OXALIC ACID SYNTHESIS IN OXALIS PES-CAPRAE (L.).

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Declaration

I hereby declare that the work presented in this thesis has been carried out by myself, and that it has not been submitted in any previous application for a degree.

Abbreviations

Amino Acids

Ala	Alanine
Asp	Aspartic acid
Asp (NH_2)	Asparagine
Cys acid	Cysteic acid
Glu	Glutamic acid
Glu (NH_2)	Glutamine
Gly	Glycine
Ileu	Isoleucine
Leu	Leucine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Tau	Taurine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Other Compounds

ATP	Adenosine 5'-triphosphate
CoA	Coenzyme A

FMN	Riboflavin phosphate
G-SH	Glutathione, reduced
NAD	Nicotinamide-adenine dinucleotide
$NADH_2$	Nicotinamide-adenine dinucleotide, reduced
NADP	Nicotinamide-adenine dinucleotide phosphate
$NADPH_2$	Nicotinamide-adenine dinucleotide phosphate, reduced.

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Preface

Part of the work described in this thesis has already been accepted or submitted for publication. The publications, in the order in which they were submitted, are listed below.

(i) Oxalic Acid Synthesis in Shoots of *Oxalis pes-caprae*.
by Adele Millerd, R. K. Morton and J. R. E. Wells
(1963). *Biochem. J.* **86**, 57.

(ii) The Role of Isocitrate Lyase in Synthesis of Oxalic Acid in Plants.
by Adele Millerd, R. K. Morton and J. R. E. Wells
(1962). *Nature, Lond.* **196**, 955.

(iii) Oxalic Acid Synthesis in Shoots of *Oxalis pes-caprae*. The Precursors of Glycollic Acid and Glyoxylic Acid.
by Adele Millerd, R. K. Morton and J. R. E. Wells
(1963). *Biochem. J.* (submitted for publication).

(iv) Enzymic Synthesis of Oxalic Acid in *Oxalis pes-caprae*.
by Adele Millerd, R. K. Morton and J. R. E. Wells
(1963). *Biochem. J.* (submitted for publication).

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GENERAL INTRODUCTION

Oxalis pes-caprae (Soursob) is among those plants which contain notable amounts of oxalic acid. Indigenous to South Africa (Salter, 1944), Oxalis was introduced into South Australia as an ornamental plant in 1839. So rapid and effective was its spread, that by 1879 it was recognised as a weed in cultivated areas (Schomburgk, 1879). The present distribution of Oxalis pes-caprae in the cultivated areas of South Australia has been reviewed by Michael (1959).

Agriculturally Oxalis presents a problem, not only because of its widespread distribution and successful competition with more favoured pasture species but also because of its high oxalic acid content which causes a chronic nephritis in sheep depastured on Oxalis dominant areas. In simple stomached animals, a hypocalcaemic syndrome has been associated with excessive oxalic acid ingestion. Experiments with sheep (Dodson, 1959) have shown that oxalic acid poisoning in these animals is associated with physical rupture of the kidney tubules due to crystallisation of oxalic acid, rather than hypocalcaemia. Losses as high as 10% as a result of Oxalis-induced nephritis have been reported in some flocks of sheep. The tolerance of sheep to oxalic acid is dependant upon

the presence in the rumen of certain bacteria capable of decarboxylating oxalic acid. These bacteria are associated with Oxalis stands in the field. They are only found in the rumen of the sheep ingesting Oxalis at the time of the year when soursob is flourishing (Dodson, 1959). The severity of oxalic acid poisoning depends on the initial inoculum of these degradative bacteria present in the rumen when sheep graze on Oxalis. Animals reared in areas in which Oxalis is not prevalent are very susceptible to nephritis when brought into pastures infested with this weed.

Measures employed to control the growth and spread of Oxalis have proved inadequate. Cultivation has never been entirely effective, although repeated cultivation at the stage when the bulb from which the plant grows is nearly exhausted exerts a temporary setback to Oxalis. Not only can broken portions of the plant regenerate, but newly formed bulbs, pulled down into the soil by a contractile root as far as eighteen inches, are well clear of the effects of cultivation.

A wide range of weedicides has been applied to mature Oxalis plants, but none has been effective in controlling growth. No approach has been made to a selective attack on the plant. The experiments described here have been designed to elucidate the mechanism of oxalic acid synthesis in Oxalis. If this biosynthesis is obligatorily coupled with the growth of the plant, then selective inhibition of an enzyme (or enzymes) essential to this synthesis may result in control of the plant. Although all plants probably contain some oxalic acid (Bennet-Clark, 1933), an excessive amount

of this acid is found in only a small number of plants (see Michael, 1959), so that inhibition of formation of this major product of metabolism in Oxalis provides a possible avenue for selective control.

The white emergent shoots of bulbs of Oxalis pes-caprae rapidly synthesise oxalic acid and contain as much as 16% of oxalic acid on a dry weight basis. The shoots have been used throughout this study since they represent the starting point of the growth of the plant, and therefore the logical stage for selective control. Moreover, the biosynthesis of oxalic acid could be studied in white Oxalis shoots without the added complications of photosynthetic reactions.

PART I. OXALIC ACID SYNTHESIS IN VIVO

A. INTRODUCTION

1. The biosynthesis of oxalic acid

(a) Synthesis of oxalic acid in animals.

The synthesis of oxalic acid in animals has been associated with glycollic acid, glyoxylic acid and glycine. In the rat, only glyoxylic acid was converted to oxalic acid to a significant extent (Weinhouse & Friedman, 1951). Although it seemed likely that glycollic acid was metabolised via glyoxylic acid, *in vivo*, the yield of oxalic acid from glycollic acid was small. Glyoxylic acid also contributed to glycine synthesis in the rat, but there was no significant conversion of glycine to glyoxylic acid or to oxalic acid. Coryell, O'Rear and Hall (1961) have found that, in the rat, glyoxylic acid was converted more readily to oxalic acid than was glycollic acid. However, relatively greater amounts of glycollic acid are converted to oxalic acid in the pyridoxine-deficient rat.

Studies *in vitro* have confirmed that glyoxylic acid may be oxidised enzymically to oxalic acid in animal tissues (Ratner, Nocito & Green, 1944; Nakada & Weinhouse, 1953; Crawhall & Watts, 1962). The wide distribution of glycine oxidase in animal tissues

(Ratner, Nocito & Green, 1944) suggested that glycine was a likely precursor for glyoxylic acid and oxalic acid in animals. Studies in vivo in the rat have substantiated this proposal (Archer, Dormer & Scowen, 1958; Calhoun, Jennings & Bradley, 1959; Gershoff & Faragalla, 1959).

(b) Synthesis of oxalic acid in plants.

Studies on the biosynthesis of oxalic acid in plants received a stimulus from the discovery of an enzyme, glycollate : O_2 oxidoreductase (glycollate oxidase, EC1.1.3.1, formerly known as, glycollic acid oxidase), first in barley leaves (Kolesnikov, 1948) and subsequently in a wide range of green plants (Clagett, Tolbert & Burris, 1949). Although this flavoprotein enzyme was considered to be incapable of oxidising glyoxylic acid (Zelitch & Ochoa, 1953; Frigerio & Harbury, 1958), Kenton & Mann (1952) and Richardson & Tolbert (1961a) have shown oxalic acid to be the end product of glyoxylic acid oxidation by this enzyme. The apparent discrepancy in these findings can probably be explained by two factors.

The oxidation of glycollic acid to glyoxylic acid, catalysed by glycollate oxidase, is accompanied by the production of hydrogen peroxide (Kenton & Mann, 1952; Zelitch & Ochoa, 1953). The subsequent fate of glyoxylic acid so formed depends on the presence of catalase. In the absence of catalase, the oxidation of glycollic acid by molecular oxygen catalysed by glycollate oxidase

results in the production of formic acid, carbon dioxide and water according to the equations :-

$$\text{Glycollic acid} + O_2 \xrightarrow{\text{Glycollate oxidase}} \text{Glyoxylic acid} + H_2O_2$$

$$\text{Glyoxylic acid} + H_2O_2 \xrightarrow{\text{Non-enzymic}} \text{Formic acid} + CO_2 + H_2O$$

In the absence of catalase, then, oxalic acid would not be identified as the end product of glycollic acid oxidation via glyoxylic acid.

The enzymic oxidation of glyoxylic acid to oxalic by glycollate oxidase is strongly inhibited by oxalic acid itself (Tolbert, Glagett & Burris, 1949; Richardson & Tolbert, 1961a). Thus, in crude enzyme preparations the oxidation of glyoxylic acid to oxalic acid may be difficult to demonstrate, but the purified enzyme, although more active with glycollic acid, nonetheless will catalyse the oxidation of glyoxylic acid to oxalic acid.

The incorporation of ^{14}C from glycollic acid into oxalic acid has been demonstrated *in vivo* in tobacco leaves (Zbinovsky & Burris, 1952; Vickery & Palmer, 1956). However, the formation of oxalic acid appeared to be a side reaction; citric acid was clearly established as the main product of metabolism. The accumulation of citric acid *in vivo* is probably due to the inhibition of citrate (isocitrate) hydrolyase (aconitate hydratase, EC 4.2.1.3, formerly known as aconitase) by glyoxylic acid (Ruffo, Adinolfi, Budillon &

Capobianco, 1962), the oxidation product of glycollic acid. The administration of [^{14}C] glycollic acid to spinach leaves (Richardson & Tolbert, 1961a) and to white Oxalis shoots (this thesis, Table 4 p. 80; see also, Millerd, Morton & Wells, 1963), both of which contain high concentrations of oxalic acid, resulted in the incorporation of ^{14}C into oxalic acid *in vivo*.

(c) Synthesis of oxalic acid in microorganisms

In contrast with the small number of direct precursors postulated for oxalic acid synthesis in animals and plants, studies with microorganisms have suggested that a large number of compounds may be involved in this synthesis. Glycollic acid and glyoxylic acid have been associated with oxalic acid production, but a number of other compounds have also been considered as direct precursors of oxalic acid in certain fungi. The oxidation of acetic acid via glycollic acid and glyoxylic acid to oxalic acid was suggested by Challenger, Subramanian & Walker (1927) and Nord and Vitucci (1947). However, the direct oxidation of acetic acid to oxalic acid does not occur (Lewis & Weinhouse, 1951b; Bomstein & Johnson, 1952; Cleland & Johnson, 1956). Similarly, the proposed coupling of two molecules of formic acid (Chrzaszcz & Zakomorny, 1933) or the hydrolysis of oxalosuccinic acid (Lynen & Lynen, 1948) do not appear to be operative in the biosynthesis of oxalic acid (Lewis & Weinhouse, 1951a).

It is difficult to present a precise mechanism for the synthesis of oxalic acid in microorganisms as many different pathways have been proposed. These metabolic pathways may be separated into three groups.

(i) The oxidation of glycollic acid via glyoxylic acid to oxalic acid.

Although it was not possible to demonstrate the oxidation of glycollic acid via glyoxylic acid to oxalic acid in all strains of Aspergillus niger, Franke & de Boer (1959) reported that A. niger, strain 315 was capable of an almost quantitative conversion of glycollic acid to oxalic acid. In other strains, a dismutation reaction occurred resulting in the synthesis of glycollic acid and of oxalic acid from glyoxylic acid. The reduction of NAD in the presence of glyoxylic acid and CoA by a partially purified enzyme from Pseudomonas oxalaticus has also been described (Quayle, Keech & Taylor, 1961).

(ii) The production of oxalic acid from oxaloacetic acid.

The biosynthesis of oxalic acid from oxaloacetic acid is well documented (Raistrick & Clark, 1919 ; Nord & Vitucci, 1947; Lynen & Lynen, 1948; Lewis & Weinhouse, 1951b; Cleland & Johnson, 1956). One molecule of oxaloacetic acid is hydrolytically cleaved to one molecule of oxalic acid and one molecule of acetic acid (Hayaishi, Shimazono, Katagiri & Saito, 1956) or oxidatively split to two molecules of oxalic acid (Cleland & Johnson, 1956). Cleland

& Johnson (1956) conclude, that in A. niger, strain 72-4, oxaloacetic acid is normally present in higher concentrations than acetyl-CoA; oxaloacetic acid not involved in the synthesis of citric acid is therefore converted to oxalic acid. Evidentally, there is a close relationship between the synthesis of citric acid and of oxalic acid in A. niger. Lewis & Weinhouse (1951a) established that ^{14}C from [^{14}C] acetic acid was incorporated into citric acid and into oxalic acid and that oxaloacetic acid was probably associated with both of these syntheses. Cleland & Johnson (1956) were able to control the production of citric acid or oxalic acid in A. niger, strain 72-4, simply by changing the pH of the culture medium. At low pH values citric acid was formed and at neutral pH values, oxalic acid synthesis was favoured.

(iii) The production of oxalic acid from sugars and related compounds.

The oxidation of hexoses or pentoses to 2-keto acids and the subsequent splitting off of oxalic acid was proposed by Allsopp (1937). More recently, Cleland & Johnson (1956) have shown that a wide range of sugars and sugar alcohols are converted to oxalic acid in A. niger, strain 72-4, at neutral pH values. The pathways involved in this synthesis were insensitive to fluoride and fluoroacetic acid and thus did not appear to involve tricarboxylic acid cycle intermediates or glycolysis intermediates beyond the triose level. The suggested scheme for the fermentation of sugars to oxalic acid at neutral pH

values required transketolase, transaldolase, pentose and tetrose isomerases and glycolytic enzymes for the conversion of hexoses to trioses.

The net reaction may be summarised as follows:-

2 Ribose ⟶ 5 Glycolaldehyde.

Glycolaldehyde thus produced was presumed to be oxidised via glycollic acid and glyoxylic acid to oxalic acid. This theory of oxalic acid formation required a complete conversion of sugar carbon to oxalic acid (except in the case of glucose or gluconate); such yields were sometimes approached with [2-^{14}C] glucose or with [6-^{14}C] glucose (Cleland & Johnson, 1956). At low pH values, this metabolic pathway was apparently inactive, and A. niger, strain 72-4 accumulated citric acid, but at neutral pH these reactions appeared to be of major importance in the production of oxalic acid by this fungus.

2. Sources of C-2 units for oxalic acid synthesis

It is apparent that in different tissues, there may be a number of pathways involved in the biosynthesis of oxalic acid. The fact that a wide range of compounds contribute to oxalic acid synthesis indicates that the immediate precursors for this synthesis may arise from a pool through which these compounds are metabolised. Intermediates of the tricarboxylic acid cycle would fulfil this

requirement; in particular oxaloacetic acid and citric acid have been associated with oxalic acid synthesis (Cleland & Johnson, 1956).

In plants and in animals glycollic acid and glyoxylic acid are considered to be the immediate precursors of oxalic acid. The proposal that glycolaldehyde, a product of the pentose phosphate cycle, acts as the source of glycollic acid (Cleland & Johnson, 1956) is only one of a number of possibilities for the synthesis of glycollic acid or glyoxylic acid. Since these two acids appear to be intimately involved in oxalic acid biosynthesis, particularly in plants, some of the reactions concerned with their formation are considered here.

(a) The precursors of glycollic acid

(i) Glycollic acid as a product of photosynthetic carbon dioxide fixation.

It is well established that glycollic acid is one of the early products of carbon dioxide fixation during photosynthesis in green algae or higher plants (Benson & Calvin, 1950; Tolbert & Zill, 1956; Tolbert, 1958). Alternating exposure with $^{14}CO_2$ and with $^{12}CO_2$ confirmed that the glycollic acid synthesised was derived from intermediates of the photosynthetic carbon reduction cycle (Tolbert & Zill, 1956). Glycollic acid has been considered as the initial product of carbon dioxide assimilation during photosynthesis (Warburg &

Krippahl, 1960; Tanner, Brown, Eyster & Treharne, 1960), but studies on ^{14}C incorporation from $^{14}CO_2$ into the products of photosynthesis have shown that glycollic acid becomes labelled too slowly to justify this claim (Schou, Benson, Bassham & Calvin, 1950; Calvin and co-workers, 1951a).

Bradbeer and Racker (1961) have demonstrated the formation of glycollic acid from fructose-6-phosphate, catalysed by crystalline D-sedoheptulose-7-phosphate : D-glyceraldehyde-3-phosphate glycolaldehyde transferase (transketolase, EC 2.2.1.1.), in the presence of ferricyanide. This synthesis was also demonstrated with a mixture of xylulose-5-phosphate, ribose-5-phosphate and ribulose-5-phosphate. It was concluded that the "active glycolaldehyde-transketolase complex" could be oxidised in the presence of ferricyanide, resulting in the formation of glycollic acid. The transient formation of glycolaldehyde from intermediates of the pentose phosphate cycle has also been suggested by the results of a number of investigations into the origin of glycollic acid (Cleland & Johnson, 1956; Bolcato, de Bernard & Leggiero, 1957; Bolcato & Leggiero, 1959), and is supported by the demonstration of a glycolaldehyde dehydrogenase isolated from pea mitochondria (Davies, 1960). However, Bradbeer & Racker (1961) showed that glycollic acid was formed from fructose-6-phosphate, in the absence of ferricyanide, either in intact chloroplasts or extracts from chloroplasts containing transketolase. The reaction was stimulated by light.

The isolation of phosphoglycollic acid as an early product

of photosynthetic carbon dioxide fixation *in vivo* (Calvin and co-workers, 1951b; Benson and co-workers, 1952) and in isolated chloroplasts (Kearney & Tolbert, 1962) established this compound as the likely immediate precursor of glycollic acid. It is evident that a large number of sugars or sugar phosphates which can enter the pentose phosphate cycle may contribute to the synthesis of glycollic acid. In particular, ^{14}C from ^{14}C labelled fructose-6-phosphate (Bradbeer & Racker, 1961), ribose (Griffith & Byerrum, 1959), from ribose-5-phosphate (Wilson & Calvin, 1955; Weissbach & Horrecker, 1955) or from ribulose-diphosphate (Pritchard, Griffin & Whittingham, 1962) have been shown to be incorporated directly into glycollic acid or into glycine. The pattern of incorporation of ^{14}C into glycine from specifically labelled pentose phosphate cycle intermediates during photosynthesis was consistent with the prior formation of glycollic acid from C-1 and C-2 carbon atoms of pentose phosphates.

Glycollic acid synthesis during photosynthetic fixation of carbon dioxide is maximal at low concentrations of carbon dioxide (Benson & Calvin, 1950; Tolbert, 1958; Pritchard, Griffin & Whittingham, 1962). This observation initiated the proposal that the primary carbon dioxide acceptor, ribulose-diphosphate, present in excess at low carbon dioxide concentrations, was split to a C_2 phosphate and a triose phosphate (Tolbert, 1958; Pritchard, Griffin & Whittingham, 1962). The C_2 moiety was oxidised to phosphoglycollic and subsequently converted to glycollic acid by

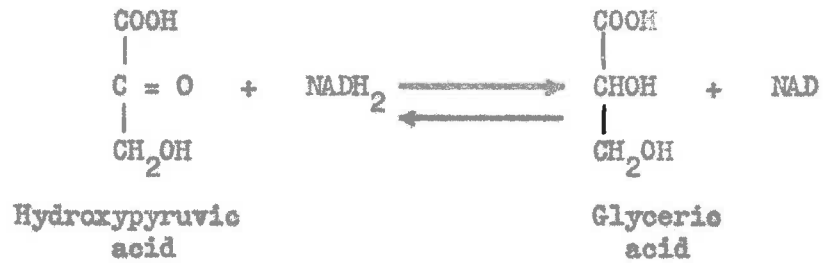
a specific phosphatase (Richardson & Tolbert, 1961b). As the carbon dioxide concentration increased, the excess of ribulose-diphosphate decreased and consequently less glycollic acid was synthesised.

Although there has been a report of rapid incorporation of carbon dioxide into glycollic acid in the dark in bean roots (Kursanov, Kryakova & Vyskrebentsera, 1953), other evidence suggests that this synthesis was essentially associated with photosynthetic reactions in the light (Griffith & Byerrum, 1959; Tolbert, 1955).

Glycollic acid is chiefly metabolised to glyoxylic acid and oxalic acid, and to lesser extent to glycine and serine in non-photosynthetic Oxalis shoots (this thesis, Table 4, p. 68). In photosynthetic tissues, glycollic acid is associated with a number of metabolic conversions. Glycine and serine were reported as the chief products of glycollic acid in vivo (Tolbert & Cohan, 1953b). Subsequently, Rabson, Tolbert & Kearney (1962) have proposed a "glycollate pathway" in which glycollic acid arising from photosynthetic intermediates is metabolised via glyoxylic acid, glycine, serine and glyceric acid to sucrose. This pathway has been confirmed by the administration of specifically labelled serine and glycollic acid to wheat leaves and soybean leaves (Jiminez, Baldwin, Tolbert & Woods, 1962). The significance of this pathway in green plants will be discussed subsequently.

(ii) Glycollic acid as a product of glyceric acid metabolism.

The possibility of glycollic acid synthesis being associated with glyceric acid metabolism arose from the findings of Milhaud, Benson & Calvin (1956) that considerable ^{14}C from [^{14}C] hydroxypyruvic acid was incorporated into glycollic acid in Scenedesmus. Stafford, Magaldi & Vennesland (1954) have isolated D-glycerate : NAD oxidoreductase (glycerate dehydrogenase, EC 1.1.1.29) from green leaves which catalyses the reduction of hydroxypyruvic acid to D-glyceric acid, according to the following equation :-



This enzyme is not active with pyruvic acid (c.f. Ballou & Hesse, 1956), but is probably similar to glycollate : NAD oxidoreductase (glyoxylate reductase, EC 1.1.1.26) isolated from green plants (Zelitch, 1953). The equilibrium of this reaction lies strongly in favour of hydroxypyruvic acid reduction; it is, therefore, unlikely that this enzyme is of physiological significance in the synthesis of hydroxypyruvic acid (or glycollic acid) from D-glyceric acid.

A number of keto acids including hydroxypyruvic acid are

substrates for mammalian L-lactate dehydrogenase. Willis & Sallach (1962) have partially purified D-glycerate dehydrogenase active with NAD or NADP; the production of hydroxypyruvic acid from glyceric acid could be demonstrated in this system. A D-α-hydroxy acid oxidase, apparently requiring no coenzyme, has been isolated from rabbit mitochondria (Tubbs, 1960a, b; Tubbs & Greville, 1961). This enzyme, which catalyses the oxidation of D-lactic acid at a greater rate than for L-lactic acid, also catalyses the oxidation of D-glyceric acid to hydroxypyruvic acid. The significance of hydroxypyruvic acid in mammalian tissue is probably in relation to serine production (Sallach, 1955; Hedrick & Sallach, 1960), rather than in the production of glycollic acid.

It was proposed by Dickens & Williamson (1956) that yeast L-lactate : cytochrome b_2 oxidoreductase (lactate dehydrogenase, EC 1.1.2.3) would oxidise L-glyceric acid to hydroxypyruvic acid. However, Morton, Armstrong & Appleby (1961) have shown that this enzyme is inactive towards both hydroxypyruvic acid and L-glyeric acid. The claim of Dickens & Williamson (1956) was based on studies with partially purified enzyme from yeast prepared according to Boeri, Cutolo, Luzzati & Tosi (1955), and suggests that yeast contains a separate enzyme capable of utilising hydroxypyruvic acid and L-glyceric acid as substrates. No connection has been shown between hydroxypyruvic acid metabolism and glycollic acid synthesis in yeast.

(iii) Glycollic acid production from glyoxylic acid.

Observations of the effect of catalytic amounts of glycollic acid and of glyoxylic acid on the stimulation of oxygen uptake by homogenates of spinach leaves led to the discovery of a specific $NADH_2$-linked glyoxylate reductase (Zelitch, 1953; 1955). This enzyme, catalyses the reduction of glyoxylic acid to glycollic acid.

Glyoxylic acid Glycollic acid

This enzyme system, coupled with the flavoprotein enzyme glycollate oxidase can transfer hydrogen from $NADH_2$ to oxygen through a series of reactions which constitute a glycollate oxidation cycle (Zelitch, 1953; Zelitch & Ochoa, 1953). Although crude preparations of glyoxylate reductase react with $NADH_2$ and $NADPH_2$, the rate of oxidation of $NADH_2$ is approximately five times greater; the crystalline enzyme from tobacco leaves (Zelitch, 1955) and an enzyme of similar specific activity from spinach leaves (Holzer & Holldorf, 1957) do not oxidise $NADPH_2$ in the presence of glyoxylic acid.

The glyoxylate reductase isolated from castor bean seedlings (Kornberg & Beevers, 1957) showed a low level of activity towards the oxidation of $NADH_2$ but was active in the presence of $NADPH_2$. The "new" glyoxylate reductase specific for $NADPH_2$ has been partially purified from tobacco and spinach leaves (Zelitch & Gotto, 1962).

Glyoxylate reductase activity has also been reported in mammals (Nakada & Weinhouse, 1953) and in microorganisms (Hullin & Hassall, 1960, 1962; Katagiri & Tochikura, 1960a).

The significance of glyoxylate reductase in microorganisms is obscure. It may be coupled with the oxidation of other substrates such as malic acid, isocitric acid or glucose-6-phosphate (Hullin & Hassall, 1962). The low level of glycollate oxidase activity in bacterial extracts (Katagiri & Tochikura, 1960b; Hullin & Hassall, 1962) renders it unlikely that glyoxylate reductase is of quantitative importance in a glycollate oxidation cycle. The presence of glyoxylate reductase and of L_s-isocitrate glyoxylate lyase (isocitrate lyase, EC 4.1.3.1., formerly known as isocitritase) adequately explain the occurrence of glycollic acid in bacterial systems when acetic acid is the sole source of carbon for growth (Ajl, 1952; Bolcato, de Bernard & Leggiero, 1957). Thus, the evidence for the direct oxidation of acetic acid to glycollic acid and subsequent oxidation of glycollic acid via glyoxylic acid to formic acid and carbon dioxide (Bolcato, 1959; Bolcato, Scevola & Tiselli, 1958) is largely invalidated.

In green plants, the two glyoxylate reductases appear to be linked with oxidative enzymes within the chloroplasts or mitochondria (Zelitch & Gotto, 1962). A similar function for glyoxylate reductase has been proposed for the transport of hydrogen from $NADH_2$ in the cytoplasm through the mitochondrial membrane of liver tissue (Schäfer & Lamprecht, 1961). In photosynthetic tissue, glyoxylic acid is

reduced to glycollic acid only in the light. Kearney & Tolbert (1962) have concluded that glyoxylate reductase activity (particularly that of the $NADPH_2$-linked enzyme (Zelitch & Gotto, 1961)) is dependent upon the supply of reduced nicotinamide nucleotide coenzymes derived from photosynthesis.

In non-photosynthetic tissues the reduction of glyoxylic acid to glycollic acid also occurs, but is not dependent upon light for activity (Kornberg & Beevers, 1957; see also, this thesis, Table 4, p.68 & p.123). Both the castor bean and Oxalis are tissues with a rather unique type of metabolism. In the former case C_2 units from fatty storage tissue are rapidly incorporated into carbohydrate through the operation of the glyoxylate cycle (Kornberg & Beevers, 1957a), and in the latter case there is rapid synthesis of C_2 units for the production of oxalic acid. The production of glycollic acid involving isocitrate lyase, L_s-isocitrate : NADP oxidoreductase (isocitrate dehydrogenase, EC 1.1.1.42) and glyoxylate reductase is indicated below.

$$\text{Isocitric acid} \xrightarrow[MgCl_2,\ GSH]{\text{Isocitrate lyase}} \text{Glyoxylic acid} + \text{Succinic acid}$$

$$\text{Isocitric acid} + NADP \xrightarrow{\text{Isocitrate dehydrogenase}} \alpha\text{-Oxoglutaric acid} + NADPH_2 + CO_2$$

$$\text{Glyoxylic acid} + NADPH_2 \xrightarrow{\text{Glyoxylate reductase}} \text{Glycollic acid} + NADP$$

Overall, the reactions may be summarised as follows :-

$$2 \text{ Isocitric acid} \longrightarrow \underset{\text{acid}}{\alpha\text{-Oxoglutaric}} + \underset{\text{acid}}{\text{Succinic}} + \underset{\text{acid}}{\text{Glycollic}} + CO_2$$

In non-photosynthetic tissues containing isocitrate lyase, the action of isocitrate dehydrogenase provides $NADPH_2$ for the reduction of glyoxylic acid. In photosynthetic plants it is likely that the reduced coenzyme is derived from photosynthetic reactions. Light is also necessary for the production of glycollic acid from pentose phosphate intermediates. This glycollic acid pool is the precursor of glyoxylic acid in such tissues. The reduction of glyoxylic acid to glycollic acid completes one turn of the glycollate oxidation cycle.

(b) The precursors of glyoxylic acid

(i) Glyoxylic acid formation from glycollic acid

Glycollate oxidase is of widespread occurrence in green plants (Clagett, Tolbert & Burris, 1949). This enzyme, which catalyses the oxidation of glycollic acid to glyoxylic acid, was identified as a flavoprotein enzyme with riboflavin phosphate as the prosthetic group (Zelitch & Ochoa, 1953). A similar enzyme has been partially purified from rat liver (Kun, Dechary & Pitot, 1954; Robinson, Keay, Molinari & Sizer, 1962) and from extracts of Pseudomonas ovalis (Chester) (Kornberg & Gotto, 1959). As previously discussed (p.5), it has now been shown that the purified enzyme

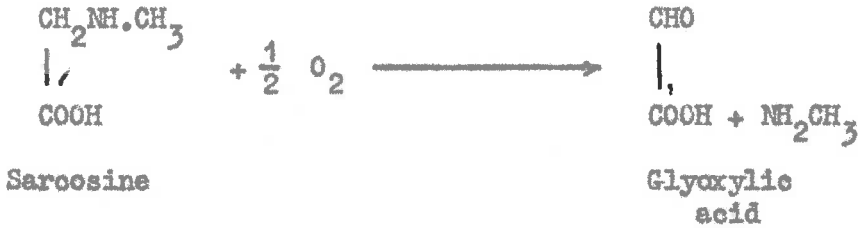
is capable of oxidising glycollic acid to glyoxylic acid and glyoxylic acid to oxalic acid (Richardson & Tolbert, 1961a).

Clagett, Tolbert & Burris (1949) and Tolbert & Burris (1950) found that glycollate oxidase activity was absent from etiolated plants and embryos. By exposing such plants to light the activity of this enzyme was readily demonstrated. Although this "activation" was thought to be associated directly with the light, Tolbert & Cohan (1953a) were able to obtain a similar response of glycollate oxidase activity in etiolated plants in the dark, by the administration of the substrate, glycollic acid. They proposed that the response in enzyme activity was due to glycollic acid; in the light glycollic acid itself was produced from the intermediates of photosynthetic carbon assimilation.

(ii) Glyoxylic acid formation from glycine.

