340\,m\mu}$.

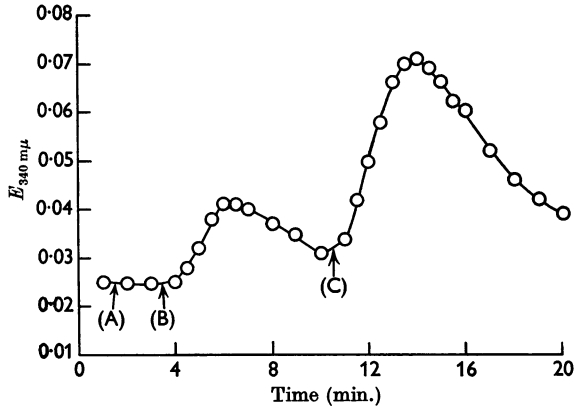


Fig. 3. Reversal of isocitrate lyase catalysed by *Oxalis* extract. The complete system contained, in 2·85 ml.: 200 μmoles of sodium phosphate, pH 8·0, 10 μmoles of $MgCl_2$, 0·5 μmole of NADP and 0·5 ml. of *Oxalis* extract, pH 8·0. (A) Succinic acid (10 μmoles), (B) glyoxylic acid (10 μmoles) and (C) isocitric acid (1 μmole) were added to the assay cell as indicated. Since the *Oxalis* extract contained isocitrate-dehydrogenase activity (see text) an increase in $E_{340\,m\mu}$ due to the reduction of NADP represented a measure of isocitrate-lyase reversal in the coupled system. Decrease in $E_{340\,m\mu}$ was due to glyoxylate-reductase activity in the *Oxalis* extract. $E_{340\,m\mu}$ values were recorded at regular intervals in an Optica CF-4 spectrophotometer.

Isocitrate dehydrogenase and glyoxylate reductase in Oxalis. The presence of isocitrate dehydrogenase in *Oxalis* extracts was demonstrated spectrophotometrically (Fig. 4). In this experiment there was no subsequent decrease in $E_{340\,m\mu}$ as had been observed in the demonstration of isocitrate-lyase reversal (Fig. 3). However, on addition of glyoxylic acid (10 μmoles) to the assay cell after 14 min., there was an immediate decrease in $E_{340\,m\mu}$ (Fig. 4), due to glyoxylate reductase in the *Oxalis* extract.

The reactions may be summarized as shown in Scheme 1.

That α-oxoglutaric acid was the product of isocitric acid oxidation was demonstrated by the chromatography of 2,4-dinitrophenylhydrazones isolated from reaction mixtures of phosphate, pH 7·4, isocitric acid, magnesium chloride, NADP and *Oxalis* extract. Reactions were stopped with trichloroacetic acid at 0 and 60 min., and keto acid derivatives were characterized by the method of El Hawary & Thompson (1953). Chromatograms showed the presence of trace amounts of α-oxoglutaric acid in the 0 min. incubation and substantial amounts in the 60 min. incubation mixture. A trace amount of keto acid derivative was detected in the positions corresponding to the 2,4-dinitrophenylhydrazone of glyoxylic acid in the 60 min. incubation extract, indicating that there was some production of glyoxylic acid from isocitric acid.

The identity of glycollic acid as the product of glyoxylic acid reduction, catalysed by glyoxylate

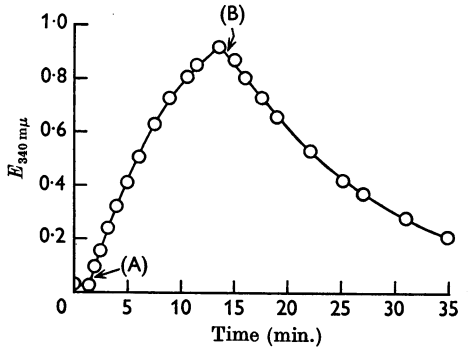
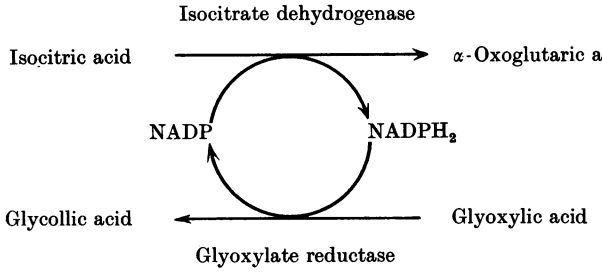