A flavin-adenine dinucleotide enzyme, glycine oxidase, (viz. sarcosine : O_2 oxidoreductase, EC 1.5.3.1.) was found in a wide range of animal liver and kidney homogenates (Ratner, Nocito & Green, 1944). This enzyme catalyses the oxidative deamination of glycine or the demethylamination of sarcosine according to the following equations.

Glycine — Glyoxylic acid



Sarcosine — Glyoxylic acid

Although Ratner, Nocito & Green (1944) claimed that glycine oxidase was distinct from D-amino acid : O_2 oxidoreductase (D-amino acid oxidase, EC 1.4.3.3), Neims & Hellerman (1962) have concluded that D-amino acid oxidase is also responsible for the oxidative deamination of glycine. The interconversion of glycine and glyoxylic acid in animal tissues is well documented (Weinhouse & Friedman, 1951, 1952; Nakada & Weinhouse, 1953; Weinhouse, 1955; Meister, 1956, 1957). Excessive tissue breakdown in the rat, following thiamine deficiency, leads to the conversion of considerable quantities of glycine to glyoxylic acid (Liang, 1962a). In microorganisms, the formation of glyoxylic acid from glycine has either been demonstrated directly (Janke & Tayenthal, 1937; Campbell, 1955), or inferred from labelling studies with [^{14}C] glycine (Dagley, Trudgill & Callely, 1961).

In plants, the conversion of glycine to glyoxylic acid does occur (Krupka & Towers, 1958) but the reverse reaction of glycine synthesis from glyoxylic acid seems to be of greater physiological significance (Tolbert & Cohan, 1953b). Since glycine and serine are inter-convertible (Wilkinson & Davies, 1958; Hauschild, 1959) serine could be metabolised via glycine to glyoxylic acid. However, <u>in vivo</u>

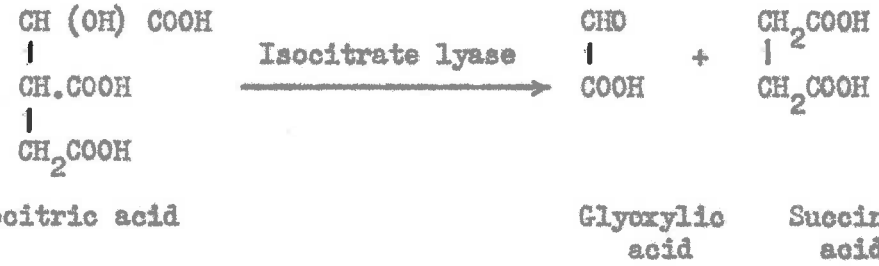
glycine is readily converted to serine but serine synthesis from glycine is slow (Wang & Burris, 1962).

(iii) Glyoxylic acid formation from Y-hydroxyglutamic acid.

Investigations with rat liver slices showed that Y-hydroxyglutamic acid was a product of hydroxyproline metabolism (Wolf & Berger, 1958). Adams and Goldstone (1960a,b) demonstrated that the initial enzymic reactions in mamalian catabolism of hydroxyproline were first, the oxidation of L-hydroxyproline to Δ^1-pyrroline-3-hydroxy-5-carboxylic acid and secondly, oxidation of the latter compound to Y-hydroxyglutamic acid. Subsequently, Maitra & Dekker (1961) postulated a stepwise conversion of Y-hydroxyglutamic acid to glyoxylic acid and alanine. A partially purified extract from rat liver requiring catalytic quantities of glutamic acid and pyridoxal phosphate catalyses this overall reaction (Dekker & Maitra, 1962). Glyoxylic acid synthesised from Y-hydroxyglutamic acid may be further metabolised to glycine (Bouthillier, Binette & Pouliot, 1961).

(iv) Glyoxylic acid formation from isocitric acid.

The enzyme isocitrate lyase catalyses the cleavage of one molecule of isocitric acid to one molecule each of glyoxylic acid and succinic acid as shown below.



Isocitric acid — Glyoxylic acid — Succinic acid

Initially, Campbell, Smith & Eagles (1953) considered citric acid to be the substrate for this reaction, but subsequently isocitric acid was shown to be the true substrate for isocitrate lyase (Saz, 1954). The presence of this enzyme was soon demonstrated in a number of microorganisms (Olsen, 1954; Smith & Gunsalus, 1954; Saz & Hillary, 1956; Kornberg & Krebs, 1957). The requirement of Mg^{2+} ions and GSH or cysteine for isocitrate lyase activity was reported by Smith & Gunsalus (1955) and later confirmed by Olsen (1959).

The first demonstration of isocitrate lyase activity in extracts other than those from microorganisms was reported by Kornberg & Beevers (1957a,b). Extracts from the fatty endosperm of germinating castor beans seedlings (Ricinus communis) catalysed the cleavage of isocitric acid to glyoxylic acid and succinic acid. The disappearance of fat was equivalent to the appearance of carbohydrate in such seedlings and isocitrate lyase was operative only during this active conversion (Carpenter & Beevers, 1959). The metabolism of plants converting fat to carbohydrate and of microorganisms growing on acetic acid as sole carbon source is essentially similar. In such cases, the combined action of isocitrate lyase and of L-malate glyoxylate-lyase (malate synthetase, EC 4.1.3.2.) can replace the oxidative steps of the

tricarboxylic acid cycle leading from isocitric acid to malic acid (Kornberg & Krebs, 1957), and account for the nett synthesis of C_4 units from C_2 units. The enzyme malate synthetase was first discovered in extracts from Escherichia coli, strain E-26, grown on acetic acid as sole carbon source (Wong & Ajl, 1956a). In the presence of isocitrate lyase (to provide glyoxylic acid) the stepwise synthesis of malic acid from acetic acid may be represented thus :

$$\text{Acetic acid} + \text{ATP} \longrightarrow \text{Acetyl-phosphate} + \text{ADP}$$

$$\text{Acetyl-phosphate} + \text{CoA} \xrightarrow{\text{Transacetylase}} \text{Acetyl-CoA} + \text{Pi}$$

$$\text{Acetyl-CoA} + \text{Glyoxylic acid} \xrightarrow{\text{Malate synthetase}} \text{Malic acid} + \text{CoA}$$

Although the production of glyoxylic acid from malic acid has been reported in tomato slices (Link, Klein & Barron, 1952) and in Rhodoseudomonas spheroides (Tuboi & Kikuchi, 1962), the equilibrium of malate synthetase is strongly in favour of malic acid synthesis.

The "short circuit" of the tricarboxylic acid cycle in the presence of isocitrate lyase and malate synthetase has been designated the "glyoxylate by-pass" or "glyoxylate cycle" (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957). The overall effect of one turn of the glyoxylate cycle is the formation of one molecule of C_4 dicarboxylic acid from two molecules of acetic acid. This, together with acetic acid, can serve as the precursor of many cell constituents. Certainly, this function appears to be of paramount importance in the

growth of microorganisms from acetic acid and in the nett conversion of fat to carbohydrate in plants. However, isocitrate lyase and malate synthetase do not always occur together, nor is their function always clearly defined. Thus, isocitrate lyase is apparently absent in animal tissues (Madsen, 1958; Olsen, 1959; Levenbrook, 1961) although malate synthetase activity has been demonstrated in animals (Ganguli & Chakraverty, 1961). In addition, isocitrate lyase activity is not necessarily associated with the utilisation of C_2 units alone as proposed by Kornberg & Beevers, (1957b) and Callely, Dagley & Hodgson (1958). This enzyme has been found in the protozoan, Tetrahymena pyriformis, during growth on a complex medium (Reeves, Papa, Seaman & Ajl, 1961).

3. Alternate metabolic pathways for compounds considered as precursors of oxalic acid

(a) Alternate metabolism by microorganisms of compounds considered as precursors of oxalic acid.

Although this section does not consider oxalic acid synthesis directly, it is concerned with precursors of oxalic acid at the C_1 and C_2 level, and their contribution to pathways other than oxalic acid biosynthesis. Furthermore, there is evidence for an oxidative breakdown of oxalic acid in plants (Zelitch & Ochoa, 1953; Giovanelli & Tobin, 1961). The intermediates involved in the catabolism of oxalic acid are also concerned in the growth of organisms

on C_1 and C_2 units as sole carbon source.

(i) Growth on C_1 units as sole carbon source.

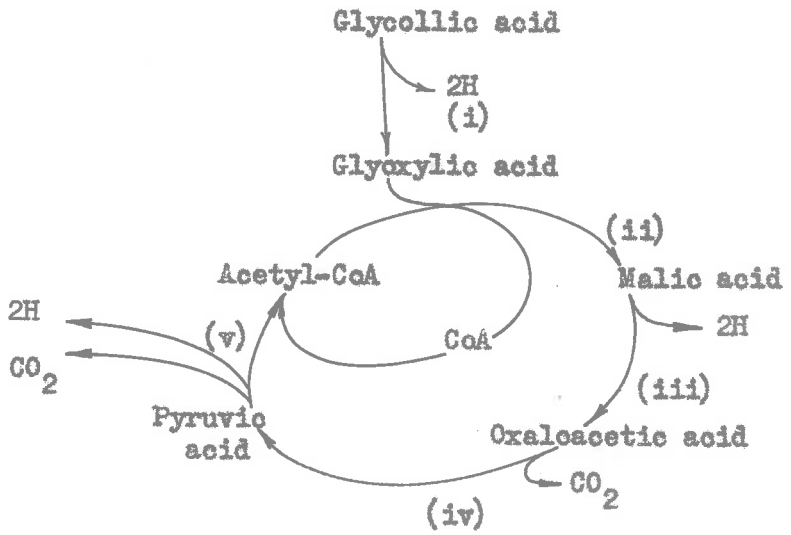
Isotopic experiments on Pseudomonas oxalaticus (OXI) grown on formic acid as sole carbon and energy source (Quayle & Keech, 1958, 1959a) indicated that this organism contained a carbon dioxide fixation cycle similar to that in photosynthetic tissue (Bassham and co-workers, 1954) and autotrophic bacteria (Santer & Vishniac, 1955; Trudinger, 1955). These indications were confirmed by the isolation of ATP : D-ribulose-5-phosphate 1-phosphotransferase (phosphoribulokinase, EC 2.7.1.19) and carboxydismutase, enzymes essential for the working of such a cycle, from these microorganisms (Quayle & Keech, 1959b). Both bicarbonate and formic acid were rapidly incorporated into 3-phosphoglyceric acid in the presence of ribulose 1:5-diphosphate or ribose-5-phosphate plus ATP. The incorporation of formic acid is preceded by an oxidation of formic acid to carbon dioxide. The carbon dioxide may be incorporated into pentose phosphate (from which glycollic acid is derived in photosynthetic tissues), or into malic acid in the presence of pyruvic acid, or into oxalacetic acid in the presence of phosphopyruvic acid.

(ii) Growth on C_2 units as sole carbon source.

When microorganisms grow on acetic acid as sole carbon source, the reactions of tricarboxylic acid cycle can provide the necessary

energy for growth and concomitant action of the glyoxylate cycle can replenish the supply of intermediates required for growth (Kornberg & Krebs, 1957). However, this combination of reactions cannot account for the growth of microorganisms from C_2 compounds which are more highly oxidised than acetic acid, such as glycollic acid.

The mechanism by which the energy requirement for growth on glycollic acid as sole carbon sourse is satisfied has been elucidated by Kornberg & Sadler (1960). A mutant of E. coli, although devoid of the condensing enzyme (Gilvarg & Davis, 1957) and hence unable to utilise acetic acid for the synthesis of citric acid, was nonetheless capable of synthesising the necessary intermediates for growth from glycollic acid alone. To account for this situation, Kornberg & Sadler (1960) proposed that glycollic acid could be oxidised via a dicarboxylic acid cycle, the essential features of which are shown below. Such a mechanism obviates the necessity for the reduction of glyoxylic acid to the oxidative level of acetic acid prior to its catabolism.



The steps involved in the proposed dicarboxylic acid cycle are as follows:

(i) The oxidation of glycollic acid to glyoxylic acid (Kornberg & Gotto, 1959).

(ii) Condensation of glyoxylic acid with acetyl-CoA to form malic acid (Wong & Ajl, 1956b).

(iii) Oxidation of malic acid to oxaloacetic acid (Mehler, Kornberg, Grissolia & Ochoa, 1948).

(iv) Decarboxylation of oxaloacetic acid to pyruvic acid and carbon dioxide, possibly via phosphopyruvate (Utter & Kurahashi, 1954).

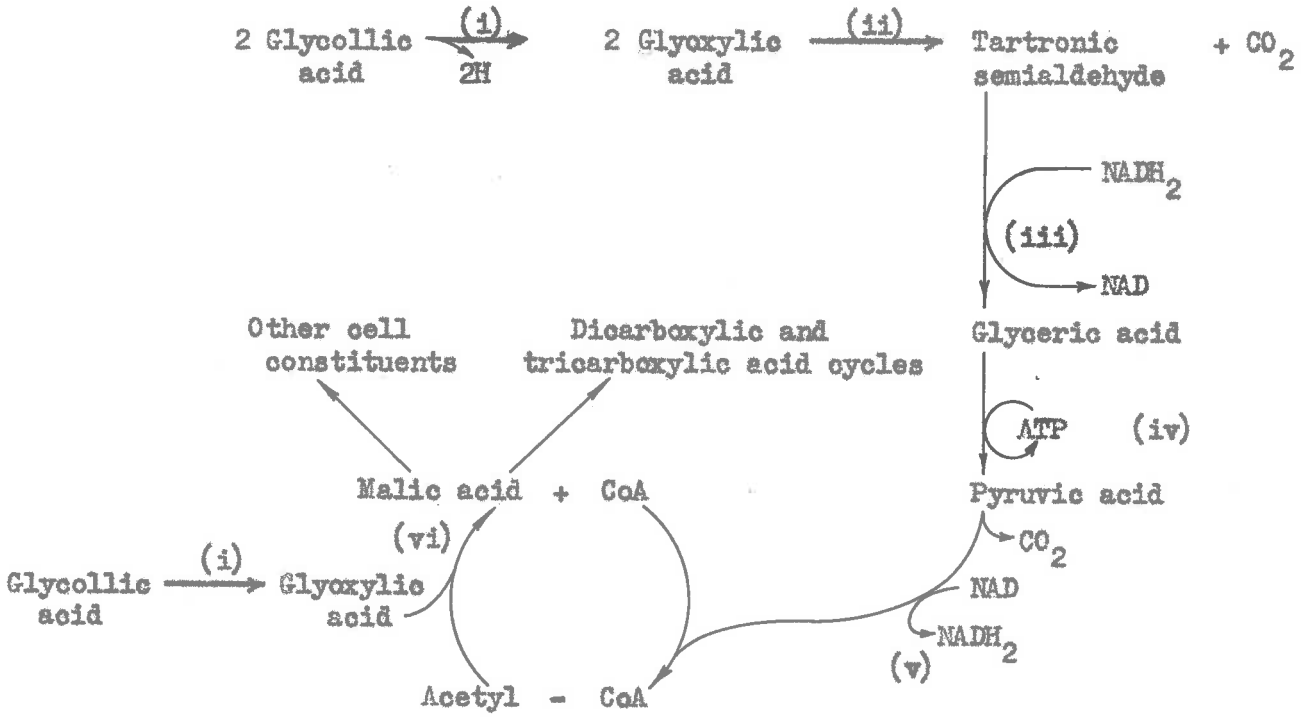
(v) The regeneration of acetyl-CoA from pyruvic acid by oxidative decarboxylation involving the pyruvate oxidase system (Korkes, 1955).

In a single turn of the tricarboxylic acid cycle, one molecule of acetyl-CoA is completely oxidised with the obligatory participation of a keto acid which is regenerated. One turn of the dicarboxylic acid cycle results in the complete oxidation of one molecule of keto acid with the obligatory participation of acetyl-CoA.

In addition to energy requirements, the growth of micro-organisms on C_2 compounds requires the net synthesis of C_3 and C_4 compounds necessary for the formation of carbohydrates and amino acid skeletons. Cellular constituents are synthesised from intermediates of the tricarboxylic and dicarboxylic acid cycles (Roberts, Abelson, Cowie, Bolton & Britten, 1955; Davis, 1955; Ehrensvärd, 1955;

Kornberg, 1959). Growth necessarily involves the removal of such intermediates from these cycles, and cannot take place unless reactions occur to effect the net formation of these compounds from the C_2 source. The key initial compound in such syntheses is glyoxylic acid. Thus, growth on oxalic acid requires an initial reductive step, probably via oxalyl-CoA (Quayle, Keech & Taylor, 1961), growth on glycollic acid an oxidative conversion (Kornberg & Gotto, 1961) and growth on glycine involves the initial deamination of glycine to glyoxylic acid (Callely & Dagley, 1959).

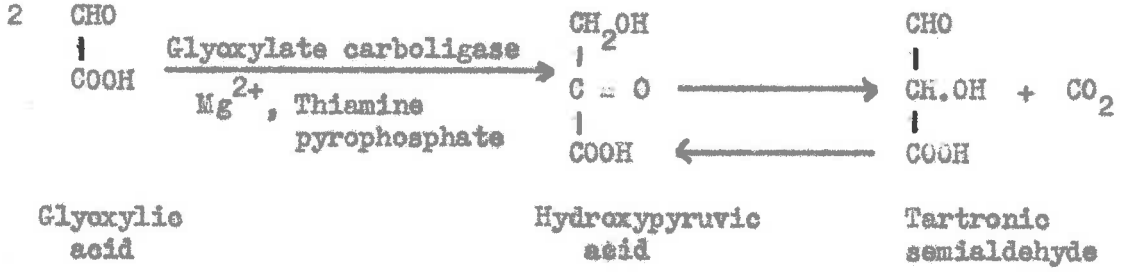
The sequence of reactions leading to the net synthesis of cell constituents from C_2 compounds more highly oxidised than acetic acid have been demonstrated in Pseudomonas grown on glycollic acid as sole carbon source (Kornberg & Gotto, 1961). This scheme is presented below:



The oxidation of glycollic acid to glyoxylic acid (i) and the condensation of glyoxylic acid and acetyl-CoA to form malic acid (vi) have been discussed previously (p. 28). The other reactions involved in the above sequence are briefly discussed here.

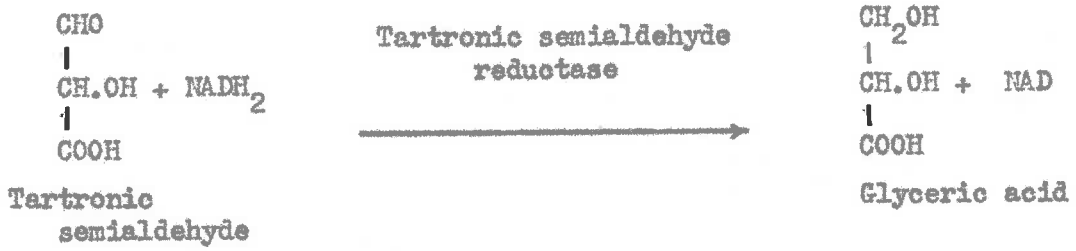
(a) The condensation of two molecules of glyoxylic acid to one molecule of tartronic semialdehyde (ii).

This condensation reaction was discovered in E. coli by Krakow & Barkulis (1956). The C_3 compound formed was originally considered to be hydroxypyruvic acid, although it was realised that this compound would be in equilibrium with its isomer, tartronic semialdehyde.



The enzyme catalysing the above reaction has been designated, glyoxylate carboligase (Krakow, Hayaishi & Barkulis, 1959). It is now considered that the enzymic product of the reaction is tartronic semialdehyde (Krakow, Barkulis & Hayaishi, 1961; Kornberg & Gotto, 1961) particularly in the light of the isolation of tartronic semialdehyde reductase from glycollic acid grown microorganisms (Kornberg & Gotto, 1961).

(b) Reduction of tartronic semialdehyde to glyceric acid (iii)



Tartronic semialdehyde

Glyceric acid

Tartronic semialdehyde reductase catalyses the reaction shown above. This enzyme, which has been crystallised from P. ovalis (Chester) by Gotto & Kornberg (1961a), is different from D-glycerate dehydrogenase isolated from plant tissues (Stafford, Magaldi & Vennesland, 1954; Holzer & Holldorf, 1957). Hydroxypyruvic acid is reduced at only 3% of the rate observed with equimolar concentrations of tartronic semialdehyde by tartronic semialdehyde reductase (Gotto & Kornberg, 1961b). Tartronic semialdehyde is not reduced by glycerate dehydrogenase.

(c) Conversion of glyceric acid to pyruvic acid (iv)

Glyceric acid is phosphorylated to phosphoglyceric acid and the subsequent formation of pyruvic acid is explained by the well-established reactions of the Embden-Meyerhof scheme.

(d) Completion of the biosynthetic cycle to form malic acid (v), (vi).

Pyruvic acid is oxidatively decarboxylated to acetyl-CoA (Korkes, 1955). This C_2 moiety condenses with glyoxylic acid (from glycollic acid, (i)) catalysed by malate synthetase (vi) with the regeneration of CoA. Malic acid thus formed can then enter the dicarbo-

xylic or tricarboxylic acid cycles which supply the necessary intermediates for growth.

(b) Alternate metabolism by plants of compounds considered as precursors of oxalic acid.

Glycollic acid has been associated in green plants with the synthesis of glycine and serine (Tolbert & Cohan, 1953a) and with oxalic acid synthesis in photosynthetic (Richardson & Tolbert, 1961a) and non-photosynthetic tissues (see this thesis, Table 4, p.68). It is clear that glycollic acid synthesised from intermediates of the photosynthetic cycle is involved in a number of other reactions. Tolbert & Zill (1956) reported that phosphoglycollic acid was rapidly excreted from the chloroplast in which it is formed, into the cytoplasm, where it was hydrolised to glycollic acid. Although glycollic acid itself would not re-enter the chloroplast, its oxidation product, glyoxylic acid would (Rabson, Tolbert & Kearney, 1962). This cyclic process provides for the transport of carbon products and assimilatory power of photosynthesis between the chloroplasts and the cytoplasm.

In green tissue, both [^{14}C] glycollic acid itself and labelled glycollic acid arising from $^{14}CO_2$ gave rise to labelled serine and glyceric acid; [^{14}C] serine was also converted to [^{14}C] glyceric acid (Rabson, Tolbert & Kearney, 1962). Mortimer (1961) and Rabson and co-workers, (1962) have concluded that a pathway exists for the assimilation of carbon dioxide via glycollic acid into glyceric acid

through a series of reactions not involving phosphoglyceric acid. Studies with specifically labelled compounds (Rabson, Kearney & Tolbert, 1962) indicated that this pathway did not involve glyoxylate carboligase and tartronic semialdehyde reductase as proposed for microorganisms (see p. 32), but rather a sequence involving the conversion of serine to hydroxypyruvic acid and reduction of this latter compound to glyceric acid, thus :

2 glycollic acid ⟶ 2 glyoxylic acid ⟶ 2 glycine ⟶ serine

serine ⟶ hydroxypyruvic acid ⟶ glyceric acid

Short term experiments (20 sec.) with $^{14}CO_2$ confirmed that phosphoglyceric acid formed directly from the photosynthetic cycle did contribute to the synthesis of glyceric acid, and to the synthesis of serine via phosphoserine (Ichihara & Greenberg, 1957). Similarly serine derived from glycollic acid, may be converted to phosphoglyceric acid via phosphoserine and phosphohydroxypyruvic acid (Hanford & Davies, 1958).

The significance of the 'glycollate pathway' of glyceric acid synthesis may be that it provides a glyceric acid pool in the cytoplasm (since glycollic acid is excreted from chloroplasts) as distinct from the pool derived from phosphoglyceric acid within the chloroplast. Jiminez, Baldwin, Tolbert & Wood (1962) have established the existence of a glycollate pathway from the early carbon products of photosynthesis to uniformly labelled sucrose. This synthesis occurs

with little participation of phosphoglyceric acid or sugar phosphates (Rabson, Tolbert & Kearney, 1962). In contrast, sucrose synthesis from the glyceric acid pool within the chloroplasts is intimately associated with the prior formation of phosphoglyceric acid and sugar phosphates.

4. Investigations into the synthesis of oxalic acid in *Oxalis*

It is apparent from the foregoing discussion that a number of compounds have been implicated as precursors of oxalic acid. The problem of this synthesis in *Oxalis* was initially approached by administering a number of such compounds labelled with ^{14}C to non-photosynthetic shoots *in vivo*. Examination of the subsequent fate of ^{14}C in specific compounds indicated that glycollic acid and glyoxylic acid were intimately involved in oxalic acid synthesis.

Investigations were then directed towards the origin of glycollic acid and of glyoxylic acid in *Oxalis* shoots. It was apparent from the administration of ^{14}C labelled glyoxylic acid that this acid contributed significantly to glycollic acid production. This result indicated that, in *Oxalis*, there may be a pathway for the synthesis of glyoxylic acid other than the oxidation of glycollic acid. It has now been shown that, in *Oxalis*, glyoxylic acid is formed from isocitric acid catalysed by isocitrate lyase.

In conformation of the pathways for oxalic acid synthesis indicated by *in vivo* studies, the reactions involved in the production of glyoxylic acid, both from glycollic acid and isocitric acid, and its subsequent oxidation to oxalic acid have been characterised with cell-free extracts from *Oxalis* shoots.

B. MATERIALS

1. Plants

Bulbs of *Oxalis pes-caprae* were harvested from the field and stored in a cold room at 2° to prevent germination until required. The bulbs were germinated in the dark at room temp. (15° - 20°) in trays containing moistened vermiculite. Bulbs harvested in February and March were used from March to October, but those stored longer than this period usually failed to germinate. The white shoots which emerged were kept moist and were allowed to grow in the dark for 7 to 10 days until approx. 3 cm long. The oxalic acid content of such shoots was usually about 16% of the dry weight.

2. Chemicals

(a) ^{14}C-labelled compounds

The following ^{14}C compounds were obtained from the Radiochemical Centre, Amersham, Bucks:- D-[$^{14}C_6$] Fructose, specific activity

19.4μ curies/mg.; D-[$^{14}C_6$] glucose, specific activity 29.7μ curies/mg.; D [2-^{14}C] glucose, specific activity 13.1μ curies/mg.; [$^{14}C_2$] glycine, specific activity 7.6μ curies/mg.; [$^{14}C_2$] glyoxylic acid monohydrate, specific activity 32.6μ curies/mg.; [$^{14}C_2$] oxalic acid, specific activity 45.5μ curies/mg.; D-[1-^{14}C] ribose, specific activity 13.0μ curies/mg.; L-[$^{14}C_3$] serine, specific activity 27.8μ curies/mg.; sodium [$^{14}C_1$] bicarbonate, specific activity 270.3μ curies/mg.; and sodium [1-^{14}C] glycollate, specific activity 19.4μ curies/mg. Calcium [3-^{14}C] glycerate, specific activity 4.0μ curies/mg. was obtained from Research Specialities Co., California and DL (+) allo [1,5-$^{14}C_2$] isocitric acid lactone, specific activity 34.6μ curies/mg. from the California Corporation for Biochemical Research.

(b) Compounds used in estimation of ^{14}C activity

The following chemicals were used for liquid scintillation counting : p -dioxane, naphthlene, toluene (A.R.; British Drug Houses Ltd.), 2,5-diphenyloxazole and p -bis-2,5-phenyloxazylbenzene (A.R. scintillation grade; Nuclear Enterprises (G.B.) Ltd., Scotland).

(c) General chemicals

Phenol, 3-methyl-propan-1-ol, pyridine, chloroform and formic acid (British Drug Houses Ltd.) were A.R. grade. Butan-1-ol (Colonial Sugar Refineries Ltd.) and propionic acid (British Drug Houses Ltd.), both L.R. grade were re-distilled before use.

Sodium glyoxylate monohydrate, DL-isocitric acid, trisodium salt (Sigma Chemical Co.); L-malic acid (California Corporation for Biochemical Research), citric acid (Univar.) and tartaric acid (British Drug Houses Ltd.) were A.R. grade. Glycollic acid, L.R. grade (British Drug Houses Ltd.) was re-crystallised. Mallinckrodt silicic acid, 100 mesh (A.R., Mallinckrodt Chemical Works) was suspended in distilled water and fine particles removed by suction before use.

C. METHODS

1. Treatment of shoots with radioactive compounds

(a) Administration of ^{14}C-labelled compounds by injection.

In the first series of *in vivo* experiments the following ^{14}C labelled compounds were administered to *Oxalis* shoots : [$^{14}C_6$] glucose, [$^{14}C_2$] glyoxylic acid, sodium [1-^{14}C] glycollate, [$^{14}C_2$] oxalic acid, sodium [$^{14}C_1$] bicarbonate, [$^{14}C_2$] glycine and [$^{14}C_3$] serine. Solutions used (final vol., 0.2 ml.) contained 100μ curies of ^{14}C labelled compound except that the solution of [$^{14}C_2$] glyoxylic acid contained 30μ curies. Glucose was dissolved in M-sodium acetate buffer, pH 5.0; acidic compounds were adjusted to pH 5.0 with 0.05 N-NaOH and sodium bicarbonate was used as supplied. With an "Aglar" microsyringe (Burroughs, Welcome Ltd.), portions (0.01 ml.) of the

appropriate solution were injected into each of twenty shoots attached to the Oxalis bulbs. The bulbs were maintained at room temp. for 1 hr.; the shoots were then detached and extracted immediately as described below. In this series of experiments, this procedure was used throughout, except that ten of the shoots injected with glucose were detached and extracted after 1 hr. and the remaining ten after 24 hr. Although no conclusive evidence is available, preliminary studies with dyes suggest that the injected compounds are rapidly transported throughout the shoot tissue.

(b) Administration of ^{14}C-labelled compounds to grooves cut in shoots.

Difficulties were experienced in ensuring uniform application of ^{14}C compounds to the shoot material by injection. In addition, the time required to inject twenty shoots was considerable. To overcome these difficulties, a narrow groove was cut lengthwise along each shoot (approx. 1 mm. x 1 mm.) with a scalpel, the slice of tissue removed, and the appropriate volume (0.02 - 0.025 ml.) of ^{14}C labelled material introduced into the groove with a micro-pipette. Preliminary experiments in which [$^{14}C_6$] fructose and calcium [$^{14}C_3$] glycerate were applied to grooves cut in Oxalis shoots and the products after extraction examined by paper chromatography and radioautography, indicated that this technique was satisfactory. Not only did more uniform application of radioisotope allow a valid comparison of the contribution of different

^{14}C labelled compounds to oxalic acid synthesis *in vivo*, but also by this technique it was possible to assess the total recovery of ^{14}C as a percentage of ^{14}C administered to *Oxalis* shoots.

In qualitative experiments, 25μ curies of [$^{14}C_6$] fructose and 25μ curies of calcium [3-^{14}C] glycerate were dissolved in 0.05 ml. water and 0.01 ml. portions of the appropriate solution applied to grooves cut in each of five shoots for each experiment. Examination of extracts from these shoots showed that the organic fraction contained excessive amounts of oxalic acid which streaked on chromatography. Thus, in a further series of quantitative experiments *in vivo* only two shoots were used for each experiment. The four ^{14}C compounds in the quantitative experiments were applied in the following manner. D-[2-^{14}C] glucose (50μ curies) was dissolved in 0.3 ml. water and 0.02 ml. was applied to each shoot. D-[1-^{14}C] Ribose (50μ curies) was dissolved in 0.19 ml. water and 0.02 ml. was applied to each shoot. Sodium [1-^{14}C] glycollate (100μ curies) was dissolved in 1.0 ml. water; 0.025 ml. was applied to each shoot. Potassium DL(+) allo [1,5-$^{14}C_2$] isocitrate (0.21 ml. containing approx. 20μ curies) was administered immediately following hydrolysis of the lactone; 0.025 ml. was applied to each shoot. Hydrolysis was effected by adding three equivalents of KOH to the lactone and the solution (final vol., 0.21 ml.) was maintained on a steam bath for 15 min. and then cooled. The final pH of the solution was 5-6 (cf. Kornberg & Beevers, 1957).

2. Pre-treatment of shoots with malonic acid

In experiments with [2-^{14}C] glucose and with [1,5-$^{14}C_2$] isocitric acid, shoots were pre-treated with malonic acid. Malonic acid, 0.1N, was adjusted to pH 4.5 with 3M-NaOH (Laties, 1949) and 0.05 ml. portions applied to grooves cut in Oxalis shoots. Control shoots were pretreated with 0.05 ml. water. These shoots were placed in the dark at room temp. for 1 hr. before the administration of the appropriate ^{14}C compound to the grooves.

3. Extraction, separation and identification of compounds containing radioisotope.

Shoots were treated with radioisotope for 1 hr., 6 hr. or 24 hr. After treatment, the shoots were extracted by dispersing in a Potter-Elvehjem homogeniser in 5-10 ml. ethanol at room temp. It was assumed that this treatment prevented any further enzymic changes since the final pH of the brei was 1-2. The suspension was centrifuged and the precipitate extracted with successive portions of water (5-10 ml.) until no radioactivity was detected in the supernatant. In the first series of experiments in which ^{14}C compounds were administered by injection, the final volume of the combined extract was approx. 40 ml., in the second series the final vol. was approx. 20 ml.

For each experiment, the combined extract was applied to a column (1 cm. x 1 cm.) of Dowex-50 (H^+form), the effluent of which

flowed directly onto a column (2 cm. x 1 cm.) of Dowex-2 (carbonate form). The columns were washed with 2 x 5 ml. of water. The material which passed through both columns contained non-ionic compounds (mainly sugars). The amino acids were then eluted from the column of Dowex-50 with either 30 ml. of 0.5 N-NH_4OH (first series of experiments) or 10-15 ml. of 4N - NH_4OH (second series) and organic acids were eluted from the column of Dowex-2 with 50 ml 0.5 N-HCl or 10-15 ml. 4 N-HCl. Elution was continued until no further radioactivity could be detected in the eluate. Each of the three fractions so obtained was made up to a standard volume and the relative distribution of ^{14}C between them obtained by plating a known volume (0.02 - 0.05 ml.) of the appropriate solution in an infinitely thin film on copper planchettes, drying under an infra-red lamp and the radioactivity determined with a Geiger-Müller end-window tube. Individual compounds containing ^{14}C were identified by paper chromatography and radioautography.

4. Paper chromatography and radioautography

(a) Preparation of paper for chromatography.

Initially, Whatman No. 1 paper (46 cm. x 57 cm.) was washed with 0.1 N-HCl in ethanol and subsequently with glass distilled water until the pH of the eluate was no longer acid. In the second series of experiments, in which ^{14}C compounds were counted on the paper, the complete washing procedure of Connell, Dixon & Hanes (1955), except for the wash with calcium acetate, was used.

(b) Preparation of extracts for paper chromatography.

For qualitative studies on the products which became labelled after the application of ^{14}C compounds to Oxalis shoots, extracts of amino acids and of organic acids were reduced in volume by a stream of air at room temp. Extracts containing a low level of radioactivity were reduced to a volume such that 0.1 to 0.2 μ curies could be spotted on chromatograms in a volume of 0.2 ml. to 0.5 ml. Where possible approx. 1 μ curie was applied to the chromatogram.

In the second series of *in vivo* experiments quantitative estimations of the ^{14}C content of specific compounds were made by direct counting of such compounds located on chromatograms. For these experiments, extracts were evaporated to dryness at 30° (external temp.) on a rotary film evaporator connected to an oil vacuum pump. The residues were dissolved in 1.0 ml. of 20% (v/v) ethanol. For amino acid extracts, 0.2 ml. portions were applied to chromatograms and for organic acids, 0.05 ml. portions of the extract were used.

(c) Solvents used for paper chromatography.

(i) General separation of amino acids and organic acids.

Mixtures of amino acids and of organic acids were separated by two dimensional descending paper chromatography using phenol saturated with water at 24° in one dimension and butan-1-ol-propionic acid - water (47 : 22 : 31 by vol.) in the other as described by Benson

and co-workers (1950). Although losses of some compounds have been reported in this system (see Rabson, Tolbert & Kearney, 1962), nevertheless, it has been widely used and therefore the relative positions of a large number of compounds separated by these solvents is well documented.

In the first series of *in vivo* experiments paper chromatography and radioautography were used as qualitative indicators of those compounds which contained radioisotope. A mixture of glyoxylic acid, glycollic acid, malic acid, citric acid, isocitric acid and tartaric acid (0.01 ml. of a mixture 0.1 N in respect of each acid) was co-chromatographed with the organic acid extracts. This enabled those acids to be more easily detected on chromatograms with indicator sprays. Chromatograms were dried in a stream of warm air and placed in contact with Kodirex X-ray film (Kodak, Australasia Ltd.) and developed at suitable intervals. Where it was required to locate compounds on the chromatograms, 0.5% (w/v) ninhydrin in butan-1-ol was used to develop amino acids, and organic acids were located with aq. 10% (w/v) potassium ferrocyanide, 0.5% (w/v) ferric ammonium sulphate in 70% (v/v) ethanol (Martin, 1955).

(ii) Separation of glycine and serine by paper chromatography.

Glycine and serine were not separated satisfactorily for quantitative purposes in the system described above. The mixture of these two amino acids was located by radioautography, eluted from two-dimensional chromatograms with water, and the eluate concentrated

in vacuo. The two compounds were then separated by ascending paper chromatography with pyridine-water (80 : 20, v/v).

(iii) Separation of citric acid and isocitric acid by paper chromatography.

Citric acid and isocitric acid mixtures were eluted from two-dimensional chromatograms with water, concentrated *in vacuo*, and the two acids separated by double development of paper chromatograms with a solvent described by Ladd & Nossal (1954), modified to contain butan-1-ol, 3-methyl-propan-1-ol, water, pyridine and 98-100% formic acid (3:6:7:4:1 by vol.) (E. Holdsworth, personal communication).

5. Quantitative separation of amino acids and organic acids from extracts of Oxalis shoots

In the first series of *in vivo* experiments in which a number of ^{14}C compounds were injected into *Oxalis* shoots, paper chromatography and radioautography was used only as a qualitative guide to the distribution of ^{14}C amongst various compounds. For a quantitative determination of this distribution, individual amino acids and organic acid were separated by column chromatography.

(a) Separation and estimation of acidic and neutral amino acids.

The separation of acidic and neutral amino acids from extracts of *Oxalis* shoots was carried out essentially as described by Moore,

Spackman & Stein (1958). Glass columns of internal diameter 1.5 cm. and having a surrounding water jacket, were packed with Dowex-50 resin (X-8, H^+ form), which had been previously graded into particules of 35-70 μ as described by Hamilton (1958). The resin bed was 150 cm. x 1.5 cm. Water from a constant temperature bath at 50° was circulated around the columns during chromatography. Portions (2.0 ml.) of extracts containing amino acids were applied to the appropriate column and elution was carried out with 0.2 M-sodium citrate buffers at pH 3.25 (approx. 340 ml.) and pH 4.25 (approx. 260 ml.) as described by Moore, Spackman & Stein (1958). The volume of the two buffers required varied; that at pH 3.25 was used until alanine had been eluted from the column and thereafter, sodium citrate pH 4.25 was the eluting solvent. The buffers were always boiled and then cooled before use to prevent bubbles forming in the resin. For reproducible results it was also necessary to ensure intimate mixing of the resin and sodium citrate buffer pH 3.25 and even packing of the resin bed before the start of an elution procedure.

The effluent from the columns passed through approx. 1 m. of fine PVC tubing of 1-2 mm. internal diameter (in which negligible mixing occurs) into a fraction collector adjusted to deliver 2 ml. samples. As carbon dioxide is evolved in the ninhydrin reaction and would therefore constitute a possible loss of $^{14}CO_2$, only even numbered fractions were assayed to determine the positions of the amino acids eluted from the columns. The ninhydrin reagent of Moore & Stein (1954), consisting of ninhydrin (20 g.) and hydrindantin (3 g.) dissolved in

750 ml. redistilled methyl cellosolve and made up to 1 l. with sodium acetate buffer, pH 5.5, was used for the assay of amino acids. To the 2 ml. fractions, 1 ml. of ninhydrin reagent was added, the mixture was placed in a boiling water bath for 20 min. and then 5 ml. of 50% (v/v) ethanol added. After vigorous shaking the optical density of each fraction was read in a 1 cm. cuvette at 570 mμ. The assay of the even numbered fractions located the position of the individual amino acids as they were eluted. The odd numbered fractions in each peak were bulked and stored at -15° before being assayed for ^{14}C activity.

(b) Separation and estimation of organic acids.

The fraction of the shoot extract containing organic acids was neutralised with 0.5 N-NaOH and then evaporated to dryness in a stream of cold air. The residue was dissolved in 0.5 N-H_2SO_4 (1.5 ml.) and the organic acids separated by chromatography on a survey column (15 cm. x 1 cm.) of silicic acid gel. Glyoxylic acid (sodium salt), glycollic acid, malic acid, citric acid, isocitric acid (tri-sodium salt) and tartaric acid (2 mg. of each compound in 0.25 ml. of 0.5 N-H_2SO_4) were co-chromatographed with 0.5 ml. portions of the acids from the plant extract. Organic acids were eluted from the columns with increasing concentrations of butan-1-ol in chloroform (water washed) in the series 5%, 15%, 25%, 35% and 50% (v/v). The procedure used was essentially as described by Bulen, Varner & Burrell (1952), except that no nitrogen pressure was exerted on the column. Fractions of 4 ml. were collected.

The organic acids in fractions collected from the column eluate were estimated by titration with 0.01 N-NaOH after the addition of 5-10 ml. of water to each sample. The end-point was taken as permanence of a pink colour in the presence a drop of 0.1% phenol red for at least 5 sec. Vigorous agitation during titration of each sample was essential. The appropriately combined fractions for each acid were evaporated to dryness on a steam bath and the residue dissolved and made up to a volume of 5 ml. with water. Samples (0.1 ml.) were used for the determination of radioactivity.

Glycollic acid and oxalic acid do not separate on the silicic acid column under the conditions used. Oxalic acid was therefore separated from mixtures of these two acids as its calcium salt. The precipitate obtained by the addition of an excess of calcium chloride was collected by centrifuging and was washed three times with dilute acetic acid previously adjusted to pH 5.5 with 0.5 N-NaOH. Glycollic acid in the supernatant and washings was then estimated as described for the other organic acids. The precipitate of calcium oxalate was dissolved in 0.5 N-HCl (2.0 ml.) and portions (0.02 ml.) were assayed for ^{14}C activity.

If the elution was carried out with 15% butan-1-ol in chloroform (300 ml.) it was found that a mixture of glyoxylic acid, glycollic acid and oxalic acid could be adequately separated by silicic acid gel chromatography. This obviated the isolation of oxalic acid as its calcium salt from mixtures of glycollic acid and oxalic acid.

- - -

In the second series of experiments in vivo, quantitative determinations of the distribution of ^{14}C in individual amino acids and in individual organic acids was also made. However, these compounds were separated by paper chromatography and the ^{14}C activity in them was determined on the paper. This latter method was less time consuming and also ensured that the compounds being assayed for radioactive content were free from contamination.

6. Techniques used for the determination of radioactivity

(a) End-window counting at infinite thinness.

This method of counting ^{14}C activity was used extensively in the earlier experiments with radioisotopes. A known volume (0.02 ml. to 0.1 ml.) of each solution containing radioactivity was plated in an infinitely thin film on copper planchettes. After drying under an infra-red lamp, the radioactivity was determined with a Geiger-Müller end-window tube, type E.W. 3H (20th Century Electronics, Ltd., England) operating at 600 volts. Under these conditions a counting efficiency of 4-5% was obtained. A minimum of 1000 counts was recorded for each sample and duplicate samples were counted three times. The plating and counting procedure was repeated when more than 3% variation in duplicate samples was recorded.

This method of counting has several disadvantages. The relatively low efficiency requires more ^{14}C activity in each experiment, otherwise the counting procedure may be very time consuming for those samples containing small amounts of radioisotope. Secondly, the plating technique is subject to relatively large errors, particularly when volumes of the order of 0.02 ml. are applied to planchettes. In liquid scintillation procedures, volumes of 0.2 ml. to 0.25 ml. are counted, thus minimising volume errors. Thirdly, considerable losses of volatile compounds may be encountered in drying samples under infra-red lamps. Nevertheless, end-window counting is a simple and convenient procedure. The count rate is not affected by the constituents in the solution to be counted; for example, organic acids eluted from silica gel columns contained phenol red indicator which would have interferred with liquid scintillation counting.

(b) Liquid scintillation counting of radioactive solutions.

The most obvious advantage of liquid scintillation counting of β particles over end-window counting, is the increase in efficiency that can be obtained; a ten-fold increase in counting efficiency is possible. This advantage is off-set to some degree by the sensitivity of liquid scintillators to changes in the composition of solutions to be counted. In particular, either high or low pH values of solutions have a profound effect on the count rate observed. Even with one

particular sample, the count rate observed may vary within a short time period; these samples often decrease to a constant counting rate on standing for approx. 20 min. Since scintillator solutions commonly consist of a phosphor (or phosphors) dissolved in an organic solvent (such as toluene) and solutions to be counted are frequently aqueous samples, only a limited amount of the sample is compatible with the scintillator. Excessive amounts (over 5%, v/v) of the sample in the scintillator results in "cloudiness" and spurious count rates.

The quenching effect of aqueous samples or constituents in solutions to be counted may be greatly reduced by the incorporation of naphthalene in the scintillator solution (Furst, Kallman & Brown, 1955). For amino acid samples eluted from Dowex-50 (H^+ form) columns with 0.2 M-sodium citrate, the liquid scintillation technique of Butler (1961) was used. The scintillator solution consisted of naphthalene (120 g.), 2,5-diphenyloxyazole (4.0 g.) and p -bis-2,5-phenyloxazylbenzene (0.5 g.) dissolved in 1 l. p-dioxane. Using 5 ml. of the scintillator, a counting efficiency of 67% was obtained with 0.2 ml./carrier-free $^{14}CO_2$. The presence of 0.2 ml. of 0.2 M-sodium citrate buffer resulted in a reduction of counting efficiency to a value of 36%. The ^{14}C content of 0.2 ml. portions of amino acid eluates was determined in 5.0 ml. of scintillator using a type EM 79514S photomultiplier tube (Ecko Electronics Ltd.) operating at 1,200 volts.

(c) Liquid scintillation counting of ^{14}C on paper.

The assay of radioactivity directly from paper chromatograms has recently been developed by Wang & Jones (1959), who observed that commercially available filter papers did not scatter or absorb a significantly large number of photons. Since the scintillator solution permeates the paper, self-adsorption effects were small resulting in approx. 55% counting efficiency for ^{14}C compounds which were insoluble in the scintillator solution and 85% efficiency for those compounds which were eluted from the paper. The same high voltage and window widths as for solution liquid scintillation counting were employed. The absorption effect due to the paper was approx. 30% of the absolute efficiency and independant of the type of compound being assayed provided no unusual buffers, proteins or other interfering compounds were present to produce quenching effects.

Geiger & Wright (1960) studied the effect of the orientation of the paper to the counting window of the photomultiplier tube. Their results indicated a variation of 5% or less in total counts for several classes of compounds over a wide counting range and they concluded that it was unnecessary for the paper to be held in a fixed orientation relative to the photomultiplier tube window. Loftfield (1960) preferred to form the paper into a cylinder completely lining the counting vial. This resulted in a 20% loss in counting efficiency compared with a paper square, but the reported reproducibility was increased. Davidson (1961) preferred to count spots on

circular discs placed flat on the surface of the counting vial. The average variation in repetitive counts reported was 1.5% whereas with vertical squares the average variation was 7.1%. The count rate using this technique was independant of the scintillator volume between 5 ml. to 15 ml. (Davidson, 1961) and also independant of the area over which the spot was spread (Nunez & Jacquemin, 1961). Bousquet & Christian (1960) and Rapkin & Gibbs (1962) have demonstrated a linear relationship between the radioactivity applied and the count rate recorded.

Measurements of the ^{14}C content of compounds separated by paper chromatography and located by radioautography were made at room temp. with type N664A scintillation counters connected to type N530F scalers (Ecko Electronics Ltd.). The scintillation counters were equipped with EM 19514S photomultiplier tubes. Unit No. 1 operated at 1,200 volts (high voltage) and 10 volts (discriminator bias). Unit No. 2 operated at 1,450 volts (high voltage) and 15 volts (discriminator bias); both units were set with an amplifier gain x 100. The scintillator solution consisted of 2,5-diphenyloxyazole (3 g.) and p-bis-2,5-diphenyloxyazylbenzene (0.2 g.) dissolved in 1 l. of toluene. Compounds containing ^{14}C were counted on paper by placing discs of paper face down on the window of the counting pots (50 ml. Quickfit pots painted with white enamel, with ground glass tops, 4.5 cm. diam.) in 5.0 ml. of scintillator. Optical contact of the base of the pot with the top of the photomultiplier tube was made with silicone oil (100 CSTKS, Swift & Co., Sydney, N.S.W.). The efficiency of each unit was

determined by pipetting standard carrier-free sodium [1-^{14}C] carbonate onto paper discs, drying and counting as described above. The efficiency of unit No. 1 was 58% and of unit No. 2 was 54%. These figures compare favourably with the 55% efficiency recorded by Wang & Jones (1959) for ^{14}C compounds which were insoluble in toluene. A standard spot was counted at regular intervals to ensure that the observed count rates from both units was strictly comparable (Stitch, 1959). A known volume (0.01 ml.) of each extract, as applied to chromatograms, was counted on paper discs so that the observed counts in specific compounds from chromatograms could be expressed as a percentage of the total ^{14}C activity applied.

The technique of counting ^{14}C activity directly from paper chromatograms has the great advantage of obviating sample preparation as is necessary with other methods. In addition, if the compounds being assayed are insoluble in toluene, samples may be removed from the counting pots after counting and the next disc counted in the same scintillator. Since the count rate is independant of the size of the spot being assayed, this technique is perfectly suited to the counting of compounds separated on paper by chromatography or electrophoresis.

D. RESULTS AND DISCUSSION

1. The distribution of ^{14}C activity from ^{14}C-labelled compounds injected into Oxalis shoots.

(a) The distribution of isotope between sugars, amino acids and organic acids.

The following compounds were injected into white shoots of Oxalis bulbs : [$^{14}C_6$] glucose, [$^{14}C_2$] glyoxylic acid, sodium [1-^{14}C] glycollic acid, [$^{14}C_2$] oxalic acid, [^{14}C] bicarbonate, [$^{14}C_2$] glycine and [$^{14}C_3$] serine. After 1 hr. (and also after 24 hr. with glucose), the shoots were detached from the bulbs and extracted with ethanol and then with water (see Methods, p. 41). After separation of each extract into non-ionic compounds (mainly sugars), amino acids and organic acids, the three fractions were made up to a known volume (40-50 ml.) and portions (0.1 ml.) plated and counted. In Table 1, the relative distribution of radioactivity in each fraction is expressed as a percentage of the total activity in the three fractions.

(b) Qualitative distribution of ^{14}C between specific amino acids and organic acids separated by paper chromatography.

Extracts containing amino acids and organic acids were reduced in volume in air and portions (0.2 ml. to 0.5 ml.) applied to paper chromatograms as described previously (p. 43). After two-dimensional chromatography, chromatograms were placed in contact with

Table 1. Distribution of ^{14}C activity in sugars, amino acids and organic acids from ^{14}C-labelled compounds injected into Oxalis shoots.

White shoots of Oxalis bulbs were injected with various ^{14}C-labelled compounds. After 1 hr. (and also after 24 hr. with glucose) the shoots were detached and extracted with ethanol and then with water. The compounds in the extracts were fractionated into sugars, amino acids and organic acids and the ^{14}C activity of each was determined. The radioactivity in each fraction is expressed as a percentage of the total activity of the three fractions.

TABLE 1

Compound injected	Distribution (%) of ^{14}C activity in shoot extracts		
	Sugars	Amino acids	Organic acids
[$^{14}C_6$] glucose*	77.1	5.7	17.2
[$^{14}C_6$] glucose †	17.3	2.2	80.5
[$^{14}C_2$] glyoxylic acid	-	13.1	86.9
[1-^{14}C] glycollic acid	-	5.0	95.0
[$^{14}C_2$] oxalic acid	-	0.9	99.1
[$^{14}C_1$] bicarbonate	-	40.7	59.3
[$^{14}C_2$] glycine	-	98.7	1.3
[$^{14}C_3$] serine	-	99.7	0.3

* After 1 hr.

† After 24 hr.

X-ray films and these were developed at suitable intervals. Amino acids and organic acids were located on chromatograms with appropriate sprays and the amount of ^{14}C in specific compounds assessed visually by comparing the degree of exposure of portions of the X-ray film corresponding to individual compounds. These qualitative results (Table 2) provided essential information for a quantitative study of the distribution of radioisotope among the compounds detected by radioautography.

(c) Quantitative distribution of ^{14}C among specific amino acids from extracts of Oxalis shoots.

Portions (2.0 ml.) of amino acid extracts which had previously been evaporated to dryness and made up to 5.0 ml. in water were applied to columns of Dowex-50 (X-8, H^+ form) and individual amino acids eluted with sodium citrate buffers as previously described (p.45). A typical elution pattern of the amino acids from Oxalis extracts is shown in Fig. 1. Chromatography of such extracts clearly showed that glutamic acid was present in far greater concentrations than any other amino acid.

Table 2. Qualitative examination of the distribution of ^{14}C in specific amino acids and organic acids in extracts after injection of ^{14}C-labelled compounds into *Oxalis* shoots.

White shoots of *Oxalis* bulbs were injected with various ^{14}C-labelled compounds. After 1 hr. (and also after 24 hr. with glucose) the shoots were detached and extracted as described in the text and Table 1. Labelled compounds in such extracts were separated by paper chromatography and detected by radioautography and classified visually as in group A (considerable incorporation) or in group B (slight incorporation of label).

TABLE 2

Compound injected	^{14}C compounds located in *Oxalis* extracts	
	Group A	Group B
[$^{14}C_6$] glucose*	Malic, citric, isocitric Asp, Glu.	Glycollic, oxalic and unidentified acids. Ala, Val, Leu, Ileu.
[$^{14}C_6$] glucose †	Oxalic, glycollic Glu.	Glyoxylic, citric, malic Asp, Ser, Ala, Val, Leu, Ileu.
[$^{14}C_2$] glyoxylic acid	Glyoxylic, oxalic, glycollic Gly, Ser.	Malic -
[1-^{14}C] glycollic acid	Glycollic, oxalic, glyoxylic Gly, Ser.	- Asp, Glu.
[$^{14}C_2$] oxalic acid	Oxalic, glycollic -	Glyoxylic Glu, Asp, Ser, Gly, Ala.
[$^{14}C_1$] bicarbonate	Malic Glu, Asp.	Citric, isocitric Ser, Ala, Val.
[$^{14}C_2$] glycine	- Gly, Ser.	Glyoxylic, malic, glycollic, oxalic Asp, Ala.
[$^{14}C_3$] serine	- Ser, Gly.	Glycollic, glyoxylic, oxalic, malic, citric -

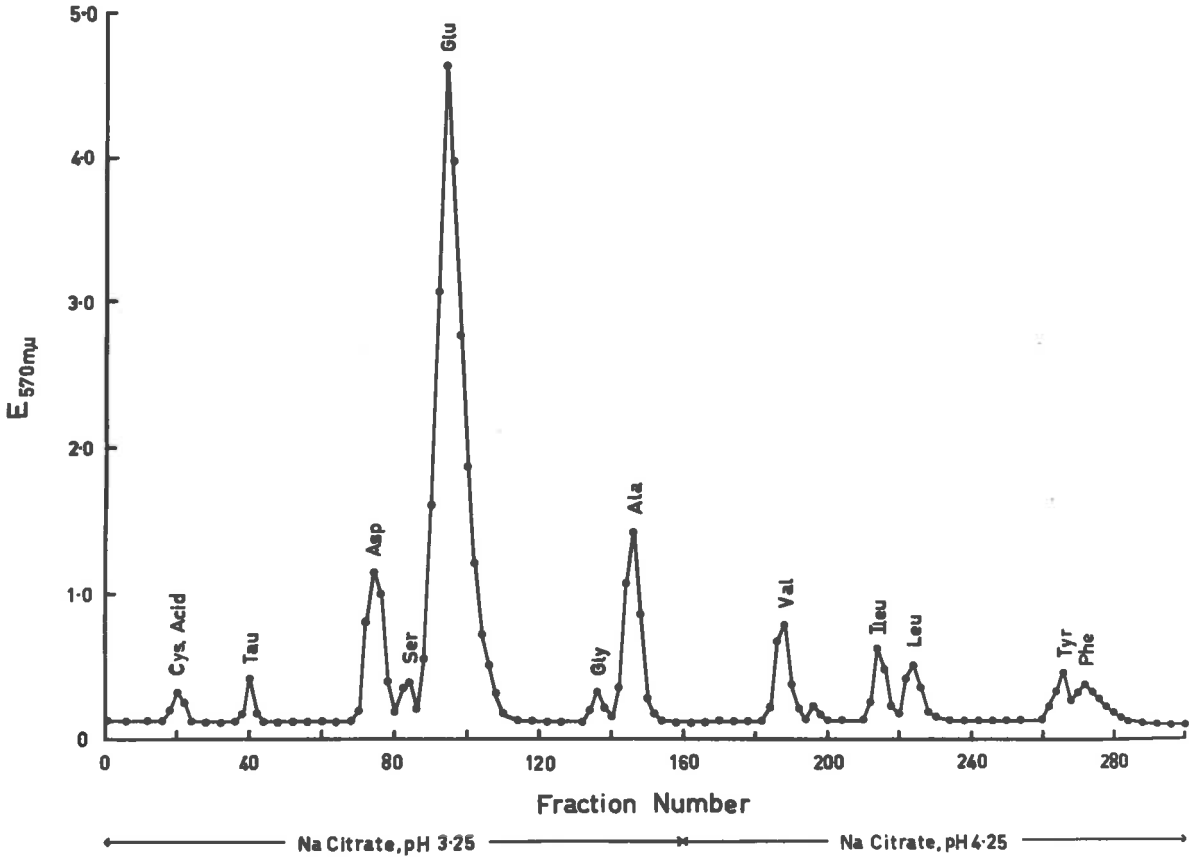
* After 1 hr.

† After 24 hr.

Fig. 1. Elution pattern of acidic and neutral amino acids of Oxalis extracts from Dowex-50 resin.

Portions (2.0 ml.) of extracts from Oxalis shoots containing amino acids were applied to columns of Dowex-50 (X-8, H^+ form) and individual amino acids eluted with sodium citrate buffers as described in the text. Even numbered fractions (2.0 ml.) were assayed with ninhydrin reagent and extinctions (1 cm.) recorded at 570 mμ.

FIG. 1


5·0
4·0
3·0
2·0
1·0
0
E 570mμ
Cys. Acid
Tau
Asp
Ser
Glu
Gly
Ala
Val
Ileu
Leu
Tyr
Phe
0
40
80
120
160
200
240
280
Fraction Number
Na Citrate, pH 3·25
Na Citrate, pH 4·25

The total ^{14}C activity applied to each column was calculated by counting portions (0.2 ml.) of the extract by the liquid scintillation technique of Butler (1961). The efficiency of counting by this method was 67% (see p. 52), whereas the efficiency of counting the eluted amino acid samples was 36% due to the quenching effect of sodium citrate buffer. The total counts recorded for 0.2 ml. portions loaded onto Dowex-50 columns were multiplied by ten to allow for the difference in volumes (as applied to the columns) and adjusted by a factor of $^{36}/_{67}$ to allow for the difference in counting efficiency. The radioisotope in individual amino acids was expressed initially as a percentage of the total ^{14}C activity loaded onto the appropriate column. However, to enable a comparison of the ^{14}C activity in individual amino acids and organic acids derived from a particular ^{14}C labelled compound, the amount of radioisotope in any compound was expressed as a percentage of the total ^{14}C activity recovered from Oxalis shoots. An example of the calculations involved is given below.

(i) For $[^{14}C_6]$ glucose (1 hr.), the total ^{14}C activity at 67% counting efficiency in 0.2 ml. sample as loaded onto Dowex-50 column = 9.691×10^3 counts/100 sec.

(ii) Since 2 ml. portions were loaded onto Dowex-50 columns, and the ^{14}C content of individual amino acids were estimated at 36% efficiency, the total ^{14}C activity loaded onto columns at 36% counting efficiency = $9.691 \times 10^3 \times \frac{2}{0.2} \times \frac{36}{67}$

$= 5.352 \times 10^4$ counts/100 sec.

(iii) The total volume of the aspartic acid peak derived from [$^{14}C_6$] glucose (1 hr.) was 20 ml. One half of this was used in detecting this compound with ninhydrin (see p. 46) and 0.2 ml. portions of the remaining 10 ml. were assayed for radioactivity. The ^{14}C activity in 0.2 ml. samples (aspartic acid) was recorded as $\underline{1.919 \times 10^2 \text{ counts/100 sec.}}$,

i.e. ^{14}C activity in total aspartic acid sample =

$$1.919 \times 10^2 \times \frac{10}{0.2} \times 2 \text{ counts/100 sec.}$$

$$= \underline{1.919 \times 10^4 \text{ counts/100 sec.}}$$

(iv) The ^{14}C content as a percentage of the total radioactivity applied to Dowex-50 columns = $\frac{1.919 \times 10^4}{5.352 \times 10^4} \times 100\%$

$$= \underline{35.85\%}$$

(v) From the results presented in Table 1 (p. 56) it is apparent that the amino acid fraction from [$^{14}C_6$] glucose (1 hr.) represented only 5.7% of the total ^{14}C activity recovered from the shoots in this experiment. Thus, the ^{14}C content of aspartic acid derived from [$^{14}C_6$] glucose (1 hr.) as a percentage of the total ^{14}C recovered from Oxalis shoots is :-

$$35.85 \times \frac{5.7\%}{100}$$

$$= \underline{2.05\%}$$

The ^{14}C content of each amino acid eluted from Dowex-50 columns was calculated in this manner and the results are presented in Table 3.