Fig. 4. Isocitrate-dehydrogenase and glyoxylate-reductase activity in *Oxalis* extract. The complete system contained, in 3·05 ml.: 200 μmoles of sodium phosphate, pH 7·4, 25 μmoles of $MgCl_2$, 0·5 μmole of NADP, 0·5 ml. of *Oxalis* extract, pH 7·4, and 5 μmoles of isocitric acid. Increases in $E_{340\,m\mu}$ consequent upon the addition of isocitric acid (A) to the assay cell were recorded at regular intervals on an Optica CF-4 spectrophotometer. Glyoxylic acid (10 μmoles) was added to the assay cell as indicated (B) (14 min.), and the glyoxylic acid-dependent oxidation of $NADPH_2$, catalysed by glyoxylate reductase of the *Oxalis* extract, was assayed by the decrease in $E_{340\,m\mu}$.



Scheme 1

reductase of *Oxalis*, was confirmed by the use of [$^{14}C_2$]glyoxylic acid. After incubation, protein was removed by centrifuging after acidification with hydrochloric acid. The supernatant was then chromatographed in phenol saturated with water at 24°. Development of X-ray film after suitable contact with the chromatograms clearly showed the enzymic formation of glycollic acid from glyoxylic acid.

The activity of *Oxalis* glyoxylate reductase was demonstrated spectrophotometrically with both $NADH_2$ and $NADPH_2$, but the activity was increased approximately 20-fold with $NADPH_2$.

Oxidation of glycollic acid and glyoxylic acid by Oxalis. Studies with [1-^{14}C]glycollic acid and [$^{14}C_2$]glyoxylic acid *in vivo* (Millerd *et al.* 1963*a*) indicated that these two acids were oxidized to glyoxylic acid and oxalic acid respectively in *Oxalis*. Preliminary manometric experiments with 'soluble protein' from *Oxalis* shoots showed a low oxygen consumption in the presence of FMN and glycollic acid and barely detectable oxygen consumption with glyoxylic acid as substrate. The same type of preparation from green *Oxalis* leaves actively oxidized glycollic acid, probably because of the presence of glycollate oxidase, which is widely distributed in green plants.

By using extracts prepared from acetone-dried powder of white *Oxalis* shoots, the oxidation of glycollic acid and of glyoxylic acid could be demonstrated spectrophotometrically by the reduction of 2,6-dichlorophenol-indophenol. No activity was obtained without the addition of FMN, and higher activities could be obtained if FMN was present throughout the extraction procedure (see Materials section).

Reactants were added to duplicate cells and $E_{600\,m\mu}$ values were measured for 30 sec. in a Beckman DK-2 recording spectrophotometer before the addition of substrate. The substrate (glycollic acid or glyoxylic acid, 10 μmoles) was added to the assay cell as indicated in Fig. 5. When the oxidation of glyoxylic acid was assayed in the presence of oxalic acid, potassium oxalate (10 μmoles) was added before the substrate. The initial rates of oxidation of glycollic acid, of glyoxylic acid and of glyoxylic acid in the presence of oxalic acid are shown in Fig. 5. As compared with glycollate oxidase of other tissues (Richardson & Tolbert, 1961), the enzyme of *Oxalis* has similar requirement for FMN but differs in the relative rates of