Table 3. The distribution of ^{14}C activity among specific amino acids from extracts of *Oxalis* shoots, after injection of ^{14}C-labelled compounds.

A number of ^{14}C-labelled compounds were injected into *Oxalis* shoots and extracts were prepared as described in the text and Table 1. The amino acids in shoot extracts were fractionated on a column of Dowex-50 (X-8, H^+ form) and the amount of radioactivity in each fraction was estimated as described in the text. The results are expressed as a percentage of the total ^{14}C activity recovered from *Oxalis* shoots.

TABLE 3

% ^{14}C activity in specific amino acids separated by column chromatography

	Cys	Tau*	Asp	Ser	Glu	Gly	Ala	Val	Ileu	Leu	Phe	Un-identified	[^{14}C] loaded onto column and recovered as specific amino acids
[$^{14}C_6$] glucose †	0.22	-	2.05	0.47	1.52	-	0.34	0.22	0.25	0.13	-	0.17	94.1
[$^{14}C_6$] glucose ‡	0.39	-	0.21	0.12	0.61	0.04	0.18	0.21	0.12	0.11	0.07	-	92.6
[$^{14}C_2$] glyoxylic acid	0.12	0.03	0.21	7.39	0.05	3.85	0.09	-	-	-	-	-	89.6
[^{14}C] glycollic acid	0.50	0.34	0.53	2.41	0.27	0.68	0.03	0.08	-	-	-	-	96.7
[$^{14}C_2$] oxalic acid	-	-	0.20	0.11	0.22	(0.22) **		0.07	-	-	-	-	90.5
[$^{14}C_1$] bicarbonate	0.57	1.38	15.10	1.38	17.42	0.16	1.55	0.86	-	-	-	-	94.4
[$^{14}C_2$] glycine	0.40	0.20	4.24	20.73	0.10	59.71	1.58	0.10	-	-	-	0.30	88.7
[$^{14}C_3$] serine	-	0.10	0.10	81.95	0.10	2.40	0.40	0.40	-	-	-	0.30	86.0

* Tentatively identified

† After 1 hr.

‡ After 24 hr.

** These two amino acids did not separate satisfactorily

(d) Quantitative distribution of ^{14}C among specific organic acids from extracts of *Oxalis* shoots

In assessing the suitability of silica gel chromatography for the separation of organic acids from extracts of *Oxalis* shoots, organic acid extracts were prepared from shoots exactly as described for experiments involving ^{14}C labelled compounds (p. 41). Portions (0.5 ml.) of such extracts (in 0.5 $\underline{N}$-H_2SO_4) were applied to silicic acid gel columns and elution carried out as described by Bulen, Varner & Burrell (1952). The amount of organic acid extract applied was approximately the same as that used for the separation of acids from extracts containing ^{14}C.

The elution pattern obtained after chromatography is shown in Fig. 2. A single peak corresponding in position to oxalic acid was found.

In this chromatographic procedure, oxalic acid and glycollic acid do not separate distinctly (Bulen, Varner & Burrell, 1952), so the fractions from the peak corresponding to oxalic acid were bulked and examined for the presence of glycollic acid. The aqueous layer containing the sodium salt (or salts) of the acid (or acids) was evaporated on a steam bath to approx. 10 ml. and $CaCO_3$ (50% excess based on titration figures) added. The mixture was made slightly acid with 0.5 $\underline{N}$ H_2SO_4 and stirred in a boiling water bath for 30 min. The white precipitate of calcium oxalate was removed by centrifugation, washed twice with water (2.0 ml.) at 70° and the supernatants evaporated

to dryness. The residue was dissolved in 0.5 N-H_2SO_4 (1 ml.) and applied to a silicic acid gel column. The column was eluted with 20% (v/v) butan-1-ol in chloroform (250 ml.) and fractions collected and titrated with 0.01 N-NaOH as previously described. A single peak, corresponding in position to glycollic acid, was found (Fig. 2, inset).

It was apparent from these preliminary experiments that, in extracts from Oxalis shoots containing organic acids, only oxalic acid and glycollic acid were detectable by silicic acid gel chromatography. Thus, for the separation of individual organic acids from extracts containing ^{14}C, carrier organic acids were co-chromatographed with such extracts. Glyoxylic acid (sodium salt), glycollic acid, malic acid, citric acid, isocitric acid (tri-sodium salt) and tartaric acid (2 mg. of each compound in 0.25 ml. of 0.5 N-H_2SO_4) were added to portions (0.5 ml.) of extracts containing ^{14}C. A typical elution pattern after development of silicic acid gel columns is shown in Fig. 3. Oxalic acid was separated from the mixture of oxalic acid and glycollic acid as its calcium salt. Calcium oxalate was dissolved in 0.5 N-HCl (2.0 ml.) and portions (0.02 ml.) assayed for ^{14}C activity. The radioactivity in all other acids was determined with 0.1 ml. portions of such acids as previously described (p. 47).

The amount of ^{14}C activity applied to each silicic acid gel columns was determined by plating and counting a known volume of each extract. Samples (0.1 ml.) of each acid eluted were also plated and counted and the radio-isotope present in individual organic acids

Fig. 2. Silicic acid gel chromatography of an organic acid extract from Oxalis shoots.

Shoots were extracted with ethanol and with water. Organic acids were eluted from a column of Dowex-2 (carbonate form) as described for in vivo experiments with ^{14}C compounds. After neutralising, extracts were evaporated to dryness, the residues dissolved in 0.5 N - H_2SO_4 and applied to silicic acid gel columns. The elution scheme is indicated by the arrows and figures showing an increase of butan-1-ol chloroform from 5% (v/v) up to 50% (v/v). Titration of the eluate showed one peak, which after treatment with $CaCO_3$ to remove oxalic acid, was re-chromatographed on silicic acid gel using 20% (v/v) butan-1-ol chloroform. The inset figure shows the peak obtained after such re-chromatography corresponding in elution pattern to glycollic acid.

FIG.2

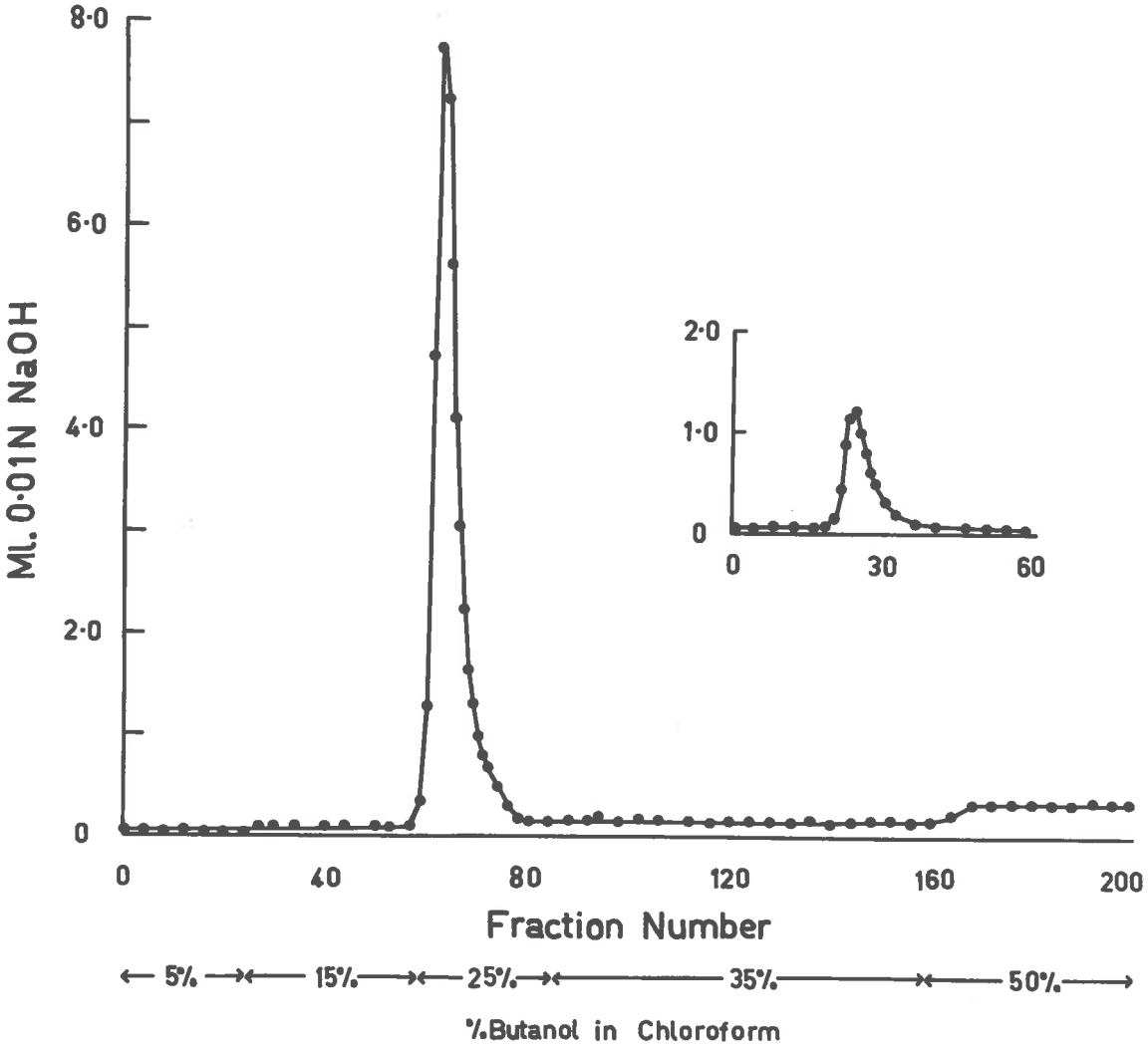
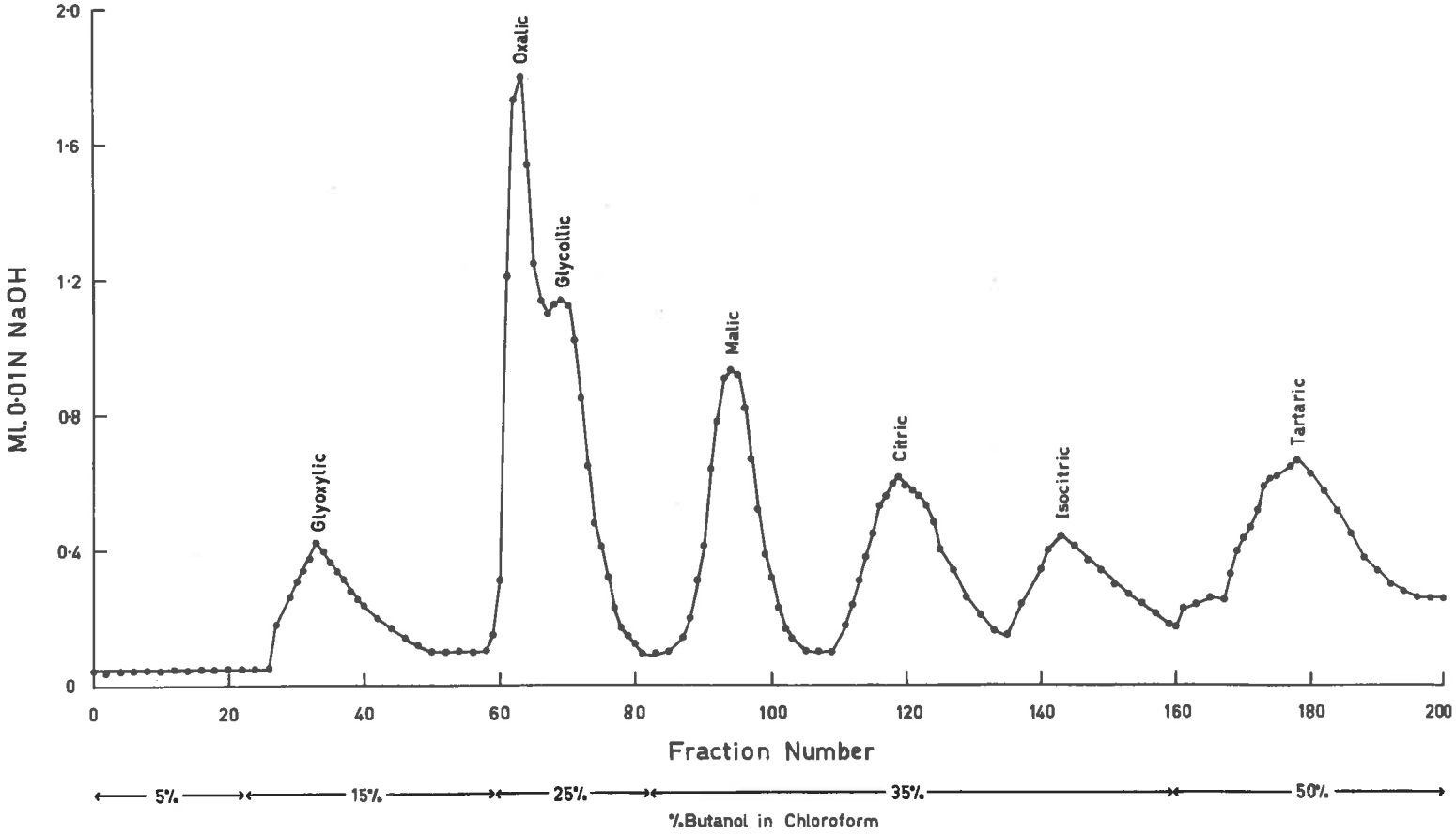

8·0
6·0
4·0
2·0
0
Ml. 0·01N NaOH
0
40
80
120
160
200
Fraction Number
2·0
1·0
0
0
30
60
← 5% →← 15% →← 25% →← 35% →← 50% →
% Butanol in Chloroform

Fig. 3. Silicic acid gel chromatography of extracts of organic acids from Oxalis shoots after injection of ^{14}C compounds, co-chromatographed with carrier acids.

Extracts from Oxalis containing organic acids were prepared after the application of a number of ^{14}C compounds to shoots. Portions (0.5 ml.) were applied to silicic acid gel columns with a mixture of glyoxylic acid, glycollic acid, malic acid, citric acid, isocitric acid and tartaric acid (total vol. 0.25 ml.) as previously described. Samples (4.0 ml.) eluted from columns were titrated with 0.01 N-NaOH and organic acids thus separated were assayed for ^{14}C activity.

The elution scheme is indicated by the arrows and figures showing an increase of butan-1-ol in chloroform from 5% (v/v) up to 50% (v/v).

FIG. 3


2·0
1·6
1·2
0·8
0·4
0
Ml. 0·01N NaOH
Oxalic
Glycollic
Malic
Citric
Isocitric
Tartaric
Glyoxylic
0
20
40
60
80
100
120
140
160
180
200
Fraction Number
5%
15%
25%
35%
50%
% Butanol in Chloroform

was expressed initially as a percentage of the total activity loaded onto the appropriate column. As was the case in calculating the radioisotope present in individual amino acids (Table 3), the ^{14}C content of organic acids was expressed as a percentage of the total ^{14}C activity recovered from Oxalis shoots. i.e. For [$^{14}C_6$] glucose (1 hr.), the amount of radioisotope present in oxalic acid as a percentage of the total ^{14}C applied to the appropriate silicic acid gel column was 6.54%. The results in Table 1 show that in 1 hr. the percentage of ^{14}C in the organic acid fraction derived from [$^{14}C_6$] glucose was 17.2%. Thus, the ^{14}C content in oxalic acid as a percentage of the total radioactivity recovered from Oxalis shoots is $6.54 \times \frac{17.2}{100}$ %, i.e. 1.12%

The percentage of ^{14}C in individual organic acids derived from ^{14}C compounds injected into Oxalis shoots is shown in Table 4.

Table 4. The distribution of ^{14}C activity among specific organic acids from extracts of *Oxalis* shoots after injection of ^{14}C-labelled compounds.

A number of ^{14}C-labelled compounds were injected into *Oxalis* shoots and extracts were prepared as described in the text and Table 1. The organic acids in shoot extracts were fractionated on a silicic acid gel column and the amount of radioactivity in each acid was estimated as described in the text. The results are expressed as a percentage of the total ^{14}C activity recovered from *Oxalis* shoots.

TABLE 4

% ^{14}C activity in specific organic acids separated by silicic acid gel chromatography

Compound injected	Glyoxylic acid	Glycollic acid	Oxalic acid	Malic acid	Citric acid	Isocitric acid	Tartaric acid	% (^{14}C) loaded onto column and recovered as specific acids
[$^{14}C_6$] glucose *	1.08	1.07	1.12	2.08	2.13	1.86	1.26	61.6
[$^{14}C_6$] glucose †	14.33	13.52	21.41	4.03	3.78	5.55	3.14	81.7
[$^{14}C_2$] glyoxylic acid	26.85	21.03	21.12	6.78	1.65	1.30	1.48	93.3
[1-^{14}C] glycollate	10.16	55.10	12.92	0.66	1.14	1.04	0.76	86.1
[$^{14}C_2$] oxalic acid	1.29	5.85	81.16	1.78	1.68	0.99	1.19	94.8
[$^{14}C_1$] bicarbonate	1.78	0.57	0.88	42.81	7.41	3.26	-	95.5
[$^{14}C_2$] glycine	0.62	0.11	0.11	0.27	0.07	0.07	0.01	97.7
[$^{14}C_3$] serine	0.05	0.10	0.05	0.04	0.04	0.02	-	97.3

* After 1 hr.

† After 24 hr.

GENERAL DISCUSSION

It is evident that emergent white shoots of *Oxalis pes-caprae* utilise carbohydrate from the starch-filled bulb for formation of oxalic acid which rapidly accumulates in the shoots. The presence of oxalic acid in shoots formed within two days of germination has been established during this work, confirming and extending earlier observations by Michael (1959). With the exception of the bulb, it appears likely that oxalic acid is a compulsory end-product of the metabolism of growing tissues of this plant.

That carbohydrate from the bulb is utilised in the production of oxalic acid is supported by the observation that within 1 hr., 17% of the activity of injected [$^{14}C_6$] glucose was recovered in the organic acid fraction and within 24 hr. about 80% was recovered in this pool (Table 1). As shown qualitatively by Table 2 and quantitatively in Table 4, using [$^{14}C_6$] glucose and a one hour exposure, there was greater incorporation of label into each of the acids of the tricarboxylic acid cycle (about 2% in each) than into glycollic acid, glyoxylic acid or oxalic acid, each of which contained approx. 1% of the recovered activity. Within 24 hr. acids of the tricarboxylic acid cycle contained 4-5% of the radioactivity, whereas glycollic acid, glyoxylic acid and oxalic acid had increased markedly in their radioisotope content containing, respectively, 13.5%, 14.3% and 21.3% of the recovered ^{14}C activity.

From the results obtained with [$^{14}C_6$] glucose it appeared likely that both glycollic acid and glyoxylic acid were intimately involved in oxalic acid synthesis in Oxalis. With [$^{14}C_2$] glyoxylic acid, in 1 hr. about 30% of the recovered activity remained in the glyoxylic acid, and glycollic acid and oxalic acid both contained about 21%. These results are consistent with a dismutation reaction as reported for extracts of A. niger by Franke & de Boer (1959). With [1-^{14}C] glycollic acid, 55% of ^{14}C activity remained in the original compound and 10.2% and 12.9% was recovered in glyoxylic acid and oxalic acid respectively (Table 4). The relationship between these three organic acids in Oxalis was further demonstrated by injecting [$^{14}C_2$] oxalic acid into shoots. Although most of the recovered activity remained in oxalic acid, glycollic acid contained 5.9% of the activity and glyoxylic acid 1.3%. Lesser, but detectable amounts of radioisotope were found in the isolated acids of the tricarboxylic acid cycle (Table 4) indicating that oxalic acid was not completely metabolically inert in Oxalis.

In confirmation of the qualitative findings (Table 2) the results in Table 3 show that from glyoxylic acid and from glycollic acid, ^{14}C was incorporated into both glycine and serine. When [$^{14}C_2$] glyoxylic acid and [1-^{14}C] glycollic acid were administered to shoots, the radioisotope found in glycine was 3.9% and 0.7% in each case whereas the radioisotope in serine derived from the same acids was 7.4% and 2.4% respectively. Similarly, when [$^{14}C_2$] glycine was injected into Oxalis shoots a large percentage of the recovered ^{14}C

activity (20.7%) was located in serine. Serine itself was comparatively inert metabolically (Tables 1, 2 and 3) and this probably accounted for the relative distribution of isotope in glycine and serine derived from glyoxylic acid and glycollic acid. The relatively low incorporation of label from [$^{14}C_2$] glyoxylic acid into glycine, (3.85%), which is unusual in plants (cf. Tolbert & Cohan, 1953b) was probably also a consequence of the relatively rapid synthesis of glycollic acid (21.0%) and oxalic acid (21.1%) from glyoxylic acid in Oxalis.

After the administration of [$^{14}C_1$] bicarbonate, the total radioactivity recovered from the shoots was only about 1% of that obtained with most other compounds used. Clearly very little ^{14}C was incorporated into plant constituents; any excess [^{14}C] bicarbonate would be lost as $^{14}CO_2$ when the shoots were extracted owing to the acid (pH 1-2) nature of the sap. The ^{14}C which was incorporated was distributed among the amino acids (40.7%) and organic acids (59.3%) respectively (Table 1). The pattern of labelling of the amino acids from bicarbonate (Table 3) shows preferential labelling of glutamic acid (17.4%) and aspartic acid (15.1%). A similar pattern was obtained after administration of [$^{14}C_6$] glucose and such incorporation can be accounted for by the well-established reactions involving transamination of α-oxoglutaric acid and oxaloacetic acid. Among the organic acids (Table 4) bicarbonate contributed most significantly to malic acid (42.8%), citric acid (7.4%) and isocitric acid (3.3%). However, there was no evidence

for rapid incorporation of label from bicarbonate into glycollic acid as occurs in photosynthetic tissue (Schou, Benson, Bassham & Calvin, 1950; Kearney & Tolbert, 1961, 1962). Furthermore, these results render it most unlikely that glycollic acid is synthesised directly from malic acid in Oxalis as has been claimed to occur in tomato slices (Link, Klein & Barron, 1952).

Under the experimental conditions described, the amount of radioactivity detected in a particular compound cannot provide precise answers on the sequence of reactions leading to the biosynthesis of oxalic acid. The level of isotope in a given compound will depend upon the rate of synthesis and breakdown of such a compound, the amount of it present (the "pool size") and also the ease with which the precursor compound can be transported to the site of synthesis. No attempt has been made here to assess such factors. However, Oxalis contains a high concentration of oxalic acid which is not rapidly metabolised. In the studies described here, ^{14}C which became incorporated into oxalic acid remained reasonably stable. Such an in vivo system can be used with advantage in an examination of possible biosynthetic pathways.

Although all ^{14}C compounds injected into Oxalis shoots gave rise to some radioisotope in oxalic acid, the contributions from bicarbonate, glycine and serine were so small that it was unlikely that any of these compounds was of physiological significance in this synthesis. The results indicated that glycollic acid and glyoxylic acid were intimately involved in oxalic acid synthesis and

that the carbon for this synthesis was derived from the sugar pool. This series of experiments did not establish the origin of glycollic acid or of glyoxylic acid. However, the pattern of labelling of isocitric acid (3.3%), glyoxylic acid (1.8%) glycollic acid (0.6%) and oxalic acid (0.9%) from [$^{14}C_1$] bicarbonate was consistent with the cleavage of isocitric acid to glyoxylic acid and succinic acid catalysed by isocitrate lyase.

2. Application of ^{14}C-labelled fructose and glyceric acid to Oxalis shoots.

The observation (Bradbeer & Racker, 1961) that fructose-6-phosphate was cleaved to glycollic acid by chloroplast preparations suggested that this transketolase-type reaction may be responsible for the formation of glycollic acid in Oxalis.

Another possible mechanism for synthesis of glycollic acid is the decarboxylation of hydroxypyruvic acid as described in suspensions of Scenedesmus by Milhand, Benson & Calvin (1956). In Oxalis shoots, serine did not give rise to levels of hydroxypyruvic acid detectable by radioautography. It is unlikely, then, that such shoots contain hydroxypyruvate-alanine transaminase active in the production of hydroxypyruvic acid from serine. Glyceric acid can also give rise to hydroxypyruvic acid, in a reaction catalysed by glycerate dehydrogenase, and this possibility was examined in Oxalis using ^{14}C glyceric acid.

Portions (0.01 ml.) of either [$^{14}C_6$] fructose or calcium [3-^{14}C] glycerate were applied to each of five shoots. The radioactive solutions were applied to grooves cut in the shoots and approx. 5μ curies was applied to each shoot. The shoots were kept in the dark for 1 hr. at room temp. and then extracted as previously described. For each experiment, the extracts were fractionated into sugars, amino acids and organic acids by column chromatography (p.42), samples from each were plated and counted and the activity in each was expressed as a percentage of the total ^{14}C recovered from the shoots. These figures are presented below.

Compound applied	Distribution (%) of ^{14}C activity in shoot extracts		
	Sugars	Amino acids	Organic acids
[$^{14}C_6$] fructose	94.1	0.8	5.1
[3-^{14}C] glycerate	1.56	0.64	97.8

It is apparent that fructose has not been rapidly incorporated into the organic acid pool. After the administration of glyceric acid, the great majority of radioactivity remained in the organic acid pool.

For each experiment, portions of the organic acid fraction were applied to paper chromatograms which were run in two dimensions, dried and put in contact with X-ray film.

Of the organic acids derived from [$^{14}C_6$] fructose, glyceric acid appeared to contain most radioisotope, lesser amounts were detected in malic acid and citric acid and isotope in glycollic acid

and oxalic acid was barely detected by radioautography.

The organic acid extract from [3-^{14}C] glyceric acid contained enough [^{14}C] oxalic acid for this acid to be easily detectable by radioautography. However, acids of the tricarboxylic acid cycle such as malic acid, citric acid, isocitric and α-oxo-glutaric acid contained more isotope than either glycollic acid or oxalic acid.

Portions of each of the organic acid extracts from [$^{14}C_6$] fructose and from [3-^{14}C] glycerate were treated with an excess of $CaCl_2$ and oxalic acid separated quantitatively as its calcium salt. After dissolving the calcium oxalate in N-HCl (2.0 ml.), portions (0.02 ml.) were plated and counted and the amount of radioisotope in such samples expressed as a percentage of the total ^{14}C activity recovered from the shoots. For [$^{14}C_6$] fructose, the amount of isotope in oxalic acid was 0.14%. Oxalic acid derived from [3-^{14}C] glyceric acid contained 2.26% of the total ^{14}C activity.

These experiments suggest that in Oxalis, glycollic acid and hence oxalic acid are not derived directly from either fructose or from glyceric acid via hydroxypyruvic acid. The qualitative results obtained by radioautography are consistent with the synthesis of oxalic acid from a tricarboxylic acid cycle intermediate (or intermediates).

3. The precursors of glycollic acid and glyoxylic acid : Application of ^{14}C-labelled compounds to grooves in Oxalis shoots.

(a) Choice of ^{14}C-labelled compounds to detect the precursors of glycollic acid and of glyoxylic acid.

In this series of experiments the following ^{14}C-labelled compounds were applied to grooves in Oxalis shoots : [2-^{14}C] glucose, [1-^{14}C] ribose, [1,5-$^{14}C_2$] isocitric acid and [1-^{14}C] glycollic acid. Cleland & Johnson (1956) showed that either [2-^{14}C] glucose or [6-^{14}C] glucose were efficiently converted to [^{14}C] oxalic acid by A. niger. If a similar pathway existed in Oxalis, the specifically labelled glucose would provide better evidence of such a pathway than the generally labelled compound. In tobacco leaves, [1-^{14}C] ribose was converted to glycollic acid (Griffith & Byerrum, 1959), and it was possible that the same situation applied to Oxalis shoots.

The application of [1-^{14}C] glycollic acid to Oxalis shoots showed that this compound was rapidly converted to both glyoxylic acid and oxalic acid (Table 4). However, it was also apparent that glycollic acid itself might be derived entirely from glyoxylic acid (see results, Table 4 for [$^{14}C_2$] glyoxylic acid). If this occurred, the focal point of interest would be an immediate precursor of glyoxylic acid other than glycollic acid. A possible mechanism was the cleavage of isocitric acid to glyoxylic acid and succinic acid,

and hence [1,5-$^{14}C_2$] isocitric acid was administered to Oxalis.

If in Oxalis, glyoxylic acid was formed from isocitric acid as well as from glycollic acid, it would be significant to assess the relative contribution of the two pathways. To obtain an indication of the relative efficiency of isocitric acid and of glycollic acid as a source of glyoxylic acid (and hence oxalic acid), [1-^{14}C] glycollic acid was administered to shoots under the experimental conditions used for [1,5-$^{14}C_2$] isocitric acid, [1-^{14}C] ribose and [2-^{14}C] glucose.

(b) The effect of pre-treatment of shoots with malonic acid.

In experiments in which [2-^{14}C] glucose and [1,5-$^{14}C_2$] isocitric acid were administered to Oxalis, some of the shoots were pretreated with malonic acid for 1 hr.(see p.41). If glyoxylic acid was a product of isocitric acid metabolism, it was hoped that the specific inhibition of succinate dehydrogenase may tend to channel more ^{14}C from isocitric acid into glyoxylic acid and oxalic acid. Malonic acid was applied to shoots 1 hr. before the appropriate ^{14}C-labelled compound; water was applied to control tissue.

Shoots were extracted 1 hr., 6 hr. and 24 hr. after the administration of [2-^{14}C] glucose and 1 hr. and 6 hr. after administration of [1,5-$^{14}C_2$]isocitric acid. The 1 hr. experiment with [1,5-$^{14}C_2$] isocitric acid was not pre-treated with malonic acid. Compounds containing isotope were separated by paper chromatography

and detected by radioautography. The ^{14}C content of individual compounds was determined on paper by a liquid scintillation technique (p. 52).

As shown in Table 5, the effect of pre-treatment with malonic acid was reflected to a small degree in the increase of ^{14}C recovered in succinic acid and in oxalic acid compared with control experiments. The overall effect of this inhibition of succinate dehydrogenase was not great enough to warrant a comparison of the ^{14}C content of all amino acids and organic acids from control and malonate-treated shoots. Neither the distribution of label between the sugars, amino acids or organic acids nor the radioisotope content of individual compounds between treated and non-treated Oxalis shoots showed significant differences. The effect of malonic acid on the ^{14}C content of succinic acid and of oxalic acid only are shown in Table 5. The values are expressed as a percentage of the total ^{14}C recovered from Oxalis shoots.

(c) Method of expressing results of in vivo experiments

An advantage of applying solutions of ^{14}C-labelled compounds to grooves in Oxalis shoots was that the amount of radioisotope which was administered could be accurately assessed. When the injection technique was used, a drop of liquid formed on the tissue when the needle had been removed; how much of this drop was sap and how much was radioactive solution could not be assessed. In contrast, isotopic

Table 5. The effect of pre-treatment of Oxalis shoots with malonic acid on the ^{14}C content of succinic acid and of oxalic acid derived from [2-^{14}C] glucose and from [1,5-$^{14}C_2$] isocitric acid.

Shoots of Oxalis were pretreated with malonic acid 1 hr. before the application of [2-^{14}C] glucose or [1,5-$^{14}C_2$] isocitric acid. Control shoots were pre-treated with an equal volume (0.05 ml.) of water. After 1 hr., 6 hr. and 24 hr. for glucose and after 6 hr. for isocitric acid, extracts were prepared from the shoots and compounds containing ^{14}C separated by paper chromatography and detected by radioautography. The ^{14}C content of individual compounds was determined on paper by a liquid scintillation technique. The results presented here show the effect of malonic acid on the incorporation of radio-isotope into succinic acid and oxalic acid.

TABLE 5.

Compound applied	Time (hr.)	% ^{14}C in specific organic acids			
		Succinic acid		Oxalic acid	
		Control	Malonate	Control	Malonate
[2-^{14}C] glucose	1	0.18	0.16	0.04	0.01
	6	0.25	0.38	0.78	1.02
	24	0.23	0.89	12.48	10.30
[1,5-$^{14}C_2$] iso-citric acid	6	4.42	4.51	4.10	4.35

solutions administered to grooves were rapidly taken up and there was no visible wastage.

With the micro-pipette used for the application of ^{14}C solutions to the shoots, a sample (0.02-0.025 ml.) of each ^{14}C solution was made up to a standard volume (50 ml.). A portion of this solution was assayed for radioactivity, and the total radioactivity applied to the shoots was calculated. It was possible, therefore, in this series of experiments to calculate results on the basis of either the total ^{14}C administered to Oxalis or the total ^{14}C recovered from the shoots. For all incubations of 1 hr. or of 6 hr. there was little difference between the total ^{14}C administered in each experiment and the total ^{14}C recovered (see Table 6), so that all results presented here are expressed on the basis of the total ^{14}C recovered from Oxalis shoots. The method of calculating these results was the same as previously described (p. 60, 61).

(d) The distribution of isotope between sugars, amino acids and organic acids from Oxalis extracts.

White shoots of Oxalis were incubated in the dark at room temp. for 1 hr. and for 6 hr. with the following compounds : [2-^{14}C] glucose, [1-^{14}C] ribose, [1,5-$^{14}C_2$] isocitric acid and [1-^{14}C] glycollic acid. Two shoots were used for each experiment and ^{14}C-labelled compounds were applied to narrow grooves cut in the shoots. After extraction of the plant material with ethanol and with water, samples of each extract were plated and counted for ^{14}C content and

were then fractionated into sugars, amino acids and organic acids by column chromatography as previously described (p.42). Samples of each of these fractions were also plated and the amount of isotope in each determined. The results in Table 6 show the amount of ^{14}C recovered in each initial extract (before fractionation) as a percentage of the total ^{14}C administered to the shoots, the distribution of this activity between sugars, amino acids and organic acids, and the percentage recovery of extracts after fractionation into the three classes of compounds.

(e) Effect of long term (24 hr.) incubation of ^{14}C-labelled compounds with Oxalis shoots.

As stated previously, the total ^{14}C administered to shoots and the total ^{14}C recovered in extracts were approximately the same when isotopes were administered for either 1 hr. or for 6 hr. In experiments with [2-^{14}C] glucose and with [1-^{14}C] ribose, incubations of 24 hr. were also carried out. The figures presented in Table 7 indicate the loss of ^{14}C over this time period. These large losses (Table 7) could be due to loss of respiratory $^{14}CO_2$ or the formation of insoluble products which were not extractable with ethanol or with water.

As shown by paper chromatography and radioautography, the general pattern of labelling of compounds for the 24 hr. period for

Table 6. Distribution of ^{14}C activity in sugars, amino acids and organic acids from ^{14}C-labelled compounds applied to grooves in *Oxalis* shoots.

White shoots of *Oxalis* were treated with a number of ^{14}C-labelled compounds and extracts prepared after 1 hr. and after 6 hr. as described in the text. The radioactivity recovered in each extract is expressed as a percentage of the total ^{14}C activity administered to the shoots. The compounds in the extracts were fractionated into sugars, amino acids and organic acids by column chromatography and the ^{14}C activity of each fraction determined. The radioactivity in each fraction is expressed as a percentage of the total ^{14}C recovered from the shoots.

TABLE 6

Compound applied	Time (hr.)	^{14}C activity recovered in shoot extract (%)	Distribution (%) of ^{14}C activity in shoot extracts			% ^{14}C activity recovered from columns
			Sugars	Amino acids	Organic acids	
[2-^{14}C] glucose	1	99.1	93.5	0.6	2.8	97.8
	6	88.7	71.8	5.8	8.0	96.5
[1-^{14}C] ribose	1	99.4	97.1	0.6	1.6	99.9
	6	94.3	84.9	3.3	5.4	99.3
[1,5-$^{14}C_2$] isocitrate	1	100.6	-	2.6	86.3	88.4
	6	97.9	-	5.7	80.3	88.0
[1-^{14}C] glycollate	1	100.3	-	3.3	90.4	93.4
	6	95.4	-	10.3	77.3	91.8

[2-^{14}C] glucose and for [1-^{14}C] ribose was similar to that observed in the shorter time periods. For this reason and because of the large percentages of ^{14}C not accounted for, the result obtained for 24 hr. incubations did not provide any additional information than had already been obtained for 1 hr. and for 6 hr. periods.

(f) Quantitative distribution of ^{14}C among specific amino acids from extracts of Oxalis shoots.

The appropriate ^{14}C-labelled compounds were applied to Oxalis shoots and extracts prepared after 1 hr. and after 6 hr. The portions of each extract containing amino acids were evaporated to dryness and then dissolved in 1.0 ml. of 20% (v/v) ethanol. Portions (0.01 ml.) of such extracts were counted on paper discs and further portions (0.2 ml.) were applied to paper chromatograms. After two dimensional chromatography in phenol saturated with water and butan-1-ol, propionic acid, water, amino acids containing ^{14}C were detected by radioautography. Areas on chromatograms containing ^{14}C-labelled compounds were identified and cut out for quantitative estimation of isotope by a liquid scintillation technique as previously described (p. 52).

The radioactivity in specific amino acids was expressed initially as a percentage of the ^{14}C activity applied to the appropriate chromatogram and finally as a percentage of the total ^{14}C activity recovered in extracts from Oxalis shoots. These figures were obtained

Table 7. Losses of ^{14}C activity from Oxalis shoots following long incubation periods (24 hr.).

A number of ^{14}C-labelled compounds were applied to grooves in Oxalis shoots as described in the text and in Table 6. For [2-^{14}C] glucose and for [1-^{14}C] ribose, incubations of 24 hr. were carried out in addition to the 1 hr. and 6 hr. time periods (see Table 6). The results presented here show the loss of ^{14}C activity over the 24 hr. time period.

TABLE 7.

Compound applied	Time (hr.)	^{14}C administered counts/100 sec.	^{14}C recovered in extract counts/100 sec.	% Loss of ^{14}C
[2-^{14}C] glucose	24	5.372×10^6	2.162×10^6	59.8
[1-^{14}C] ribose	24	7.980×10^6	4.657×10^6	41.6

after application of the appropriate factor for the percentage of ^{14}C in each of the amino acid fractions (Table 6).

In the two dimensional system described glycine and serine did not separate sufficiently well for determination of the radioactivity in each compound. The ^{14}C content of the mixture of the two was assessed, after which the compounds were eluted from the appropriate chromatograms with water and concentrated. Portions were chromatographed on paper with a solvent of pyridine-water (80 : 20, v/v) and a good separation was obtained (see Fig. 4). Glycine and serine were located by radioautography and the amount of radioactivity in each was determined. The ratio of ^{14}C activity of the two compounds was related to the total ^{14}C activity originally recorded for the mixture before elution.

The ^{14}C content of individual amino acids is shown in Table 8.

(g) Quantitative distribution of ^{14}C among specific organic acids from extracts of Oxalis shoots.

Extracts were prepared from Oxalis shoots 1 hr. and 6 hr. after the application of a number of ^{14}C-labelled compounds. The fractions of these extracts containing organic acids were evaporated and made up 1 ml. in 20% (v/v) ethanol. Subsequent procedures were carried out exactly as for paper chromatography of amino acid extracts (p. 83) except that 0.05 ml. portions were applied to chromatograms.

Fig. 4. Separation of glycine and serine by paper chromatography.

Mixtures of glycine and serine were eluted from paper chromatograms used for the separation of mixtures of amino acids from Oxalis extracts. After concentration in vacuo the mixtures of glycine and serine were separated by ascending paper chromatography in pyridine : water (80 : 20 v/v).

FIG. 4

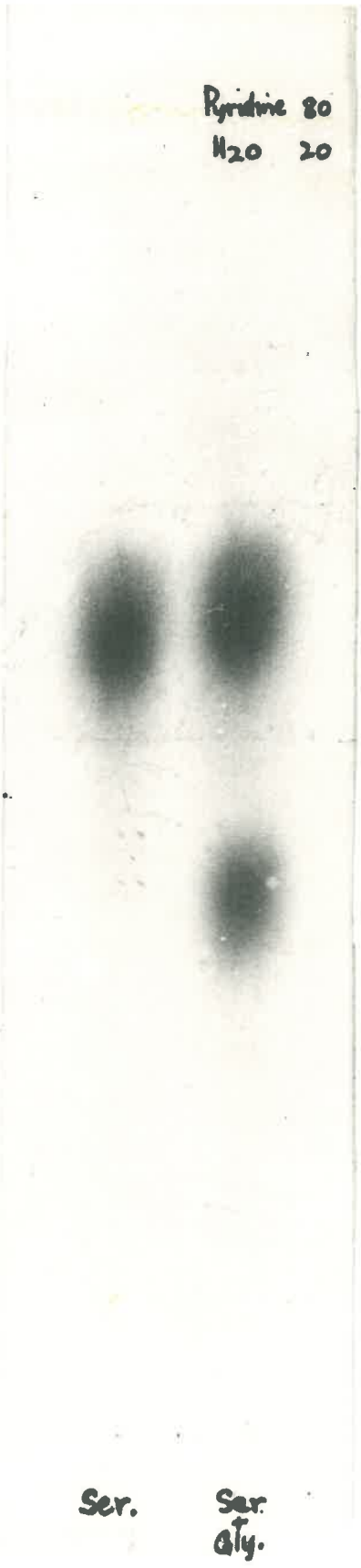


Table 8. The distribution of ^{14}C among specific amino acids from extracts of Oxalis after application of ^{14}C-labelled compounds to grooves in shoots.

Compounds were administered to shoots and extracts prepared after 1 hr. and after 6 hr. as described in the text. The amino acids in these extracts were separated by paper chromatography and the amount of radioactivity in each compound was estimated on paper by a liquid scintillation technique. The results are expressed as a percentage of the total ^{14}C activity recovered from Oxalis shoots.

Where the radioactivity in a compound was not detected, the entry is shown thus " —"; the lower limit of detection was approx. twice the background count corresponding to approx. 0.001% of the ^{14}C activity applied.

TABLE 8.

% ^{14}C activity in specific amino acids separated by paper chromatography

Compound applied	Time (hr.)	Origin*	Asp	Glu	Ser	Gly	Asp (NH_2)	Glu (NH_2)	Thr	Ala	β-Ala	Tyr	Pro	Phe	Val	Peptides	Unidentified	% ^{14}C recovered from chromatograms
[2-^{14}C] glucose	1	0.003	0.17	0.20	0.03	0.01	-	0.01	-	0.15	-	-	-	-	0.03	-	-	92.4
	6	0.08	0.73	1.50	0.40	0.10	-	0.28	0.04	0.61	-	0.02	0.06	-	0.65	-	0.69	88.4
[1-^{14}C] ribose	1	0.01	0.07	0.09	0.05	0.01	-	0.11	-	0.25	-	-	-	-	-	-	-	89.4
	6	0.13	0.72	0.87	0.16	0.07	-	0.06	0.13	0.68	-	0.22	-	0.19	-	-	0.06	98.7
[1,5-$^{14}C_2$] isocitrate	1	0.51	0.51	0.68	-	-	-	0.08	-	-	-	-	-	-	0.10	-	0.51	93.2
	6	0.06	1.34	2.32	0.01	-	-	0.48	0.02	0.10	0.06	0.10	-	-	0.33	-	0.68	97.1
[1-^{14}C] glycollate	1	0.02	0.09	0.17	1.55	0.81	-	0.03	-	0.05	-	-	-	-	0.05	0.08	0.18	91.8
	6	0.03	0.14	1.02	6.40	1.63	0.07	0.23	-	0.10	-	-	-	-	0.03	0.17	0.07	96.1

*Material remaining at origin after development of chromatograms.

Originally 0.2 ml. portions were applied but excessive amounts of oxalic acid present in these extracts caused streaking on chromatograms. Compounds containing ^{14}C were located by radioautography, identified and counted.

Areas on chromatograms containing a mixture of citric acid and isocitric acid were eluted with water, concentrated, and the two acids separated on paper by double development with a solvent consisting of butan-1-ol, 3-methyl-propan-1-ol, water, pyridine and formic acid (3 : 6 : 7 : 4 : 1 by vol. - see p. 45). Separation of these two acids is shown in Fig. 5.

The ^{14}C content of organic acids was calculated in the same manner as for amino acids (p. 61); the results (Table 9) are expressed as a percentage of the total ^{14}C recovered from *Oxalis* shoots.

The quantitative results of the previous series of *in vivo* experiments (Tables 1 to 4) indicated that the carbon for oxalic acid synthesis in *Oxalis* shoots was derived from the sugar pool, or derivatives thereof, and that glycollic acid and glyoxylic acid were intimately associated with the biosynthesis of oxalic acid. The second series of experiments described here (Tables 6 to 9) have extended the previous findings and have supplied information concerning the precursors of glycollic acid and of glyoxylic acid.

Fig. 5. Separation of citric acid and isocitric acid by paper chromatography.

Areas on chromatograms (used in the general separation of organic acids) containing a mixture of citric acid and isocitric acid were eluted with water. After concentration, these extracts were applied to paper for chromatography and mixtures of citric acid and isocitric acid were separated by double development with a solvent consisting of butan-1-ol, 3-methyl-propan-1-ol water, pyridine, formic acid (3 : 6 ; 7 : 4 : 1 by vol.).

Citric acid is identified as the faster moving component.

FIG. 5

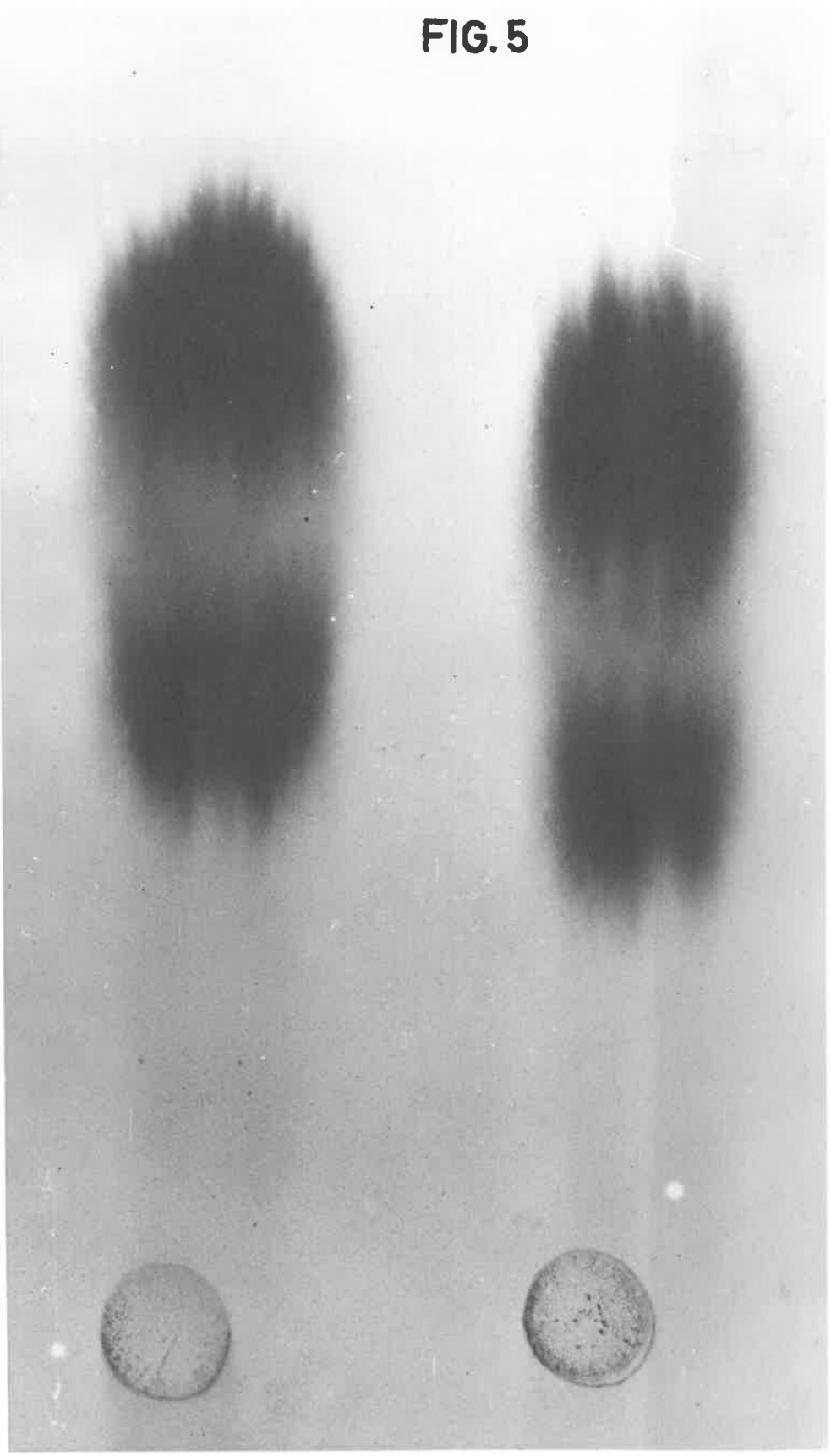


Table 9. The distribution of ^{14}C among specific organic acids from extracts of *Oxalis* after application of ^{14}C-labelled compounds to grooves in shoots.

Compounds were administered to shoots and extracts prepared after 1 hr. and after 6 hr. as described in the text. The organic acids in these extracts were separated by paper chromatography and the amount of radioactivity in each compound was estimated on paper by a liquid scintillation technique. The results are expressed as a percentage of the total ^{14}C activity recovered from *Oxalis* shoots.

Where the radioactivity in a compound was not detected, the entry is shown thus " —— "; the lower limit of detection was approx. twice the background count corresponding to approx. 0.001% of the ^{14}C activity applied.

TABLE 9.

Compound applied	Time (hr.)	% ^{14}C activity in specific organic acids separated by paper chromatography														% ^{14}C recovered from chromatograms
		Origin*	Phosphate esters	Glyceric acid	Citric acid	Isocitric acid	α-Oxoglutaric acid	Succinic acid	Malic acid	Fumaric acid	†Phosphoglycollic acid	Glycollic acid	Glyoxylic acid	Oxalic acid	Unidentified	
[2-^{14}C] glucose	1	0.14	0.76	0.52	0.11	0.05	-	0.18	0.16	-	-	0.03	-	0.04	0.29	82.8
	6	0.40	1.83	1.75	0.86	0.40	0.23	0.25	0.55	0.09	-	0.08	-	0.78	0.24	93.4
[1-^{14}C] ribose	1	0.06	0.27	0.08	0.03	0.01	-	-	0.03	-	-	0.02	-	0.01	0.74	86.9
	6	0.29	0.52	0.39	0.24	0.13	-	0.04	0.35	-	-	0.03	-	0.41	2.61	91.8
[1,5-$^{14}C_2$] isocitrate	1	1.29	-	-	5.05	54.44	12.52	6.39	-	-	-	0.78	0.35	2.16	0.69	96.9
	6	1.93	-	-	34.03	15.19	6.58	4.42	1.12	0.08	-	0.96	0.48	4.10	1.57	89.0
[1-^{14}C] glycollate	1	4.88	-	-	0.34	0.20	0.54	-	0.81	-	0.63	60.21	1.99	2.98	1.08	81.5
	6	1.86	-	-	1.19	0.82	0.54	0.93	0.85	0.54	0.93	30.77	4.56	20.03	0.85	82.6

* Material remaining at origin after development of chromatograms.

† Tentatively identified

GENERAL DISCUSSION

A comparison of the distribution of the isotope from ^{14}C-labelled compounds administered to grooves in *Oxalis* shoots showed that there was a greater incorporation of radioactivity into amino acids and into organic acids from [2-^{14}C] glucose than from [1-^{14}C] ribose (Table 6). This difference was more evident after exposure for 6 hr. The application of [1,5-$^{14}C_2$] isocitric acid for 1 hr. resulted in a greater incorporation of isotope into the amino acid pool than from incubation for 1 hr. with [2-^{14}C] glucose. After 6 hr., the incorporation was the same (5.8% and 5.7%, respectively, Table 6.)

It is evident that glycollic acid contributed significantly to the amino acid pool. When [1-^{14}C] glycollic acid was administered to *Oxalis* shoots, 3.3% of the ^{14}C activity applied was located in the amino acid fraction within 1 hr.; 10.3% of the activity was present in the amino acid pool after 6 hr. (Table 6). These figures represent the largest incorporation into amino acids from any of the ^{14}C-labelled compounds administered.

As shown in Table 6, 99-100% of the ^{14}C activity administered to *Oxalis* shoots was recovered after incubations for 1 hr. Recoveries from the series in which shoots were extracted 6 hr. after the application of isotope varied from 88.7% to 97.9%. The separation of extracts into sugars, amino acids and organic acids by column chromatography resulted in negligible losses of ^{14}C for [2-^{14}C] glucose and

for [1-^{14}C] ribose (which were not adsorbed by cationic or anionic columns) whereas recoveries from [1,5-$^{14}C_2$] isocitric acid and [1-^{14}C] glycollic acid were lower (88.0% to 93.4%).

After administration of [2-^{14}C] glucose for 1 hr. and for 6 hr. most radioisotope in the amino acid fraction was located in glutamic acid, aspartic acid and alanine (Table 8). The same pattern of incorporation was evident in these amino acids from [1-^{14}C] ribose, except that in 1 hr. there was greater incorporation of isotope into alanine than into glutamic acid or aspartic acid (Table 8). Such incorporation can be accounted for by transamination of α-oxoglutaric acid, oxaloacetic acid and pyruvic acid.

As shown in Table 9, the incorporation of ^{14}C into organic acids from [2-^{14}C] glucose and from [1-^{14}C] ribose was somewhat similar. Initially, phosphate esters contained most ^{14}C; subsequently glyceric acid and acids of the tricarboxylic acid cycle became labelled. The relatively greater incorporation of ^{14}C into glyceric acid from [2-^{14}C] glucose (0.52% and 1.75%) rather than from [1-^{14}C] ribose (0.08% and 0.39%) was reflected in generally higher levels of ^{14}C in tricarboxylic cycle acids derived from [2-^{14}C] glucose. The level of isotope in oxalic acid derived from [2-^{14}C] glucose was also higher (0.04% and 0.78%) when compared with that derived from [1-^{14}C] ribose (0.01% and 0.41%). The biosynthesis of oxalic acid from [2-^{14}C] glucose or from [1-^{14}C] ribose does not appear to involve glycollic acid, in which there was a low level of radioactivity. The formation of glycollic acid from ribose, demonstrated in photosynthetic tissue (Griffith &

Byerrum, 1959) does not appear to be a significant pathway in non-photosynthetic Oxalis shoots. In the longer time period, more ^{14}C from both [2-^{14}C] glucose and from [1-^{14}C] ribose was incorporated into oxalic acid and acids of tricarboxylic acid cycle, particularly into citric acid, isocitric acid and malic acid (Table 9). These results suggested that one or more of those acids may be involved in oxalic acid synthesis. Experiments with [^{14}C] bicarbonate have already shown (this thesis, Table 4 p. 68; see also, Millerd, Morton & Wells, 1963) that, in Oxalis, malic acid did not contribute to oxalic acid production.

Glutamic acid and aspartic acid contained most of the ^{14}C activity in the amino acid fraction in 1 hr. and in 6 hr. after the application of [1,5-$^{14}C_2$] isocitric acid (Table 8). These results infer that Oxalis shoots contain isocitrate dehydrogenase and L-glutamate:NAD oxidoreductase (glutamate dehydrogenase, EC 1.4.1.2), activity. Certainly there was considerable radioisotope located in α-oxoglutaric acid after the administration of [1,5-$^{14}C_2$] isocitric acid (Table 9). The activity found in aspartic acid in this experiment could be a result of transamination of oxaloacetic acid. However, no ^{14}C activity was detected in malic acid (Table 9) or alanine (Table 8) after the administration of [1,5-$^{14}C_2$] isocitric acid for 1 hr. It is possible therefore, that within 1 hr., very little of the ^{14}C administered had passed right through the tricarboxylic acid cycle. However, if the concentration of endogenous glutamic acid is high, oxaloacetic acid would be rapidly removed, in turn stimulating the

oxidation of malic acid; it is possible therefore, to go through the cycle without significant amounts of malic acid being detected. The oxidation of glutamic acid to aspartic acid proposed by Krebs & Bellamy (1960) is supported by some experimental evidence (Jones & Gutfreund, 1961). Alternatively, oxaloacetic acid may have been formed by reversal of citrate oxaloacetate-lyase (citrate synthase, EC 4.1.3.7, formerly known as citrate condensing enzyme) since within 1 hr., citric acid contained 5% of the total ^{14}C from [1,5-$^{14}C_2$] isocitric acid (Table 9).

The distribution of ^{14}C among organic acids derived from [1,5-$^{14}C_2$] isocitric acid (Table 9) shows that, within 1 hr. some 12.5% of the recovered ^{14}C activity had been incorporated into α-oxoglutaric acid and 6.4% of such activity into succinic acid. From this experiment it was not possible to assess whether all the ^{14}C activity in succinic acid was derived from α-oxoglutaric acid or partly from isocitric acid by the action of isocitrate lyase. Since considerable radioisotope was found in oxalic acid (2.2%) and glycollic acid (0.8%) it seems likely that at least some isocitric acid was split into glyoxylic acid and succinic acid. The relatively low level of isotope in glyoxylic acid (Table 9), was probably due to its instability during the preparation of extracts for chromatography and more particularly the instability of this acid during paper chromatography in butan-1-ol, propionic acid, water (Kearney & Tolbert, 1962).

The percentage incorporation of ^{14}C into amino acids (Table 8) and into organic acids (Table 9) after treatment of Oxalis shoots with

[1,5-$^{14}C_2$] isocitric acid for 6 hr. showed trends similar to those observed after 1 hr. The decrease of isotope in α-oxoglutaric acid after 6 hr. was associated with an increase in the ^{14}C activity in glutamic acid (Table 8). Similarly a decrease in the ^{14}C level of succinic acid and an increase in ^{14}C in malic acid and fumaric acid (6 hr.) indicated a wider distribution of isotope through tricarboxylic acid cycle intermediates in the longer time period.

In the organic acid fraction (Table 9), the most striking change in the distribution of ^{14}C from [1,5-$^{14}C_2$] isocitric acid in the 6 hr. period was the conversion of isocitric acid to citric acid, rather than continued production of α-oxoglutaric acid or glyoxylic acid from isocitric acid.

That the incorporation of isotope from [1,5-$^{14}C_2$] isocitric acid into oxalic acid was only doubled between the 1 hr. and the 6 hr. incubation periods (Table 9) may be due to a number of factors:-

(i) Since the isocitric acid employed was not uniformly labelled, redistribution of the carbon atoms of this acid during the operation of the tricarboxylic acid cycle would decrease the frequency of ^{14}C being incorporated into glyoxylic acid and oxalic acid.

(ii) Isocitrate lyase purified from yeast (Olsen, 1959) exhibited a marked decrease in activity with DL(+) allo-isocitric acid compared with L_s-isocitric acid. With DL(+) allo-isocitric acid at four times the concentration of the L_s-isomer the enzyme had only 20% of the activity. Thus, the use of DL(+) allo [1,5-$^{14}C_2$] isocitric acid in these experiments may have resulted in partial inhibition of isocitrate

lyase of Oxalis shoots. Inhibition of this enzyme may also be effected by tricarboxylic acid cycle intermediates, particularly succinic acid (Kornberg, Gotto & Lund, 1958; Kornberg, Collins & Bigley, 1960).

(iii) Glyoxylic acid itself has been associated with inhibition of the tricarboxylic acid cycle. In particular, glyoxylic acid is inhibitory to the decarboxylation of pyruvic acid (Liang, 1962b) and to oxygen uptake of intact rat-liver mitochondria in the presence of L-glutamic acid, α-oxoglutaric acid or pyruvic acid (Ruffo, Adinolfi, Budillon & Capobianco, 1962a,b). The inhibitory effect of glyoxylic acid on citric acid oxidation (D'Abramo, Romano & Ruffo, 1958) was shown in vitro to be due to the interaction of glyoxylic acid with oxaloacetic acid to form a C_6 compound (Ruffo, Romano & Adinolfi, 1959). Chromatographic evidence (Ruffo, Romano, Adinolfi & Verga, 1960), indicated that the new compound was probably a C_6 tricarboxylic acid. It has been isolated and identified as α-hydroxy-β-oxalosuccinic acid (oxalomalic acid). Glyoxylic acid and oxaloacetic acid combine non-enzymically at pH 7.4 or more alkaline pH values and the resulting compound is strongly inhibitory to citrate (isocitrate) hydro-lyase, (aconitate hydratase EC 4.2.1.3, formerly known as aconitase). This inhibition causes an accumulation of citric acid (Bade, 1962; Ruffo, Testa, Adinolfi & Pelizza, 1962). It is likely that glyoxylic acid formed from isocitric acid in Oxalis would cause inhibition of citric acid oxidation since it would be near the active site of aconitate hydratase in the mitochondria. The failure to observe similar inhibition by glyoxylic acid derived from glycollic acid may be due to the removal of this compound (by oxidation) before the formation of

sufficient oxalomalic acid at the site of action of aconitate hydratase.