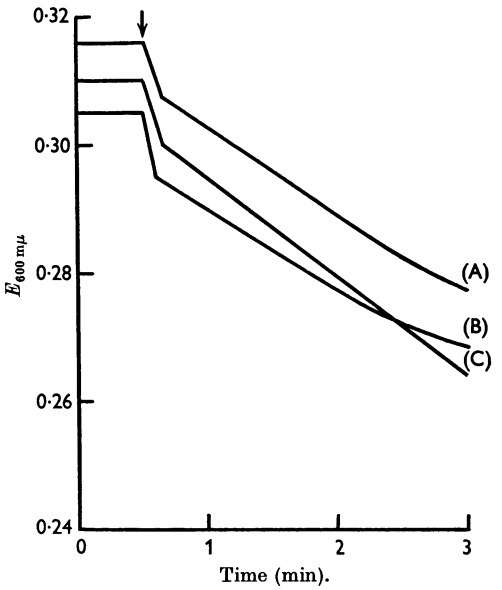


Fig. 5. Oxidation of glycollic acid and of glyoxylic acid by *Oxalis* extracts. The complete system contained, in 2·6 ml.: 200 μmoles of sodium phosphate, pH 8·0, 0·1 μmole of 2,6-dichlorophenol-indophenol and 0·5 ml. of *Oxalis* extract, pH 8·0, containing 50 μM-FMN. Reactants were added to duplicate cells and $E_{600\,m\mu}$ was followed for 30 sec. in a Beckman DK-2 recording spectrophotometer before the addition of substrate. The decrease in $E_{600\,m\mu}$ consequent upon the addition of 10 μmoles of glyoxylic acid (A), 10 μmoles of glycollic acid (B) and 10 μmoles of glyoxylic acid after the addition of 10 μmoles of oxalic acid to the appropriate assay cell (C) was recorded for 3 min. on a Beckman DK-2 recording spectrophotometer. Time of addition of substrate in each case is indicated by the arrow.

Table 1. *Identification of oxidation products of glycollic acid and glyoxylic acid catalysed by* Oxalis *extracts*

[1-^{14}C]Glycollic acid and [$^{14}C_2$]glyoxylic acid were incubated with *Oxalis* extracts and reactions were stopped at 0 and 60 min. as described in the text. After chromatography of the reaction mixtures, the ^{14}C activity in glycollic acid, glyoxylic acid and oxalic acid was determined. Results are expressed as a percentage of the total radioactivity recovered from appropriate chromatograms.

Substrate	Time (min.)	^{14}C activity in organic acids separated by paper chromatography (%)			
		Origin*	Glycollic acid	Glyoxylic acid	Oxalic acid
[1-^{14}C]Glycollic acid	0	1·7	93·6	3·1	1·6
	60	2·3	44·9	43·0	9·8
[$^{14}C_2$]Glyoxylic acid	0	3·1	3·2	91·6	2·1
	60	3·5	4·8	59·3	32·4

* Material remaining at origin after development of chromatograms.

oxidation of glycollic acid and glyoxylic acid and in the absence of oxalic acid inhibition of the oxidation of glyoxylic acid. There was no reduction of NAD or NADP in the presence of *Oxalis* extract and glycollic acid or glyoxylic acid.

The products of oxidation of glycollic acid and of glyoxylic acid in the system described above were identified by radioautography after incubation with [1-^{14}C]glycollic acid and with [$^{14}C_2$]glyoxylic acid. The mixtures were incubated at 30° and the reactions were stopped at 0 and 60 min. by the addition of hydrochloric acid (final concn. 2·5N). Denatured protein was removed by centrifuging, the supernatants were dried on a rotary film evaporator and the residues were dissolved in 0·25 ml. of 20% (v/v) ethanol. Portions (0·1 ml.) were applied to chromatograms and these were developed in phenol saturated with water at 24°. Losses of glyoxylic acid during chromatography in this solvent are small (Kearney & Tolbert, 1962). Compounds containing ^{14}C were detected by radioautography and the amount of radioisotope in glycollic acid, glyoxylic acid and oxalic acid was determined by a liquid-scintillation technique as described by Millerd, Morton & Wells (1963*b*). The ^{14}C activity in each compound was expressed as a percentage of the total radioactivity located on the appropriate chromatogram. The results in Table 1 clearly show enzymic incorporation of ^{14}C from [1-^{14}C]glycollic acid into glyoxylic acid. There was less incorporation of ^{14}C into oxalic acid. Incubation of [$^{14}C_2$]glyoxylic acid with *Oxalis* extract (Table 1) resulted in substantial incorporation of ^{14}C into oxalic acid and a small incorporation of ^{14}C into glycollic acid. The results in Table 1 (zero-time incubations) showed contamination of the ^{14}C-labelled starting material (from 1·6 to 3·2%). Contaminations of the same order have been noted previously for [$^{14}C_2$]glyoxylic acid (Kearney & Tolbert, 1962).