To compare the contribution of glycollic acid and of isocitric acid to oxalic acid biosynthesis, [1-^{14}C] glycollic acid was administered to Oxalis shoots.

Within 1 hr. the only amino acids to contain a significant amount of isotope were glycine and serine (Table 8). Amongst the organic acids (Table 9), glyoxylic acid contained 2.0% and oxalic acid 3.0% of the recovered activity. Tricarboxylic acids contained a lesser amount of radioisotope. After treatment of the shoots with [1-^{14}C] glycollic acid for 6 hr. most ^{14}C in the amino acid fraction was again located in glycine (1.6%) and serine (6.4%). Glutamic acid (1.0%) was the only other amino acid which contained a significant level of ^{14}C. The most notable feature of the ^{14}C distribution in the organic acids (Table 9) was the large incorporation of isotope into oxalic acid (20.0%). Thus, in 1 hr. the percentage incorporation of ^{14}C into oxalic acid from [1,5-$^{14}C_2$] isocitric acid and from [1-^{14}C] glycollic acid was comparable (2.2% and 3.0% respectively). However, after 6 hr. there was a much greater incorporation from [1-^{14}C] glycollic acid into oxalic acid. The lower recovery of ^{14}C from organic acid chromatograms from the experiment using [1-^{14}C] glycollic acid (Table 9) was probably due to sublimation of glycollic acid from the paper to the X-ray film (Kearney & Tolbert, 1962). The significance of glycollic acid in serine biosynthesis has been noted previously by Rabson, Tolbert & Kearney (1962). In white Oxalis shoots,

after the administration of [1-^{14}C] glycollic acid for 6 hr., serine contained more ^{14}C than any other compound derived from glycollic acid with the exception of oxalic acid.

E. CONCLUSIONS

The experiments in this section describe studies to determine the precursors of oxalic acid in non-photosynthetic shoots from the starchy bulbs of Oxalis pes-caprae. The application of a number of ^{14}C compounds to shoots has indicated that glycollic acid and glyoxylic acid are intimately associated with this synthesis.

In photosynthetic tissues, both bicarbonate and ribose contribute to the synthesis of glycollic acid whereas, in non-photosynthetic Oxalis shoots neither of these compounds appeared to be of significance in the formation of glycollic acid. In vivo, glyoxylic acid served as an efficient precursor of glycollic acid.

Oxalic acid synthesis appears to be associated with the oxidation of glyoxylic acid which may arise either from the oxidation of glycollic acid or from the cleavage of isocitric acid.

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PART 2. OXALIC ACID SYNTHESIS IN VITRO

A. INTRODUCTION

The application of ^{14}C-labelled compounds to *Oxalis* shoots and examination of the subsequent distribution of radioisotope among specific compounds, (Part 1), indicated a number of metabolic reactions associated with oxalic acid metabolism. The experiments *in vivo* with [$^{14}C_6$] glucose showed that both glycollic acid and glyoxylic acid were intimately involved in oxalic acid synthesis. Application of glyoxylic acid and of glycollic acid labelled with ^{14}C confirmed that *Oxalis* shoots contained an oxidative mechanism for the conversion of glycollic acid via glyoxylic acid to oxalic acid. The oxidation of glycollic acid and of glyoxylic acid catalysed by glycollate oxidase purified from photosynthetic tissue has been described (Richardson & Tolbert 1961a). Clagett, Tolbert & Burris (1949), found glycollate oxidase activity in a wide range of photosynthetic tissues but reported that it was not present in embryos or etiolated plants.

Following the administration of [$^{14}C_2$] glyoxylic acid to *Oxalis* shoots, ^{14}C was rapidly incorporated not only into oxalic acid, but also into glycollic acid (see Table 4, p. 68), suggesting that glyoxylic acid was also readily reduced to glycollic acid. An

$NADH_2$-linked glyoxylate reductase isolated from green leaves has been associated with glycollate oxidase in a glycollate oxidation cycle (Zelitch, 1953; Zelitch & Ochoa, 1953). More recently (Zelitch & Gotto, 1962), an $NADPH_2$-linked glyoxylate reductase has also been found in photosynthetic tissue.

The incorporation of ^{14}C from [$^{14}C_2$] oxalic acid into glyoxylic acid and into glycollic acid (Table 4, p. 68) indicated that oxalic acid was not completely metabolically inert in Oxalis. The reduction of oxalic acid via oxalyl-CoA has been described in extracts of P. oxalaticus (Quayle, Keech & Taylor, 1961).

In photosynthetic tissues, carbon dioxide and a number of hexoses or pentoses associated with the photosynthetic carbon reduction cycle, have been considered as precursors of glycollic acid (see Part 1, p. 11). In white Oxalis shoots however, neither [^{14}C] bicarbonate, [$^{14}C_6$] fructose or [1-^{14}C] ribose contributed significantly to glycollic acid synthesis in vivo. It is apparent that, in these shoots, the reduction of glyoxylic acid represented the major pathway for the synthesis of glycollic acid. Clearly then, it was necessary to postulate a source for the synthesis of glyoxylic acid other than the oxidation of glycollic acid.

The application of [$^{14}C_2$] glycine to Oxalis resulted in very small incorporation of radioisotope into glyoxylic acid or oxalic acid. However, the pattern of distribution of ^{14}C from labelled glucose, carbon dioxide and isocitric acid was consistent

with a cleavage of isocitric acid to glyoxylic acid and succinic acid. This reaction, catalysed by isocitrate lyase, has been considered to be confined strictly to those plant tissues actively converting fat to carbohydrate (Carpenter & Beevers, 1959). Although this condition does not apply to Oxalis, such shoots would be active in the synthesis of C-2 units (glyoxylic acid) for the production of oxalic acid.

The experiments reported in this Section concern in vitro studies of reactions involved in the metabolism of oxalic acid and related compounds in Oxalis. The enzymic system involved in the oxidation of glycollic acid and of glyoxylic acid has been characterized. A preliminary examination of the reactions involved in the utilisation of oxalic acid has been made. In addition, the demonstration of isocitrate lyase activity in cell-free extracts of Oxalis shoots has established the significance of this pathway in the production of oxalic acid in Oxalis.

B. MATERIALS

1. Plants

Bulbs of Oxalis pes-caprae were harvested from the field and germinated on trays containing moistened vermiculite. The shoots which emerged were kept well watered and allowed to grow in the dark for 1 to 5 months. The shoots grew vigorously for at least six

months although they received water only and were kept in complete darkness. A photograph of the plant material, approximately four months after germination is shown in Plate 1.

2. Chemicals

All inorganic reagents used were of A.R. grade. DL(+) allo isocitric acid (tri-sodium salt, 42% D form), GSH, sodium glyoxylate monohydrate and α-oxoglutaric acid were A.R. grade (Sigma Chemical Co.). CoA, ferricytochrome c (horse-heart, type II, 65%), riboflavin phosphate (FMN) and adenosine triphosphate (ATP) were also products of Sigma Chemical Co. Catalase (beef liver; crude) was obtained from Nutritional Biochemicals Corporation. Semicarbazide - HCl, 2:4-dinitrophenylhydrazine (British drug Houses Ltd.) and potassium oxalate (Judex Chemical & Pharmaceutical Co.) were A.R. grade. Glycollic acid (re-crystallised before use), succinic acid (British Drug Houses Ltd.) cysteine-HCl (E. Merck & Co.) and trichloracetic acid (May & Baker) were L.R. grade.

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

Norite 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 - Dichlorophenolindophenol. 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 x 50 ml.) and extracted with 100 ml. of 2% sodium bicarbonate. 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 & Soehne) by reduction with ethanol and alcohol : NAD oxidoreductase (alcohol dehydrogenase, EC 1.1.1.1) as described by 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 & 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 from 3 min., cooled and centrifuged. The supernatant containing $NADPH_2$ was stored at -15°.

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

Yeast alcohol dehydrogenase. The crystalline suspension of the enzyme was obtained from C. F. Boehringer & Soehne.

Isocitrate dehydrogenase. This was prepared from pig heart and purified to the end of step 2 as described by Ochoa (1955).

Succinate dehydrogenase (Succinate : (cytochrome c) oxidoreductase, EC 1.3.99.1). A mitochondrial preparation containing succinate dehydrogenase activity was isolated from rat heart muscle as described by Keilin & Hartree (1940).

C. METHODS

1. Isolation of enzymically active proteins from Oxalis

(a) Ammonium sulphate fractionation of soluble proteins from Oxalis shoots.

Shoots (25-50 g.) were detached from the bulbs. All subsequent procedures were carried out at 2°. The shoots were cut into small pieces (2-3 cm.) and ground in a pre-chilled glass mortar in 3 volumes of 0.2 M - phosphate buffer, pH 8.3. The brei was centrifuged at 36,000 g. for 10 min. and the supernatant volume measured. The supernatant was brought to 70% saturation with solid

ammonium sulphate. The required amount of ammonium sulphate was added slowly over a period of approx. 30 min. and the mixture was kept slowly stirred with a glass rod. Stirring was continued until approx. 15 min. after the added salt had all dissolved.

The mixture was centrifuged at 20,000 g. for 15 min., the supernatant discarded and the brown precipitate was dissolved in 0.02M - phosphate buffer, pH 7.8 (usually 10 ml.) The protein solution was dialysed against approx. 200 volumes of 10^{-3} M phosphate buffer pH 7.8 for 1 to 2 hr. Denatured protein was removed by centrifugation (10,000 g., 10 min.) and portions (1 to 2 ml.) of the soluble protein were used for the manometric assay of oxygen uptake in the presence of either glycollic acid or glyoxylic acid.

(b) Preparation of acetone powders of the particulate fraction of Oxalis shoots.

Shoots of Oxalis which had been grown in the dark were detached from their bulbs. All subsequent steps were carried out at 2°. For these preparations approx. 200 g. of shoots were used; however, extraction in 3 volumes of 0.2 M phosphate buffer pH 8.3 was carried out in stages so that 30-35 g. of shoots were extracted in approx. 100 ml. of buffer. Under these conditions the brei was kept just above pH 7.0 but a check was always made that the pH did not fall below this level due to excess 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 Corporation, New York). The supernatant was discarded and the grey-brown precipitate homogenised for 15 sec. in 40 ml. of cold acetone at - 15° in a glass-teflon (Potter Elvehjem type) homogeniser pre-chilled to -15°. The suspension was centrifuged at 2,000 g. for 5 min. and the yellow supernatant discarded. The grey pellet was re-homogenised twice in 40 ml. of acetone at -15° and the clear supernatants discarded. The final white pellet was spreed over a large area of a glass centrifuge tube which was placed in a vacuum dessicator containing P_2O_5 and paraffin shavings. Excess acetone was removed *in vacuo*.

The yield of fine white powder obtained was approx. 1 mg./g. of fresh weight of shoots. The powder was extracted immediately before use with 0.1 M-phosphate at pH 7 to 8, depending on the required pH of the reaction being studied. In general, approx. 200 mg. of powder were 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 the addition of FMN (50 μM final concentration) 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 powder resulted in extracts with increased activity in the oxidation of glycollic acid and glyoxylic acid.

2. General assay procedures

(a) Manometric estimation of oxygen consumption.

Oxygen consumption was determined at 30° using the standard Warburg technique (Umbreit, Burris & Stauffer, 1957). All reactions were carried out in duplicate flasks (15 ml. capacity); the total volume of reactants was 2.2 ml.

(b) Spectrophotometric measurements.

Extinctions were 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 extinction at a given wave-length, the Beckman spectrophotometer was preferred so that the co-enzyme or the 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.

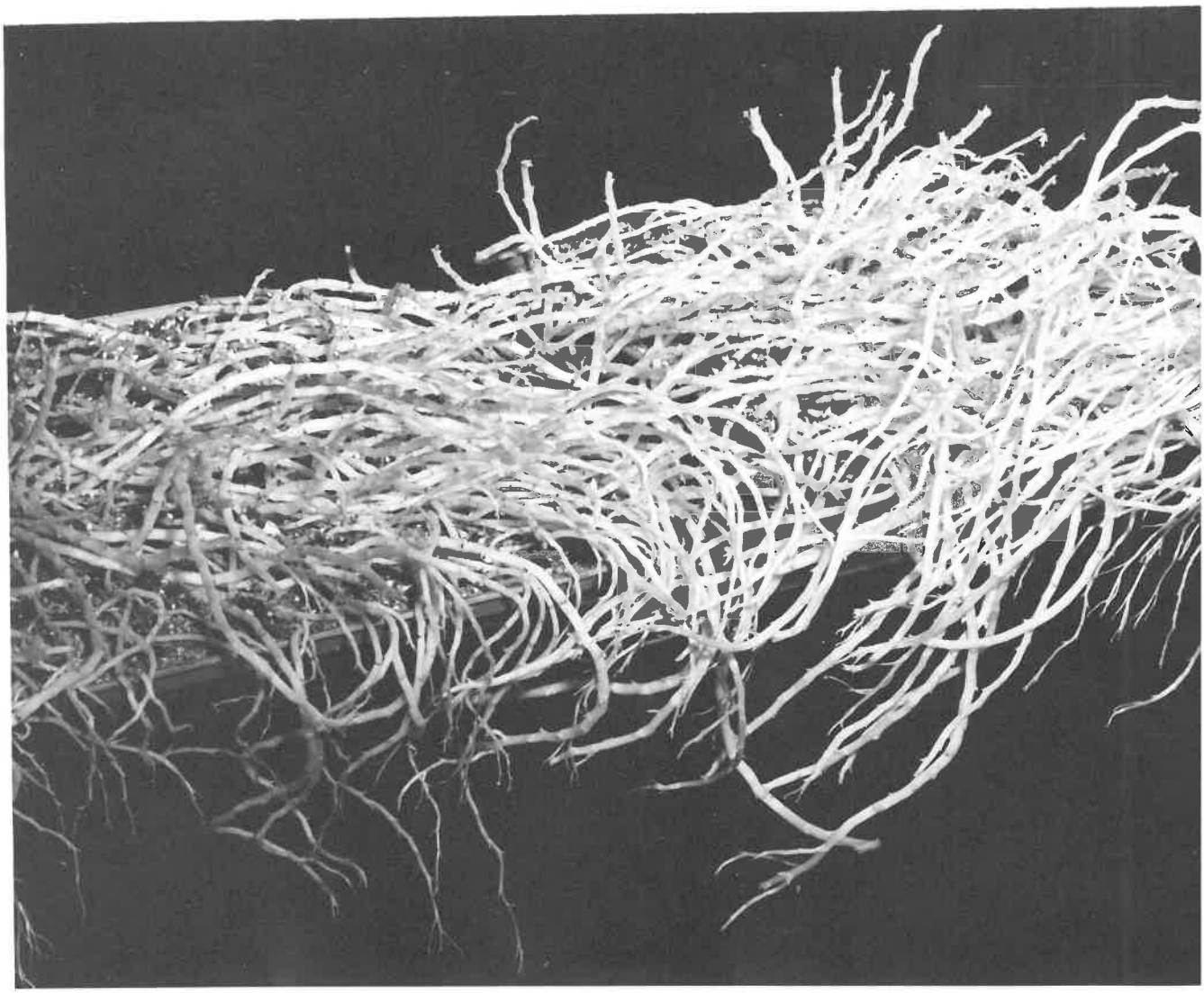
(c) Estimation of soluble protein.

The protein content of enzymic extracts was determined

Plate 1. *Oxalis* shoots used for the preparation of cell-free extracts for enzymic assays.

Bulbs of *Oxalis pes-caprae* were harvested from the field and germinated on trays containing moistened vermiculite. These shoots were kept in complete darkness and continued to grow vigourously for periods of up to six months. This picture was taken approximately four months after the germination of the shoots.

PLATE 1



spectrophotometrically by measuring extinctions of such extracts at 260 mμ and 280 mμ (Warburg & Christian, 1941).

D. RESULTS AND DISCUSSION

1. Oxidation of glycollic acid and of glyoxylic acid by cell-free extracts of Oxalis shoots.

(a) Manometric assays of the oxidation of glycollic acid and of glyoxylic acid by extracts of Oxalis shoots.

(i) Comparison of the rates of oxygen uptake in the presence of glycollic acid with soluble protein from white Oxalis shoots and from green leaves of Oxalis.

Glycollate oxidase of green plants is associated with the soluble protein fraction of these tissues (Zelitch, 1953). Extracts of soluble protein were therefore prepared from white Oxalis shoots (see p. 105) and portions assayed for their ability to catalyse the oxidation of glycollic acid. A number of preparations from shoot tissue showed a very low level of oxygen consumption in the presence of glycollic acid. These results indicated that either the oxidative mechanism was not particularly

active in these tissues or that the extraction procedure used destroyed most of the enzymic activity. In order to examine the second possibility, soluble protein was prepared from 25 g. of Oxalis shoots and from 25 g. of Oxalis leaves harvested from the field. The final volume of each protein solution was 10 ml. The oxygen consumption consequent upon the oxidation of glycollic acid catalysed by the extract from non-photosynthetic shoots and by the extract from green Oxalis leaves is shown in Fig. 6. These results clearly show that the soluble protein from photosynthetic tissue of Oxalis is very active in catalysing the oxidation of glycollic acid. This activity is probably due to the presence of glycollate oxidase which is widely distributed in green plants. The soluble protein from non-photosynthetic Oxalis shoots showed a low level of activity towards the oxidation of glycollic acid (see Fig. 6).

(ii) Effect of FMN on rate of oxygen uptake by soluble protein from Oxalis shoots in the presence of glycollic acid and of glyoxylic acid.

The rate of oxygen uptake by soluble protein extracts from white Oxalis shoots in the presence of glycollic acid was very low (Fig. 6). In an attempt to increase this activity, FMN (final concentration 50 μM) was added to incubation mixtures.

The rate of oxygen uptake by these extracts is shown in Fig. 7. It is apparent that added FMN increased the rate of oxygen

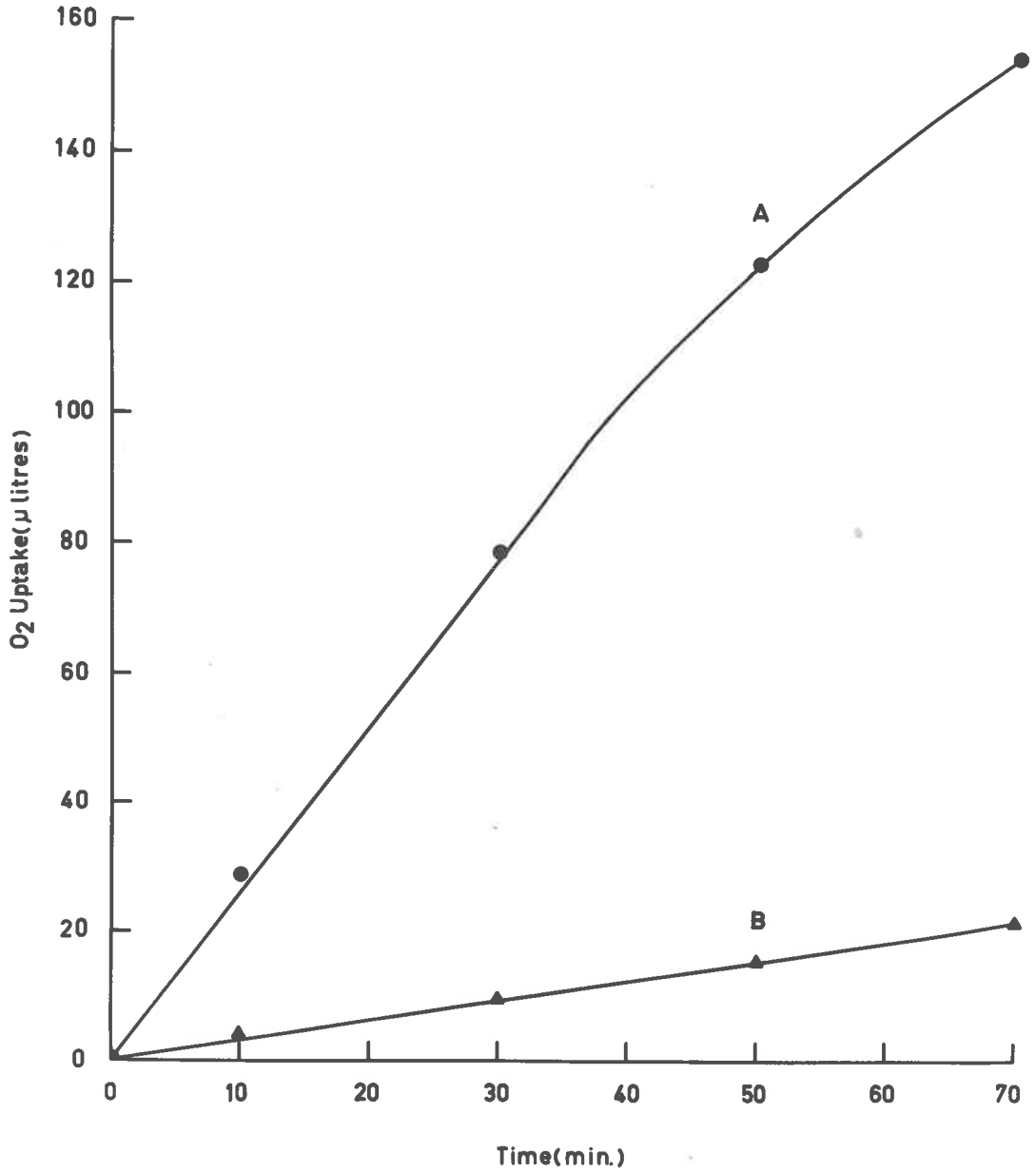
Fig. 6. Oxidation of glycollic acid by soluble protein extracts from *Oxalis* shoots and from green *Oxalis* leaves.

Soluble protein extracts were prepared from white *Oxalis* shoots and from green *Oxalis* leaves as described in the text. Reaction vessels contained *Oxalis* extract (1.8 ml. in 0.02M-phosphate pH 7.8), catalase (approx. 20 µg.) and water or glycollic acid (40 µmoles, pH 7.5). The final volume was 2.2 ml. Values plotted were corrected for oxygen consumption in the absence of substrate.

A. Oxygen uptake with extract from green leaves.

B. Oxygen uptake with extract from white shoots.

FIG. 6


160
140
120
100
80
60
40
20
0
O_2 Uptake(μ litres)
A
B
0
10
20
30
40
50
60
70
Time(min.)

consumption by soluble Oxalis extracts both for glycollic acid and glyoxylic acid. In this respect, the extracts are similar to glycollate oxidase. In the presence of added FMN, the soluble Oxalis protein catalysed the oxidation of glycollic acid at approximately five times the rate of glyoxylic acid (Fig. 7 A, C.). Glycollate oxidase from photosynthetic tissues is also more active towards the oxidation of glycollic acid (Richardson & Tolbert, 1961a). In the absence of added FMN, no oxygen uptake was observed with glyoxylic acid as substrate (Fig. 7, D.).

Stimulation of oxygen uptake was observed in similar experiments with FAD, but not with riboflavin or AMP. It is likely that FAD was broken down to FMN by the crude plant preparation.

(iii) The oxidation of glycollic acid and of glyoxylic acid by a soluble protein fraction and by a particulate fraction from Oxalis shoots.

Although glycollate oxidase activity is associated with the soluble protein of photosynthetic tissue it was possible that this situation may not apply to non-photosynthetic Oxalis shoots. Furthermore, there was no conclusive evidence that the oxidative mechanism for the oxidation of glycollic acid and glyoxylic acid in Oxalis was identical with glycollate oxidase activity. Accordingly, a preparation of soluble protein (precipitated with ammonium sulphate) and a suspension of the particulate fraction from the same shoots

Fig. 7. The effect of added FMN on the rate of oxygen uptake by soluble protein extracts from Oxalis in the presence of glycollic acid and of glyoxylic acid.

The reaction vessels contained soluble protein from Oxalis shoots (1.8 ml. in 0.02 M-phosphate, pH 7.8), catalase (approx. 20 µg), FMN (final concn. 50 µM), and water, glycollic acid (40 µmoles) or glyoxylic acid (40 µmoles). The total volume was 2.2 ml. Both control and assay flasks were made up with and without FMN. Oxygen uptake figures were adjusted for oxygen consumption observed with the appropriate controls.

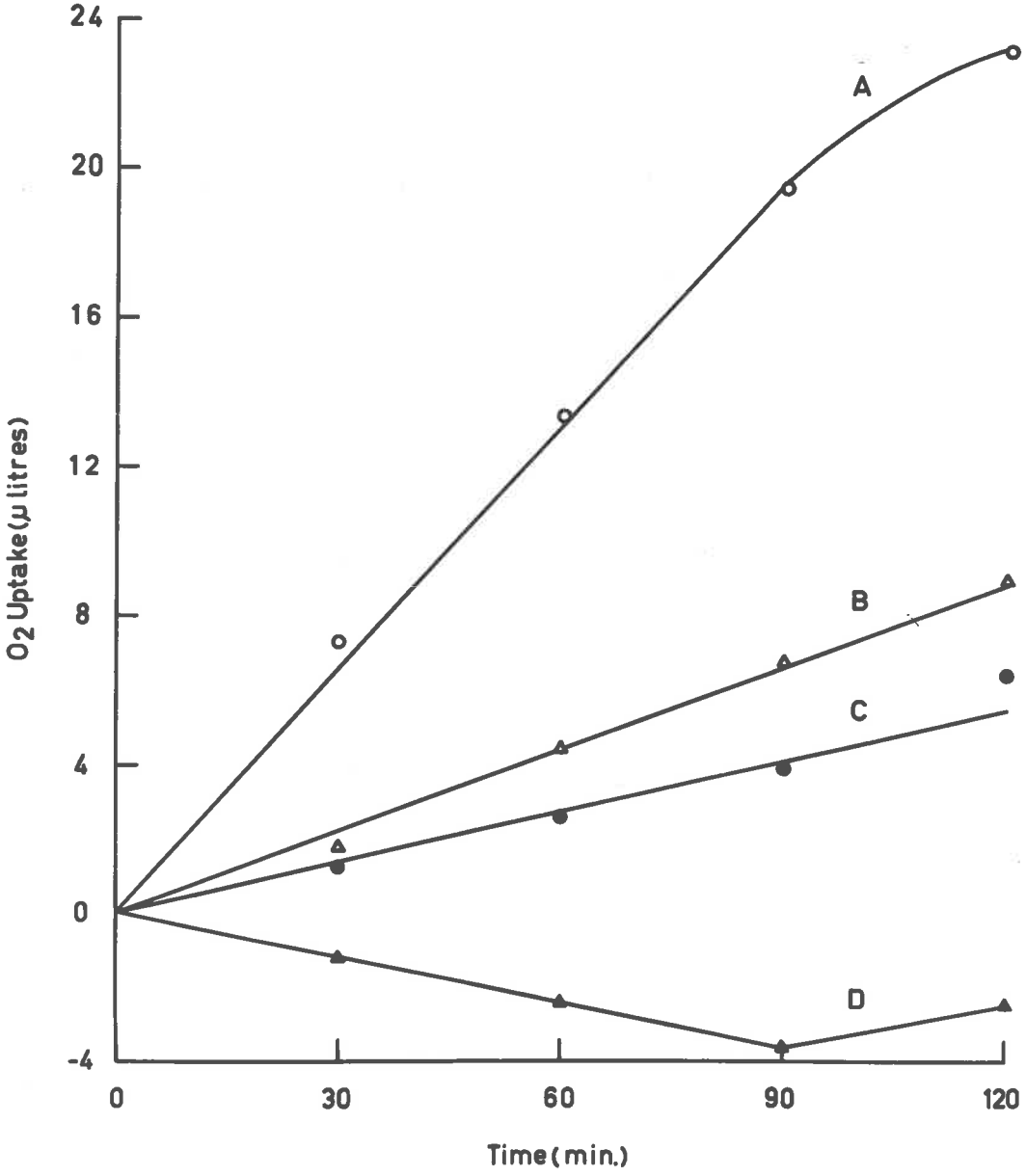
A. Glycollic acid + FMN

B. Glycollic acid

C. Glyoxylic acid + FMN

D. Glyoxylic acid

FIG. 7



were assayed for their ability to oxidise glycollic acid and glyoxylic acid. The particulate fraction consisted of the precipitate obtained after centrifuging the original brei (after screening through cheese cloth) at 36,000 g. (see p. 107). This precipitate was carefully suspended in 0.02 M-phosphate pH 7.8. Both the 0.2 M-phosphate pH 8.3, used in the original extraction and 0.02 M-phosphate used to dissolve the soluble protein or to suspend the particulate fraction, contained FMN at a concentration of 50 μM.

The corrected values for the uptake of oxygen by the soluble protein and by the particulate fraction of Oxalis shoots in the presence of glycollic acid and of glyoxylic acid are shown graphically in Fig. 8. The rate of oxygen uptake was considerably greater for the particulate fraction with glycollic acid, and with glyoxylic acid when compared with the soluble extract. Moreover, there was little difference in the rate of oxidation of glycollic acid, and of glyoxylic acid by the particulate fraction (Fig. 8.)

Since the particulate fraction of Oxalis shoots was more active towards the oxidation of glycollic acid and of glyoxylic acid, it was considered that isolation of this fraction in a medium containing sucrose (0.4 M) may preserve enzymic activity. In fact the reverse was the case; less activity towards the two oxidation reactions was observed when the particulate fraction was prepared

with 0.2 M-phosphate containing 0.4 M-sucrose.

(iv) The oxidation of glycollic acid and of glyoxylic acid catalysed by extracts of acetone powders of the particulate fraction of Oxalis shoots.

Because of the relatively low activity of all extracts from shoots, the manometric technique was not sufficiently sensitive. Spectrophotometric assay which would provide the necessary sensitivity, could not be used with the crude particulate preparation as used for manometry. Consequently, an acetone powder was prepared from this fraction (see p. 106) and this was extracted with either 0.02 M-phosphate or 0.1 M-phosphate (both pH 7.8, containing FMN, 50 μM) and the soluble protein was assayed for oxygen uptake in the presence of glycollic acid and glyoxylic acid. Preliminary experiments showed that a more active preparation was obtained by extracting with 0.1 M-phosphate.

The rate of oxygen uptake of protein extracted from acetone powders with glycollic acid and with glyoxylic acid is shown in Fig. 9. Activities were similar with both substrates as found with the particulate fraction before preparation of the acetone powder (Fig. 8). Clearly, the treatment of the particulate fraction with acetone and subsequent extraction of the powder did not destroy the activity of Oxalis protein.

Fig. 8. Rates of oxidation of glycollic acid and of glyoxylic acid catalysed by a soluble protein fraction and by a particulate fraction from *Oxalis* shoots

Extracts of soluble protein and a suspension of the particulate fraction of *Oxalis* shoots were prepared as described in the text. Each flask contained the appropriate *Oxalis* extract (1.8 ml. in 0.02M-phosphate, pH 7.8, containing FMN, 50 μM), catalase, KOH and substrate as previously described. Oxygen uptake figures were adjusted for changes in control flasks.

A. Oxidation of glyoxylic acid by the particulate fraction.
B. Oxidation of glycollic acid by the particulate fraction.
C. Oxidation of glycollic acid by soluble protein.
D. Oxidation of glyoxylic acid by soluble protein.

FIG. 8

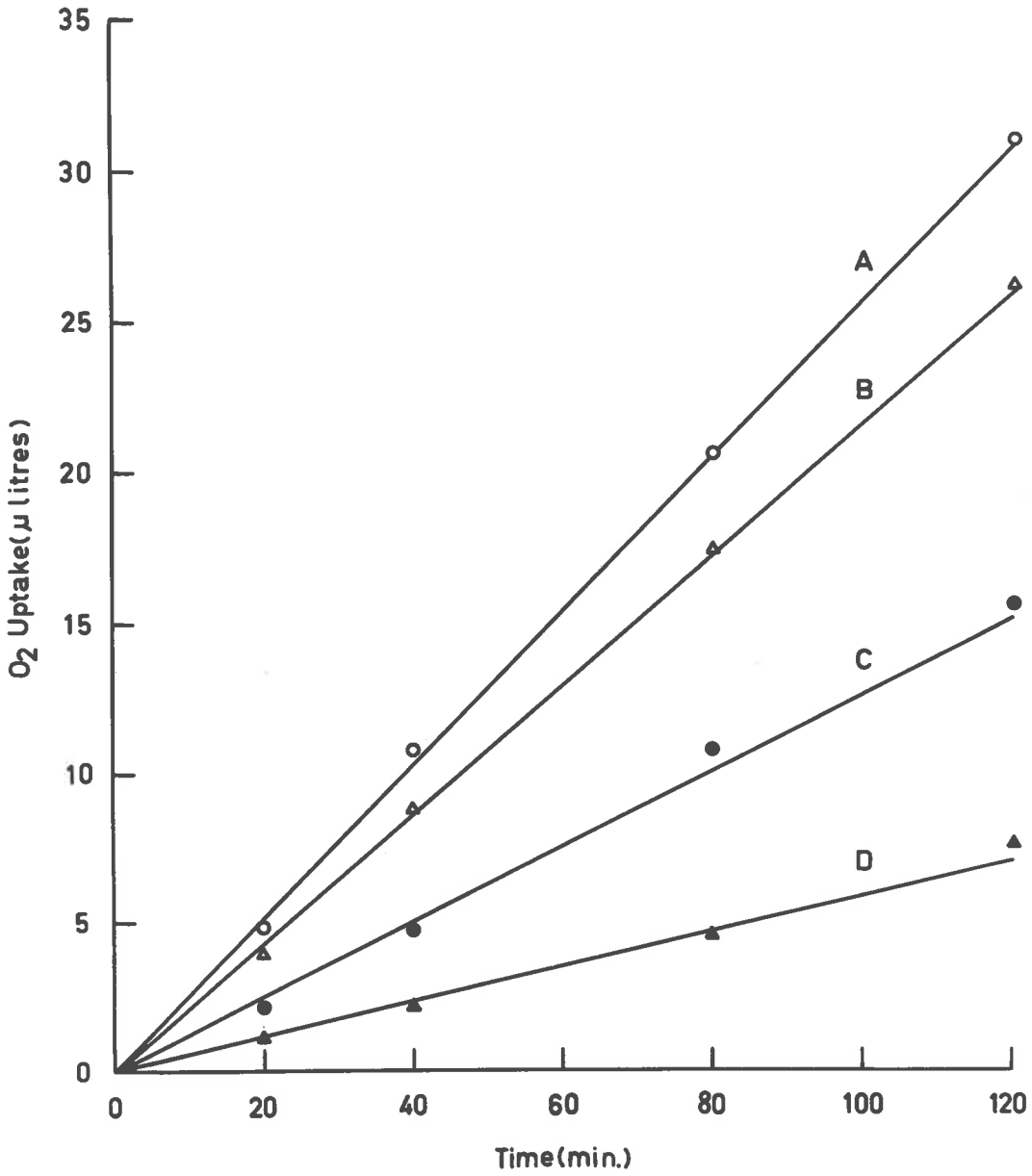

35
30
25
20
15
10
5
0
O_2 Uptake(μ litres)
A
B
C
D
0
20
40
60
80
100
120
Time(min.)

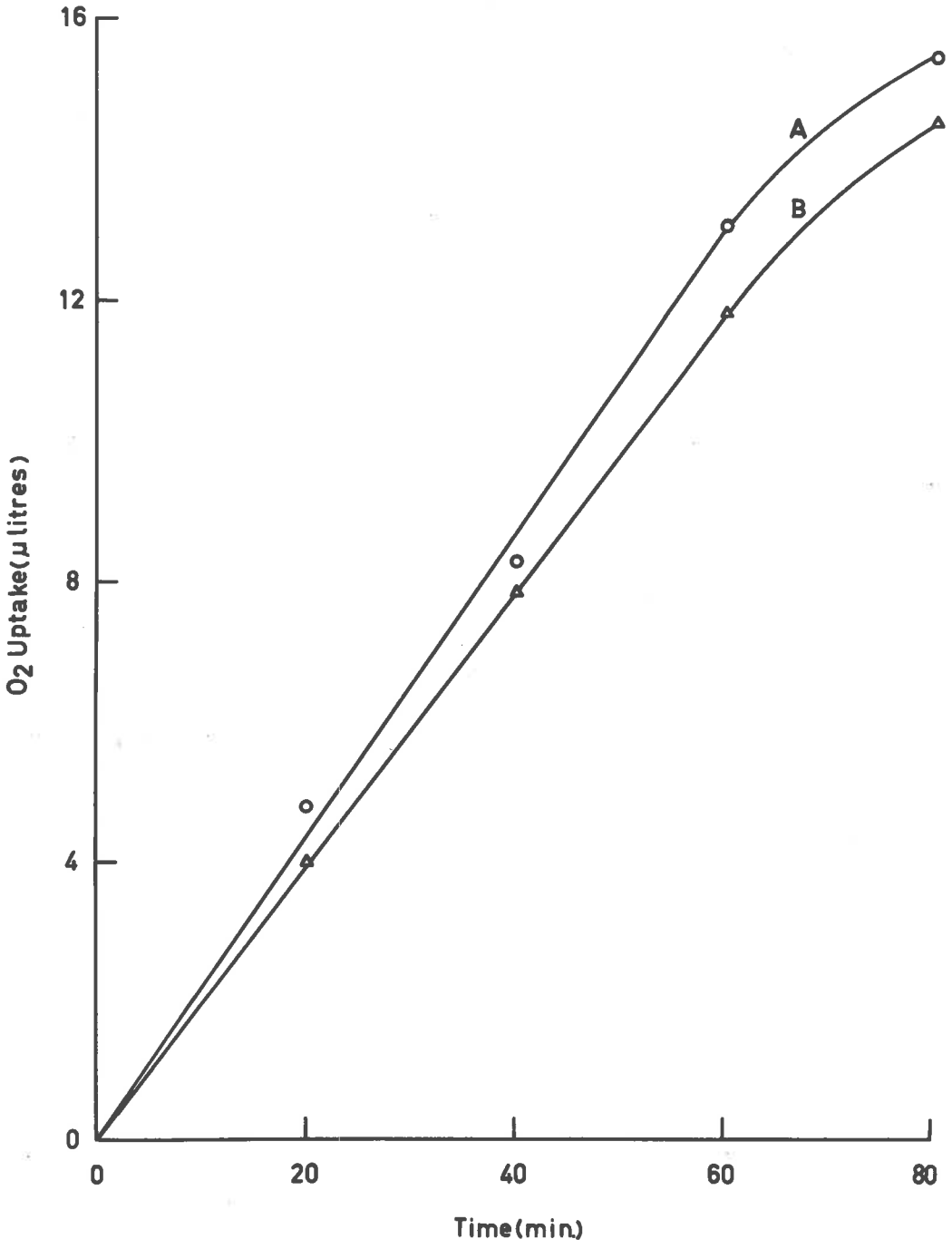
Fig. 9. The oxidation of glycollic acid and of glyoxylic acid catalysed by extracts of acetone powders of the particulate fraction of Oxalis shoots.

Acetone powders were prepared from the particulate fraction of Oxalis shoots and subsequently extracted with 0.1 M-phosphate, pH 7.8 containing FMN (50 μM). Portions (1.8 ml.) were assayed for oxidative activity in the presence of glycollic acid and of glyoxylic acid. Flasks contained Oxalis extract, catalase, KOH and substrate as previously described. Values plotted were adjusted for oxygen consumption in the absence of substrate.

A. Oxygen uptake in the presence of glycollic acid.

B. Oxygen uptake in the presence of glyoxylic acid.

FIG. 9



(b) Spectrophotometric assay of the oxidation of glycollic acid and of glyoxylic acid by extracts of acetone powders of the particulate fraction of Oxalis shoots.

The oxidation of glycollic acid and of glyoxylic acid by extracts prepared from acetone powders of white Oxalis shoots was demonstrated spectrophotometrically by the reduction of 2:6-dichlorophenolindophenol. No activity was obtained without the addition of FMN; higher activities could be obtained if FMN was present throughout the extraction procedure (see p.107). Reactants were added to duplicate cells and extinctions were measured at 600 mμ for 30 sec. in a Beckman DK-2 recording spectrophotometer before the addition of substrate.

Since oxalic acid was reported to be a strong inhibitor of the oxidation of glyoxylic acid by glycollate oxidase (Richardson & Tolbert, 1961a) a comparison of the rates of oxidation of glycollic acid, of glyoxylic acid, and of glyoxylic acid in the presence of oxalic acid was made.

The initial rates of oxidation of glycollic acid, of glyoxylic acid, and of glyoxylic acid in the presence of oxalic acid, as measured by reduction of 2:6-dichlorophenolindophenol, are shown in Fig. 10. The substrate (10 μmoles) was added as indicated by the arrow. In confirmation of the manometric assays (Fig. 8,9), the extract from the particulate fraction of Oxalis shoots oxidised

glycollic acid and glyoxylic acid at approximately equal rates. Moreover, the oxidation of glyoxylic acid by this extract was not inhibited by substrate concentrations of oxalic acid.

When NAD or NADP replaced 2:6-dichlorophenolindophenol in this assay system, no reduction of these nucleotides was detected.

(c) Identification of the products of oxidation of glycollic acid and of glyoxylic acid catalysed by extracts of acetone powders of the particulate fraction of Oxalis.

The products of oxidation of glycollic acid and of glyoxylic acid by extracts of Oxalis shoots were identified by radioautography after incubation with [1-^{14}C] glycollic acid or with [$^{14}C_2$] glyoxylic acid. Reaction mixtures contained Oxalis extract (1.0 ml. at pH 8.0, containing 50 μM FMN), glycollic acid or glyoxylic acid (10 μmoles) and [1-^{14}C] glycollic acid (approx. 2.5 μcuries) or [$^{14}C_2$] glyoxylic acid (approx. 2.6 μcuries). Mixtures were incubated at 30° and the reactions were stopped at 0 and 60 min., by the addition of HCl (final concn. 2.5-N). 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 paper chromatograms and these were developed in phenol saturated with water at 24°. Losses of glyoxylic acid during chromatography in this solvent are small

Fig. 10. Spectrophotometric assay of the oxidation of glycollic acid, of glyoxylic acid, and of glyoxylic acid in the presence of oxalic acid, by extracts of Oxalis.

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 extinctions at 600 mμ followed for 30 sec. in a Beckman DK-2 recording spectrophotometer before the addition of substrate. The decrease in extinction at 600 mμ consequent upon the addition of 10 μmoles of glyoxylic acid (A), of 10μmoles of glycollic acid (B) and of 10 μmoles of glyoxylic acid after the addition of 10 μmoles of oxalic acid to the appropriate assay cell (C) were recorded for 4 min. The time of addition of substrate in each case is indicated by the arrow.

FIG.10

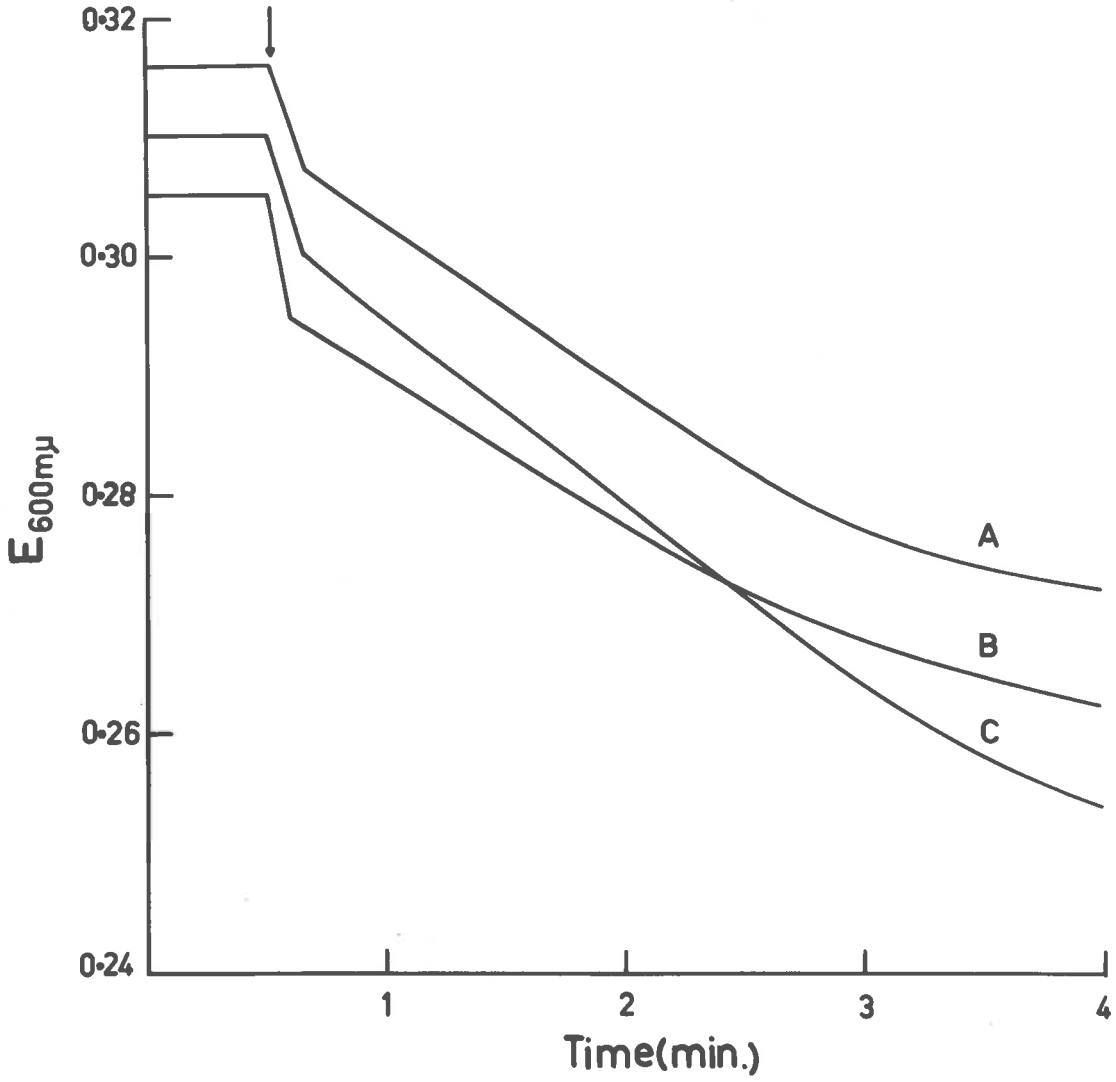


Table 10. Identification of the products of oxidation of glycollic acid and of glyoxylic acid catalysed by extracts of acetone powders of the particulate fraction of Oxalis shoots.

Acetone powders of the particulate fraction of Oxalis shoots were extracted with 0.1 M-phosphate, pH 8.0 containing 50 μM FMN. Portions of this extract (1.0 ml.) were incubated with glycollic acid or glyoxylic acid (10 μmoles) and [1-^{14}C] glycollic acid (approx. 2.5 μcuries) or [$^{14}C_2$] glyoxylic acid (approx. 2.6 μ curies). 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, in glyoxylic acid and in oxalic acid was determined. The results are expressed as a percentage of the total radioactivity recovered from appropriate chromatograms.

(Kearney & Tolbert, 1962).

Compounds containing ^{14}C were detected by radioautography and the amount of radioisotope in glycollic acid, in glyoxylic acid and in oxalic acid was determined by a liquid scintillation technique as previously described (see p. 52). The ^{14}C activity in each compound was expressed as a percentage of the total radioactivity located on the appropriate chromatogram. The results (Table 10) 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 extracts (Table 10) resulted in substantial incorporation of ^{14}C into oxalic acid and a small incorporation of ^{14}C into glycollic acid. The results in Table 10 (zero time incubations) also showed contamination of the ^{14}C starting material (from 1.7% to 3.5%). Contaminations of the same order have been noted previously for [$^{14}C_2$] glyoxylic acid (Kearney & Tolbert, 1962).

2. Reduction of oxalic acid by cell-free extracts of Oxalis shoots.

Since ^{14}C was detected in glycollic acid and in glyoxylic acid after the administration of [$^{14}C_2$] oxalic acid (Table 4, p. 68) it appeared that oxalic acid was not metabolically inert in Oxalis shoots. The reduction of oxalic acid by an extract of an acetone powder of the particulate fraction of Oxalis was demonstrated spectrophotometrically.

TABLE 10

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.

The following components in an assay of oxalic acid reduction were contained in a volume of 3.15 ml. : phosphate buffer (150 μmoles at pH 7.4), cysteine (5 μmoles at pH 7.0), ATP (10 μmoles), $MgCl_2$ (10 μmoles), CoA (approx. 0.7 μmoles), $NADPH_2$ (0.6 μmoles), Oxalis extract (0.5 ml. at pH 7.4), oxalic acid (10 μmoles) and succinic acid (10 μmoles). All reactants except oxalic acid and succinic acid were added to duplicate cells. The addition of oxalic acid alone to the assay cell did not cause a decrease in ε_{340}. However, on addition of succinic acid to both cells, the presence of oxalic acid resulted in a linear decrease in extinction at 340 mμ (Fig. 11). 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 P. oxalaticus (Quayle, Keech & Taylor, 1961).

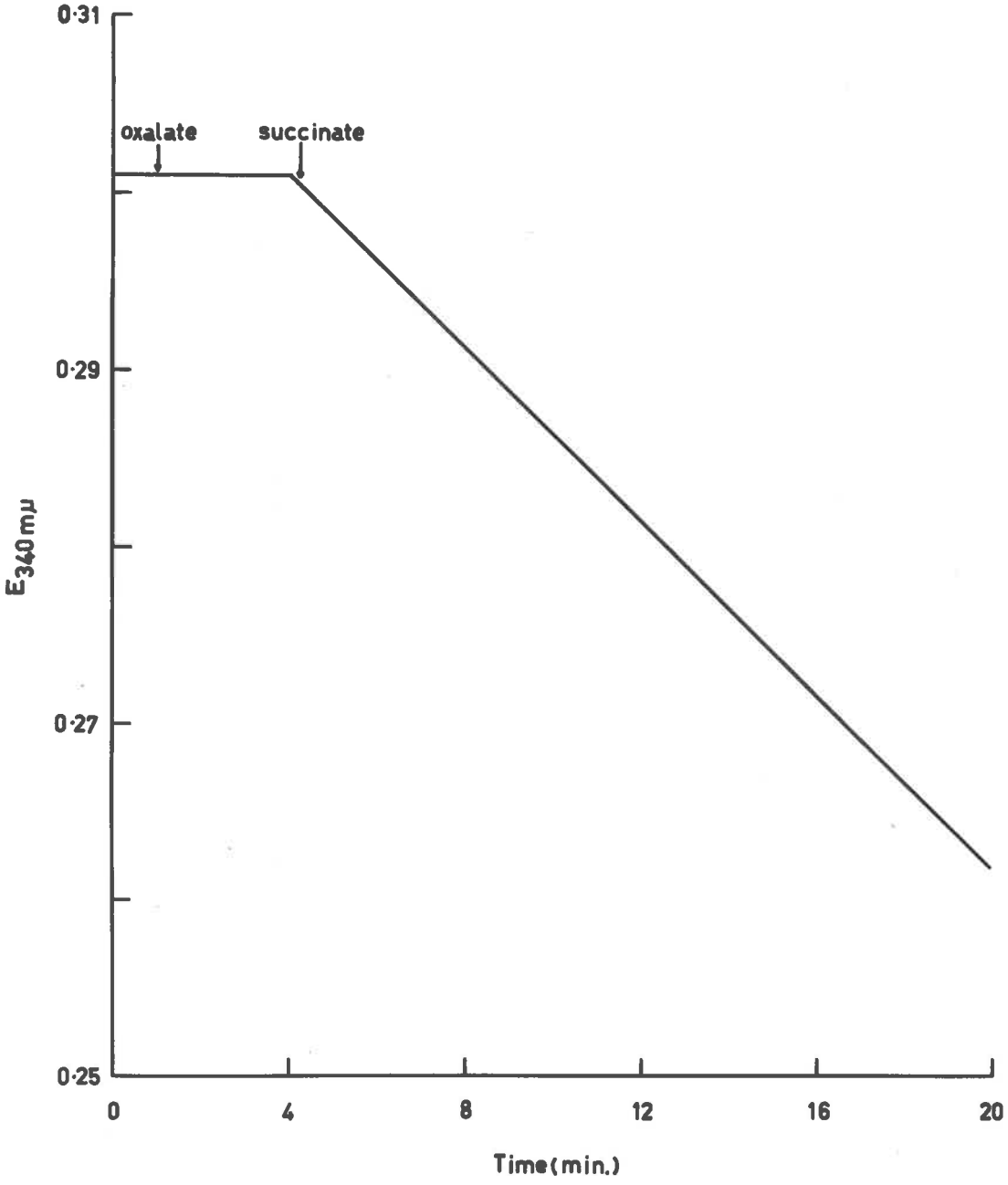
3. Reduction of glyoxylic acid by cell-free extracts of Oxalis shoots.

The application of [$^{14}C_2$] glyoxylic acid to Oxalis shoots resulted in the incorporation of considerable radioisotope into glycollic acid (Table 4, p. 68). In confirmation of these results, extracts from acetone powders of the particulate fraction

Fig. 11. Reduction of oxalic acid catalysed by an extract of an acetone powder of the particulate fraction of *Oxalis* shoots.

The complete system contained in 3.15 ml., 150 μmoles of sodium phosphate, pH 7.4, 0.5 μmoles of CoA, 0.6 μmoles of $NADPH_2$ and 0.5 ml. of *Oxalis* extract, pH 7.4. Oxalic acid (10 μmoles) or succinic acid (10 μmoles) were added as indicated. Decreases in extinction at 340 mμ were recorded with a Beckman DK-2 recording spectrophotometer.

FIG.11


0·31
0·29
0·27
0·25
oxalate
succinate
$E_{340m\mu}$
0
4
8
12
16
20
Time(min.)

of Oxalis shoots readily oxidised $NADPH_2$ in the presence of glyoxylic acid. The glyoxylic acid - dependant oxidation of $NADH_2$ was also demonstrated, but the rate of reaction with $NADH_2$ was only 5% of that with $NADPH_2$.

Glyoxylate reductase activity was assayed spectrophotometrically with the following reaction mixture : phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (25 μmoles), 0.5 ml. of Oxalis extract (pH 7.4), glyoxylic acid (10 μmoles, pH 7.5), and $NADH_2$ or $NADPH_2$ (0.6 μmoles). The reaction was started by the addition of glyoxylic acid. Decreases in extinction at 340 mμ consequent upon the addition of substrate were recorded. The relative rates of oxidation of $NADH_2$ and of $NADPH_2$ by Oxalis extracts in the presence of glyoxylic acid are shown in Fig. 12.

The identity of glycollic acid as the product of glyoxylic acid reduction was confirmed by the use of [$^{14}C_2$] glyoxylic acid. Reaction mixtures contained in a total volume of 3.8 ml. : phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (25 μmoles), Oxalis extract (1.0 ml., pH 7.4), glyoxylic acid (20 μmoles, pH 7.5), [$^{14}C_2$] glyoxylic acid (approx. 1.6 μcuries) and $NADPH_2$ (1.0 μmole). The reaction mixtures were incubated at 30° and stopped at 0 and 60 min. by the addition of HCl (final concn. 2.5 N). After removal of protein by centrifuging the supernatants were dried on a rotary film evaporator. Residues were dissolved in 0.5 ml. of 20% (v/v) ethanol. Portions (0.05 ml.) were applied to chromatograms and

these were developed with a solvent of phenol saturated with water at 24°. Compounds containing ^{14}C were identified by radioautography and the amount of radioactivity in glyoxylic acid, in glycollic acid and in oxalic acid was determined by counting on paper as previously described. The results (Table 11) are expressed as a percentage of the total ^{14}C activity located on the chromatograms.

4. Isocitrate lyase activity in cell-free extracts of Oxalis shoots.

Experiments in vivo with non-photosynthetic Oxalis shoots indicated that glycollic acid formation was not associated with the cleavage of hexose or pentose phosphates; in photosynthetic tissues these sugars are associated with the synthesis of glycollic acid. The substantial incorporation of radioisotope from [$^{14}C_2$] glyoxylic acid into glycollic acid (Table 4, p.68) suggested that glyoxylate reductase was active in Oxalis. The in vitro activity of this enzyme was readily demonstrated with extracts from shoots (p.123).

The above results suggested that, in Oxalis, there was an alternate pathway for the synthesis of glyoxylic acid not involving the oxidation of glycollic acid. Although isocitrate

Fig. 12. The oxidation of $NADH_2$ and of $NADPH_2$ by extracts of Oxalis shoots in the presence of glyoxylic acid.

The glyoxylic acid-dependant oxidation of $NADH_2$ and of $NADPH_2$ was demonstrated with cell-free extracts of Oxalis shoots. The components in the reaction mixture for the assay of glyoxylate reductase activity were as follows : phosphate buffer (200 µmoles at pH 7.4), $MgCl_2$ (25 µmoles), Oxalis extract (0.5 ml. at pH 7.4), glyoxylic acid (10 µmoles) and $NADH_2$ or $NADPH_2$ (0.6 µmoles). The reaction was started by the addition of glyoxylic acid as indicated by the arrow :

A. Oxidation of $NADH_2$.

B. Oxidation of $NADPH_2$.

FIG.12

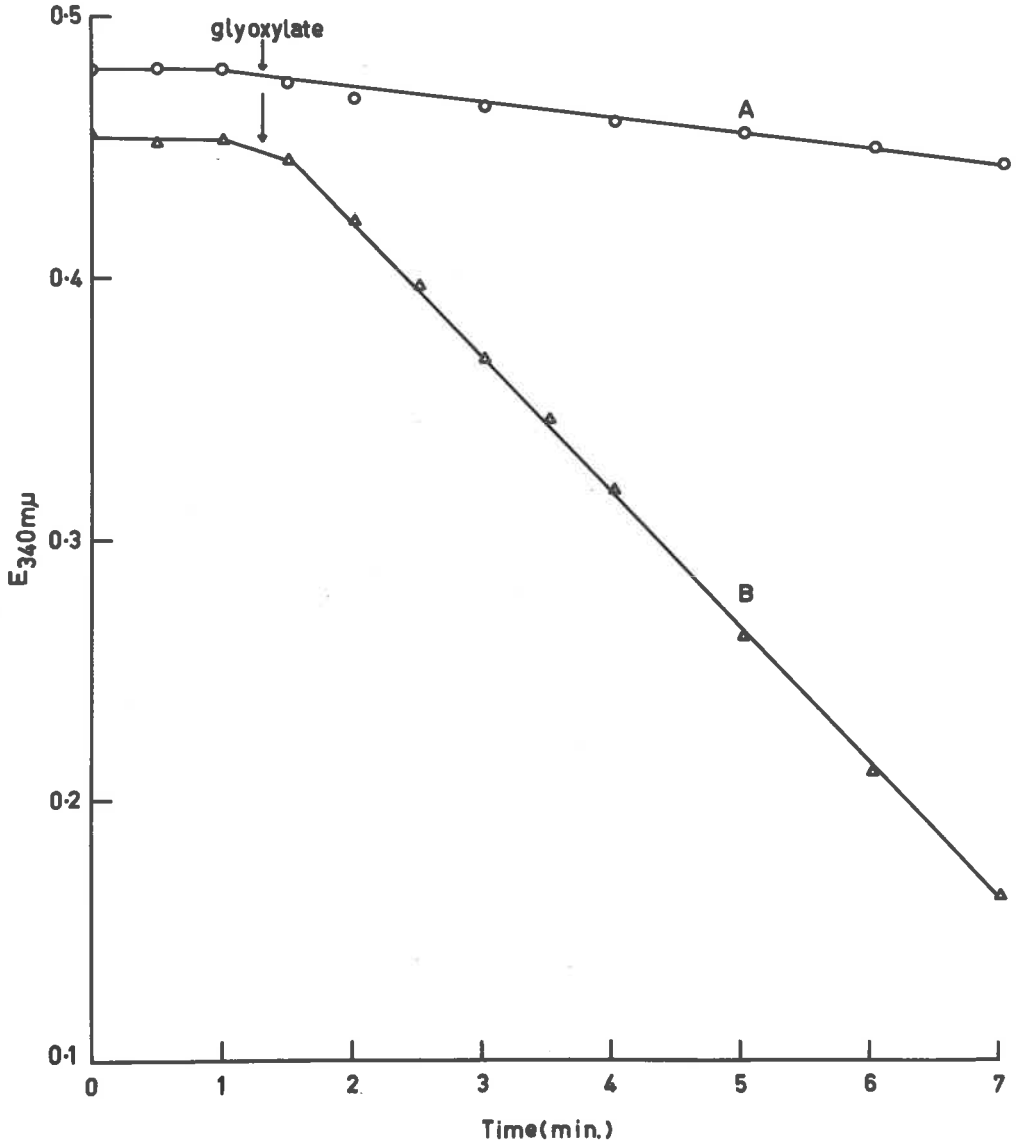


Table 11. Identification of glycollic acid as the product of reduction of glyoxylic acid catalysed by extracts of *Oxalis* shoots.

The reaction mixture contained in 3.8 ml., phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (25 μmoles), *Oxalis* extract (1.0 ml., at pH 7.4), glyoxylic acid (20 μmoles at pH 7.5), [$^{14}C_2$] glyoxylic acid (approx. 1.6 μcuries) and $NADPH_2$ (1.0 μmole). Reactions were stopped at 0 and 60 min. and the products which contained ^{14}C were detected by radioautography. The radioactivity in individual compounds was expressed as a percentage of the total activity located on chromatograms.