Reduction of oxalic acid in Oxalis. Since ^{14}C was detected in glycollic acid and glyoxylic acid after the administration of [$^{14}C_2$]oxalic acid to *Oxalis* shoots (Millerd *et al.* 1963*a*) it appeared that oxalic acid was not metabolically inert. The reduction of oxalic acid by extracts of *Oxalis* was demonstrated spectrophotometrically. The addition of oxalic acid alone to the assay cell did not cause a decrease in $E_{340\,m\mu}$. However, on addition of succinic acid to both cells, the presence of oxalic acid resulted in a linear decrease in $E_{340\,m\mu}$. The requirement for succinic acid indicated that the reaction probably proceeded through the intermediate formation of oxalyl-CoA. Such a system has been described in *Pseudomonas oxalaticus* (Quayle, Keech & Taylor, 1961).

DISCUSSION

The production of glyoxylic acid from isocitric acid, catalysed by isocitrate lyase, has been described in plants but has been considered to be confined rather strictly to those tissues converting fat into carbohydrate (Carpenter & Beevers, 1959). Although this situation does not apply in *Oxalis* shoots, there is a rapid synthesis of C_2 units for oxalic acid production.

The synthesis of C_2 units is markedly different in photosynthetic and non-photosynthetic tissues. It is well established that phosphoglycollic acid and glycollic acid are early products of carbon dioxide fixation during photosynthesis. In non-photosynthetic *Oxalis* shoots, carbon dioxide does not contribute significantly to glycollic acid synthesis (Millerd *et al.* 1963*a*). Furthermore, the origin of glycollic acid in *Oxalis* can be ascribed, at least in part, to glyoxylate-reductase activity rather than to a transketolase-type reaction involving hexose or pentose phosphates.

Both isocitrate lyase (Fig. 2) and isocitrate dehydrogenase (Fig. 4) are present in *Oxalis*. Thus *in vitro* it is difficult to demonstrate the presence of glyoxylic acid in incubation mixtures containing isocitric acid, magnesium chloride and NADP. The oxidation of isocitric acid results in the reduction

of NADP; any glyoxylic acid formed by isocitrate-lyase activity is readily reduced to glycollic acid by a NADP-linked glyoxylate reductase. In the absence of added NADP and of cysteine (to act as a trap for glyoxylic acid), it was possible to demonstrate the production of glyoxylic acid from isocitric acid (Fig. 2).

In vivo, glyoxylic acid is converted chiefly into glycine and serine in plants (Tolbert & Cohen, 1953). In *Oxalis*, however, glyoxylic acid is more readily converted into glycollic acid and into oxalic acid (Millerd *et al.* 1963*a*). The oxidation of glycollic acid and glyoxylic acid catalysed by glycollate oxidase isolated from a number of green plants has been studied by Richardson & Tolbert (1961). These authors found that glycollic acid was oxidized at a rate that was considerably greater than that of glyoxylic acid and the oxidation of glyoxylic acid was strongly inhibited by oxalic acid. This inhibition provides an explanation for the diversion of glyoxylic acid to glycine rather than to oxalic acid in the systems studied by Richardson & Tolbert (1961). In contrast, the flavin-linked oxidase (or oxidases) isolated from *Oxalis* shoots catalysed the oxidation of glycollic acid and of glyoxylic acid at comparable rates. Moreover, the presence of oxalic acid in substrate concentrations had no significant effect on the rate of oxidation of glyoxylic acid by this extract (Fig. 5). Apparently the oxidase (or oxidases) from *Oxalis* differs from glycollate oxidase isolated from photosynthetic tissues. Indeed, glycollate oxidase has been reported to be absent in embryos and etiolated plants (Clagett, Tolbert & Burris, 1949). Glycollic acid is oxidized to glyoxylic acid *in vivo* in *Oxalis* (Millerd *et al.* 1963*a*) and by *Oxalis* extracts. It may be considered therefore as a precursor for the biosynthesis of oxalic acid. Because, in *Oxalis*, biosynthesis of glycollic acid from a source other than glyoxylic acid was not apparent (Millerd *et al.* 1963*b*), it is likely that isocitric acid is the main precursor of glyoxylic acid, glycollic acid and oxalic acid. Oxalic acid appears to be a non-functional and undesirable end product of metabolism in plants. It may be slowly oxidized to carbon dioxide (Finkle & Arnon, 1959). or decarboxylated to formate and carbon dioxide (Giovanelli & Tobin, 1961). In *Oxalis*, oxalic acid is not completely inert. A $NADPH_2$- and CoA-dependent reduction of oxalic acid reported here probably involves the intermediate formation of oxalyl-CoA as described in cell-free extracts of *Pseudomonas oxalaticus* (Quayle *et al.* 1961).

SUMMARY

1. The enzymic reactions involved in the synthesis of oxalic acid and the metabolism of related compounds have been studied with cell-free extracts of *Oxalis pes-caprae*.

2. The extracts contain isocitrate-lyase activity, which accounts for the biosynthesis of glyoxylic acid, the immediate precursor of oxalic acid in *Oxalis*.

3. In *Oxalis*, the combined actions of isocitrate dehydrogenase and glyoxylate reductase result in the synthesis of glycollic acid. The former provides reduced nicotinamide–adenine dinucleotide phosphate necessary for the reduction of glyoxylic acid. Both enzymes have been demonstrated in *Oxalis* extracts.

4. Glycollic acid and glyoxylic acid were oxidized by a flavin-linked enzyme. This enzyme differed from glycollate oxidase in the relative rates of oxidation of the two substrates and the lack of inhibition of oxalic acid on the oxidation of glyoxylic acid. The lack of inhibition observed may account for the preferential oxidation of glyoxylic acid to oxalic acid rather than a diversion to glycine synthesis in *Oxalis*.

5. Extracts catalyse a reduced nicotinamide–adenine dinucleotide phosphate- and coenzyme A-dependent reduction of oxalic acid. It is probable that oxalyl-coenzyme A is formed as an intermediate in this reduction.

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The Degradation of Cotton Cellulose by the Extracellular Cellulase of *Myrothecium verrucaria*

2. THE EXISTENCE OF AN 'EXHAUSTIBLE' CELLULASE*

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A cellulolytic micro-organism almost certainly attacks cotton fibre by means of an extracellular enzyme, but filtrates derived from actively growing cultures have been reported to have little action on cotton that has not been made more accessible by swelling or partial degradation (Reese, 1956); this fact has not been explained. It has been suggested that there exist several 'cellulases', some of which preferentially attack substrates of short chain length whereas others are more active towards materials of higher degrees of polymerization. In none of this work, which has been reviewed by Gascoigne & Gascoigne (1960) and Selby (1963), has extensive degradation of unswollen cotton by cell-free extracts of the micro-organisms been demonstrated. It is the purpose of the present paper to describe how such degradation has been achieved and to provide additional evidence that more than one enzyme species is involved in the hydrolytic breakdown of unswollen cotton by cell-free filtrates from *Myrothecium verrucaria*.

The mode of enzymic attack on a soluble substrate can readily be determined by comparing the resulting changes in suitably chosen physical and chemical properties. With an insoluble substrate, however, the location of chemically identical linkages will affect both their ease of scission and the contribution the scission makes to the measured changes in the properties of the substrate. In fibrous cotton certain parts of each cellulose molecule are so arranged relative to parts of others that a region of high lateral order and strong interchain bonding occurs; such regions are termed 'crystalline'. Between them the chains are disordered to various extents and the interchain bonding is weaker; these regions, which have been termed 'amorphous', are more accessible to chemical attack because they are more easily swollen and penetrated (Howsmon & Sisson, 1954). On a larger scale the bundles of cellulose chains are grouped together in a microfibrillar structure (Preston, 1962). Such a structure will contain holes between the fibrils and these may be the smallest cavities into which cellulase can penetrate, whereas chemical reagents of relatively small molecular bulk can also attack the regions of high lateral disorder and the surface of the crystalline regions. The resistance to penetration by cellulase will be one of the chief factors regulating enzymic hydrolysis (Walseth, 1952; Reese, Segal & Tripp, 1957), and the consequent immobility of the enzyme within the substrate is presumably the cause of the localized attack observed (Selby, 1961).

Halliwell (1962) has stated that the lack of solubilization of undegraded cotton by culture filtrate from *M. verrucaria* 'may be due to the enzymic production of physical changes in cellulose which are not reflected in the formation of soluble products'. The nature of these physical changes is not made clear but as an example he cites his demonstration that hydrocelluloses lose more weight when heated with N-sodium hydroxide at 100° for 15 min. than does an undegraded cotton. It is well established, however, that treatment with hot dilute alkali causes chemical degradation of β-(1→4)-glucans containing free reducing groups, with consequent loss of weight (Clibbens, Geake & Ridge, 1927; Corbett, 1959). Under the conditions of alkali treatment used the free reducing groups produced by the exposure of cotton to culture filtrate

* Part 1: Selby (1961).