TABLE 11

Substrate	Time (min.)	% ^{14}C activity in organic acids separated by paper chromatography			
		Origin*	Glycollic acid	Glyoxylic acid	Oxalic acid
[$^{14}C_2$] glyoxylic acid	0	2.8	4.2	89.8	3.2
[$^{14}C_2$] glyoxylic acid	60	3.6	31.1	59.4	5.9

* Material remaining at the origin after development of chromatograms.

lyase activity in plants was considered to be confined to those tissues converting fat to carbohydrate (Carpenter & Beevers, 1959) the distribution of ^{14}C after the administration of [2-^{14}C] glucose, $^{14}CO_2$ and [1,5-$^{14}C_2$] isocitric acid to Oxalis shoots (see Table 4, p. 68 and Table 9, p. 90) was consistent with the formation of glyoxylic and succinic acid from isocitric acid.

(a) Keto acid formation from isocitric acid catalysed by extracts of Oxalis shoots.

Keto acid formation from DL-isocitric acid catalysed by Oxalis extracts, was measured spectrophotometrically by following the increase in extinction at 252 mμ due to the formation of semicarbazone (Olsen, 1959). The reaction mixture contained, phosphate buffer (200 μmoles at pH 6.0), $MgSO_4$ (10 μmoles), GSH (5 μmoles), semicarbazide (40 μmoles) and DL-isocitric acid (5 μmoles). All reactants were adjusted to pH 6.0 immediately before use; it was also necessary to prepare fresh solutions of GSH, semicarbazide and DL-isocitric acid (by hydrolysis of lactone) each day. Semicarbazide was omitted from the reference cell and isocitric acid from the control. The reaction was started by the addition of 0.25 ml. of Oxalis extract (pH 7.0); the final volume was 3.0 ml.

Extinctions of both the control cell and of the assay cell were recorded at 252 mμ (Fig. 13). Initial increases in extinction at 252 mμ were observed in the control cell and in the

assay cell for approximately 5 min. after the start of the reaction. Thereafter, ϵ_{252} remained constant in the control cell but increased linearly in the assay cell. In a typical assay (Fig. 13), the increase in extinction due to keto acid formation from isocitric acid was 0.084/mg. of protein/min.

(b) Keto acid formation from isocitric acid catalysed by extracts of Oxalis shoots after removal of nicotinamide nucleotide co-enzymes from the extract.

The increase in extinction at 252 mμ observed in the above assay system could have been due to the formation of α-oxoglutaric acid from isocitric acid catalysed by isocitrate dehydrogenase. In order to minimise this possibility, Oxalis extracts were treated with 5 mg./ml. of Norite SX-2 charcoal, to remove nicotinamide nucleotide co-enzymes. 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 extinction due to semicarbazone formation was 0.055/mg. of protein/min.

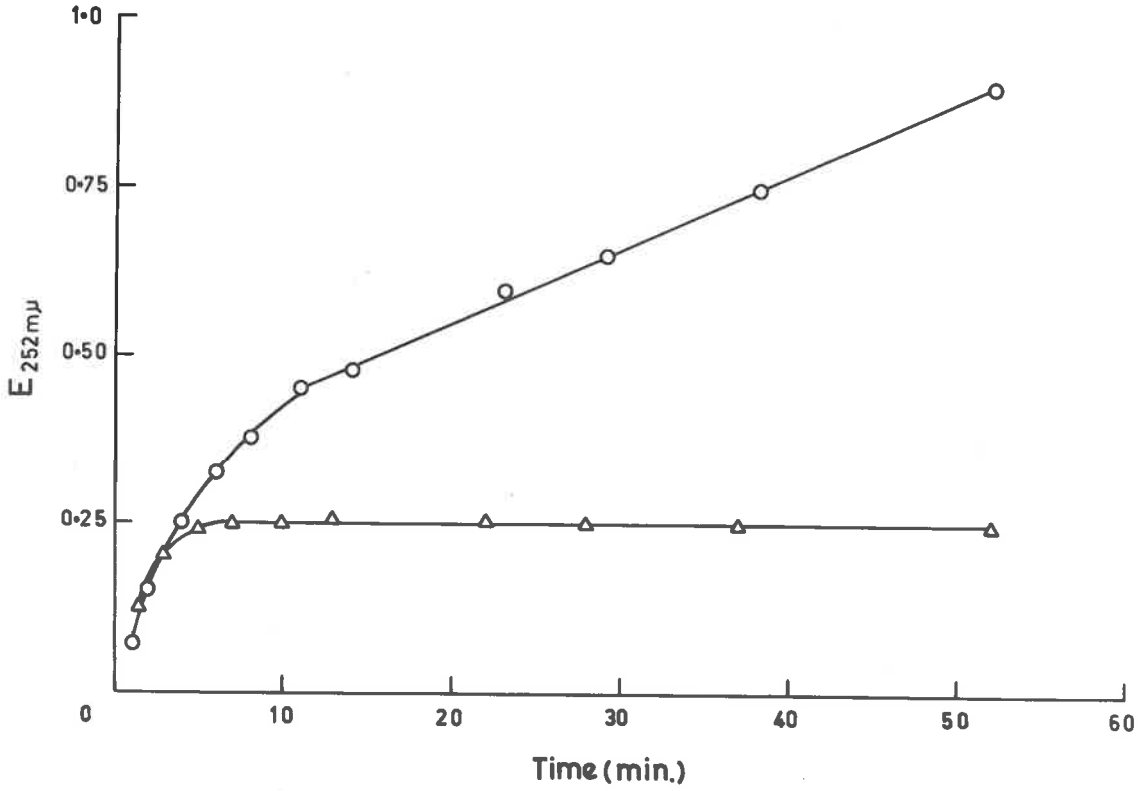
(c) Characterisation of keto acid (or acids) formed from isocitric acid by the spectrum of 2:4-dinitrophenylhydrazone derivatives.

Fig. 13. Keto acid formation from DL-isocitric acid catalysed by extracts of acetone powders from the particulate fraction of Oxalis shoots.

The complete system (O) contained in 3 ml., phosphate buffer (200 μmoles at pH 6.0), $MgSO_4$ (10 μmoles), GSH (5 μmoles), semicarbazide (40 μmoles), Oxalis extract (0.25 ml. at pH 7.0) and DL-isocitric acid (5 μmoles). All reactants were adjusted to pH 6.0 before use. Keto acid formation was measured in an Optica CF-4 spectrophotometer as the rate of change in extinction at 252 mμ 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 (Δ), DL-isocitric acid was omitted.

FIG.13



Total keto acids were estimated (Friedman, 1955) from an incubation mixture consisting of *Oxalis* extract (5.0 ml. in 0.1 M-phosphate at pH 7.5), DL-isocitric acid (40 μmoles at pH 7.5), cysteine (25 μmoles at pH 7.5) and $MgSO_4$ (50 μmoles at pH 7.5) in a total volume of 8.5 ml. The reaction was started by the addition of substrate and the incubation was carried out at 30°.

At time 0, 60 and 120 min., 2 ml. portions of the reaction mixture were removed into 2 ml. of 10% (w/v) 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 2N-HCl. A reference incubation mixture contained trichloroacetic acid and 0.1 M-phosphate at pH 7.5.

As glyoxylic acid combines with cysteine (particularly in the absence of a trapping agent) and this complex is not hydrolised by 2N-HCl at room temperature, the portions to be assayed were autoclaved with 2:4-dinitrophenylhydrazine at 15 lbs. for 15 min. (Rao & Ramakrishnan, 1962). Subsequent procedures for the isolation of 2:4-dinitrophenylhydrazones were carried out 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 extinction at 435 mμ. The spectra of the 2:4-dinitrophenylhydrazones obtained from the incubation

mixture are presented in Fig. 14 (A,B,C.).

The spectra of the 2:4-dinitrophenylhydrazone derivatives of α-oxoglutaric acid and of glyoxylic acid were also determined. Incubations with 0.3 μmoles of α-oxoglutaric acid, with 0.3 μmoles of glyoxylic acid and with a mixture of the two acids were carried out for 120 min., exactly as for the enzymic reaction described above, except that 0.1 M-phosphate, pH 7.5 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. 14 (D,E,F.).

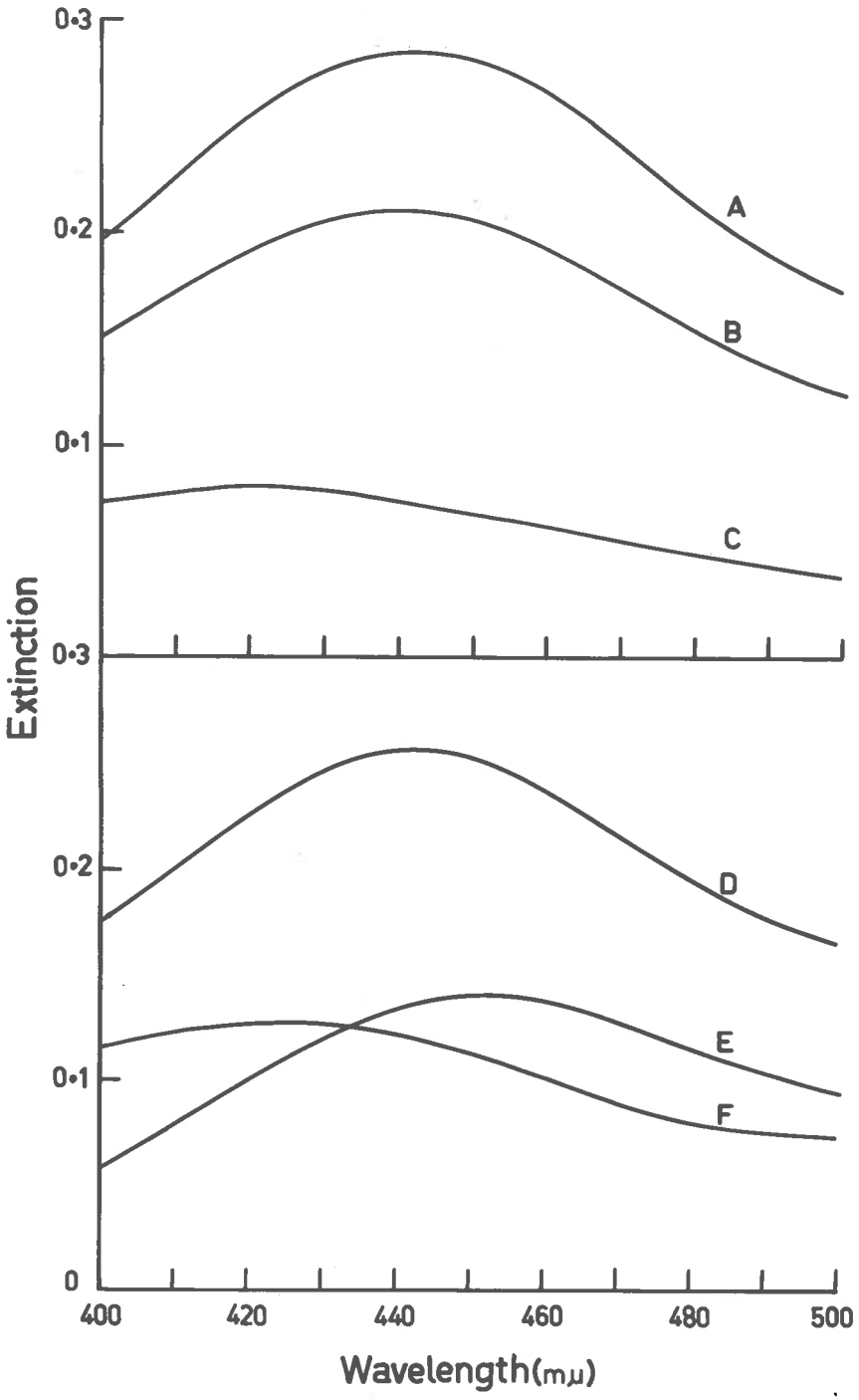
Comparison of the spectra of enzymically formed 2:4-dinitrophenylhydrazones (Fig. 14, A,B,C) and those of the reference keto acids (Fig. 14, D,E,F) clearly showed that glyoxylic acid had been formed from isocitric acid. The spectra of the 2:4-dinitrophenylhydrazones obtained at zero time (enzymic reaction, curve C) and with α-oxoglutaric acid (reference mixture, curve F) were 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 extinction at 252 mμ in the semicarbazone assay in the absence of isocitric acid (Fig. 13). The presence of α-oxoglutaric acid in Oxalis extracts was demonstrated subsequently by paper chromatography.

Although the glyoxylic acid derivative by itself had an extinction maximum at 450 mμ under the conditions used (Fig. 14 E),

Fig. 14. 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-phosphate at pH 7.5), cysteine (25 μmoles), $MgSO_4$ (50 μmoles) and DL-isocitric acid (40 μmoles). 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.

FIG.14


0·3
0·2
0·1
0·3
0·2
0·1
0
Extinction
A
B
C
D
E
F
400
420
440
460
480
500
Wavelength(mμ)

the presence of the 2:4-dinitrophenylhydrazone of α-oxoglutaric acid resulted in an extinction maximum at 440 mμ (Fig. 14, D). The 2:4-dinitrophenylhydrazone derivatives prepared from enzymically formed keto acids (after 60 min. and after 120 min.), had maxima at approximately 440 mμ (Fig. 14, A, B).

(d) The stoicheiometry of the reaction catalysed by isocitrate lyase from extracts of Oxalis shoots.

(i) Measurement of glyoxylic acid formed from isocitric acid.

The formation of glyoxylic acid from isocitric acid, catalysed by Oxalis extracts, was measured by the semicarbazide method in a reaction mixture as previously described (p. 129). The reaction was stopped after 53 min. (see Fig. 13) by the addition of HCl (final concentration 1 N); the reaction mixture was transferred to small centrifuge tubes and denatured protein removed by centrifuging (20,000 g., 10 min.). The supernatant was adjusted to pH 6.5 - 7.0 with NaOH and portions assayed for succinic acid.

(ii) Measurement of succinic acid formed from isocitric 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 reaction mixture contained phosphate buffer (200 μmoles at pH 7.4), potassium cyanide (4 μmoles at pH 7.5), ferricytochrome c (0.5 μmoles) and 0.1 ml. of a preparation of succinate dehydrogenase. The portions to be assayed for succinic acid contained GSH; this was oxidised by aerating these samples vigourously 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. The reference cuvette contained no ferricytochrome c. No increase in extinction at 550 mμ 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. (Fig. 15).

The amount of glyoxylic acid formed from isocitric acid in the above experiment was calculated by using ε_{252} mμ = 12.4 x 10^3 (Olsen, 1959). The increase in extinction due to glyoxylic acid formation from isocitric acid (assay-control) was 0.659 when the reaction was stopped after 53 min. The volume of the reaction mixture was 2.5 ml. The amount of glyoxylic acid formed was calculated as :-

$$\frac{\Delta E \times v}{12.4} \text{ μmoles,}$$

where ΔE is the change in extinction at 252 mμ (corrected for the changes in the control cuvette) and v is the final volume of the reaction mixture. In this experiment the amount of glyoxylic acid formed was :-

$$\frac{0.659}{12.4} \times 2.5 \text{ μmoles}$$

$$= \underline{0.13 \text{ μmoles}} \text{ of glyoxylic acid}$$

The amount of succinic acid formed from isocitric acid was calculated in the same way as described for glyoxylic acid. For ferricytochrome c a value of ε (reduced minus oxidised) = 21.1×10^3 was used (Van Gelder & Slater, 1962). The total increase in extinction at 550 mμ due to the reduction of ferricytochrome c in the 0.25 ml. portion assayed for succinic acid was 0.129. The total volume of this reaction mixture was 2.8 ml. Allowing for volume changes and for the reduction of two molecules of ferricytochrome c per molecule of succinic acid oxidised, the total amount of succinic acid formed from isocitric acid was calculated as :-

$$\frac{0.129 \times 2.8}{2 \times 21.1} \times \frac{3.0}{0.25} \text{ μ moles}$$

$$= \underline{0.10 \text{ μmoles}} \text{ of succinic acid.}$$

These results are in agreement with the stoicheiometry of the reaction catalysed by isocitrate lyase.

(e) The reversal of isocitrate lyase activity catalysed by extracts of Oxalis shoots.

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 extinction at 340 mμ thus :

$$\text{Glyoxylic acid} + \text{Succinic acid} \xrightarrow[MgCl_2]{\text{Isocitrate lyase}} \text{Isocitric acid} \quad \ldots\ldots (1)$$

and

$$\text{Isocitric acid} + \text{NADP} \xrightarrow[MgCl_2]{\text{Isocitrate dehydrogenase}} \alpha\text{-Oxoglutaric acid} + NADPH_2 + CO_2 \quad (2)$$

In the presence of isocitric acid, $MgCl_2$, Oxalis extract and NADP, there was a rapid increase in extinction at 340 mμ (see next section). 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.

A reaction mixture of phosphate buffer (200 μmoles at pH 8.0), $MgCl_2$ (10 μmoles), NADP (0.5 μmoles) and Oxalis extract

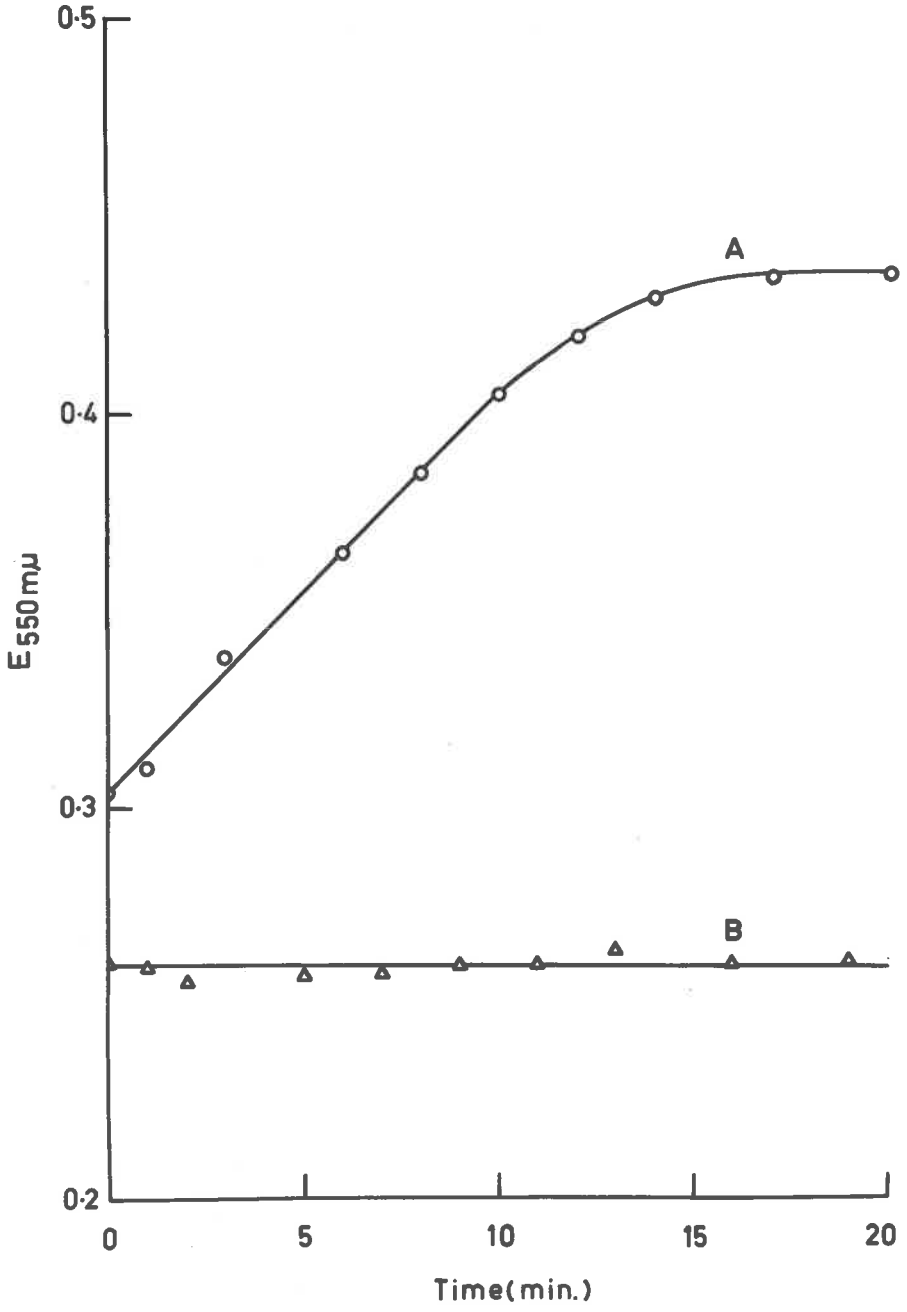
Fig. 15. Assay for succinic acid by the specific reduction of ferricytochrome c catalysed by succinate dehydrogenase.

Succinic acid formed from isocitric acid in reaction mixtures containing Oxalis extracts was estimated by the specific reduction of ferricytochrome c catalysed by succinate dehydrogenase. The reaction mixture contained in a volume of 2.8 ml., phosphate buffer (200 μmoles at pH 7.4) potassium cyanide (4 μmoles at pH 7.5), ferricytochrome c (0.5 μmoles) and 0.1 ml. of a preparation of succinate dehydrogenase. The reaction was started by the addition of 0.25 ml. portions of a reaction mixture which had been assayed for the formation of glyoxylic acid from isocitric acid catalysed by isocitrate lyase of Oxalis extracts (see p.135).

A. Reduction of ferricytochrome c by sample from assay (with isocitric acid) mixture.

B. Reduction of ferricytochrome c by sample from control (without isocitric acid) mixture.

FIG.15



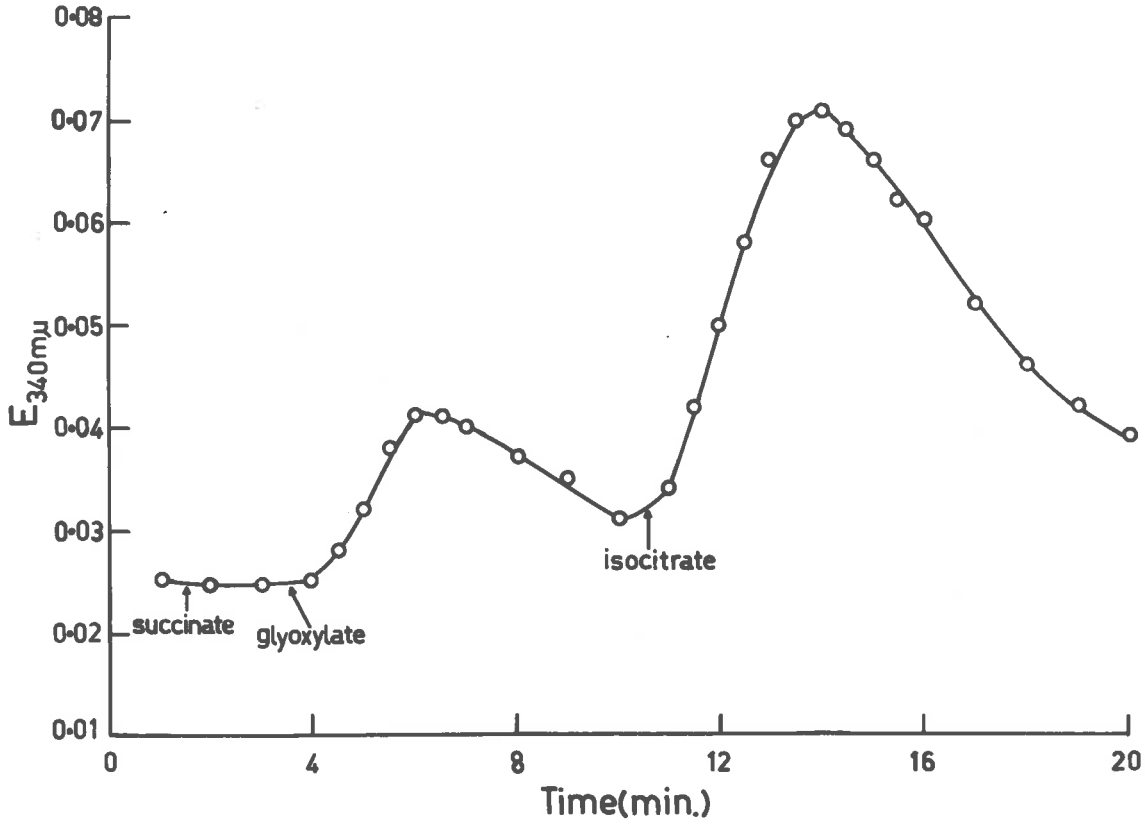
(0.5 ml. at pH 8.0) was set up in duplicate cells. Succinic acid (10 μmoles) and glyoxylic acid were added to the assay cell as shown in Fig. 16. The final volume of the reaction mixture was 2.85 ml. Extinctions at 340 mμ were recorded at regular intervals. There was no increase in extinction on the addition of succinic acid, but there was a slow increase in E 340 mμ after the subsequent addition of glyoxylic acid to the assay cell. After a further 2 min. there was a steady decrease in extinction. On the addition of isocitric acid to the assay cell (approx. 1 μmole) there was a further increase in E 340 mμ for approximately 2 min., and then a decrease, in a pattern similar to that obtained on the addition of succinic acid and glyoxylic acid. Thus, although NADP was reduced initially, the $NADPH_2$ so formed was either re-oxidised or rapidly destroyed. When the Oxalis extract was left at 25° for 3 hr. and the above assay was repeated, there was a slow steady increase in extinction at 340 mμ.

These results suggested that it was possible to demonstrate the reversibility of isocitrate lyase activity in Oxalis extracts. However, the presence of glyoxylate reductase activity also present in extracts (p. 123), masked the assay by oxidising $NADPH_2$ as soon as it was formed from NADP in the isocitrate dehydrogenase reaction.

Fig. 16. Reversal of isocitrate lyase activity catalysed by extracts of *Oxalis* shoots.

The reversal of isocitrate lyase activity catalysed by extracts of *Oxalis* was assayed in a coupled reaction in which the oxidation of isocitric acid formed from the condensation of glyoxylic acid and succinic acid was followed by increases in extinction at 340 mμ consequent upon the reduction of NADP. The complete system contained in 2.85 ml., phosphate buffer (200 μmoles at pH 8.0), $MgCl_2$ (10 μmoles), NADP (0.5 μmoles) and 0.5 ml. of *Oxalis* extract (pH 8.0). Succinic acid (10 μmoles), glyoxylic acid (10 μmoles) and 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 extinction at 340 mμ due to the reduction of NADP represented a measure of isocitrate lyase reversal. Decrease in ε_{340} mμ was due to glyoxylate reductase activity in the *Oxalis* extract. Extinctions at 340 mμ were recorded at regular intervals in an Optica CF-4 spectrophotometer.

FIG.16

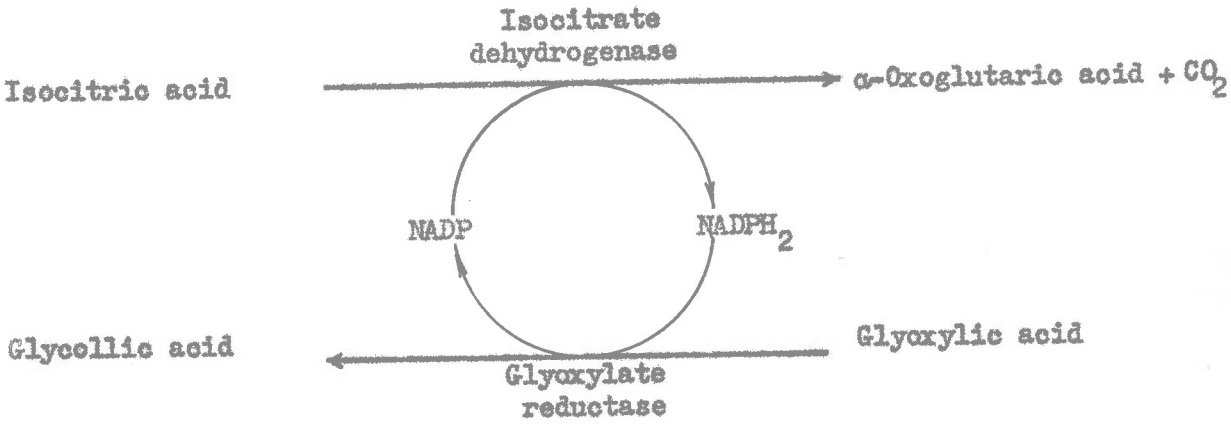

0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
$E_{340m\mu}$
succinate
glyoxylate
isocitrate
0
4
8
12
16
20
Time(min.)

5. Isocitrate dehydrogenase activity in cell-free extracts of Oxalis shoots

Since Oxalis shoots contained isocitrate lyase activity, it was necessary to determine whether isocitric acid was compulsorily cleaved to glyoxylic acid and succinic acid by these tissues or if isocitric acid was also oxidised to α-oxoglutaric acid. The administration of [1,5-$^{14}C_2$] isocitric acid to Oxalis shoots indicated that isocitrate dehydrogenase activity was present, since considerable radioisotope was located in α-oxoglutaric acid (Table 9, p. 90).

The presence of isocitrate dehydrogenase in Oxalis extracts was demonstrated spectrophotometrically. Cells contained phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (25 μmoles), NADP (0.5 μmoles) and Oxalis extract (0.5 ml. at pH 7.4). Isocitric acid (5 μmoles at pH 7.0) was added to the assay cell to start the reaction and extinctions at 340 mμ were recorded (Fig. 17). A steady increase in E 340 mμ was observed. In this experiment there was no subsequent decrease in extinction at 340 mμ as had been observed in the demonstration of isocitrate lyase reversal (Fig. 16). However, on addition of glyoxylic acid (10 μmoles) to the assay cell after 14 min., there was an immediate decrease in extinction at 340 mμ, due to glyoxylate reductase in the Oxalis extract.

The reactions may be summarised as follows :



The spectrophotometric assay of the two reactions is shown in Fig. 17.

That α-oxoglutaric acid was the product of isocitric acid oxidation was demonstrated by the chromatography of 2:4-dinitrophenylhydrazones isolated from the following reaction mixture : phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (50 μmoles), NADP (1 μmole), Oxalis extract (1.0 ml. at pH 7.4) and isocitric acid (20 μmoles at pH 7.0). The final volume was 4.6 ml. Duplicate reaction mixtures were made up; one reaction was stopped at 0 min. and the other at 60 min. by the addition of 2.0 ml. of 10% trichloroacetic acid. The reaction mixtures were incubated at 30°.

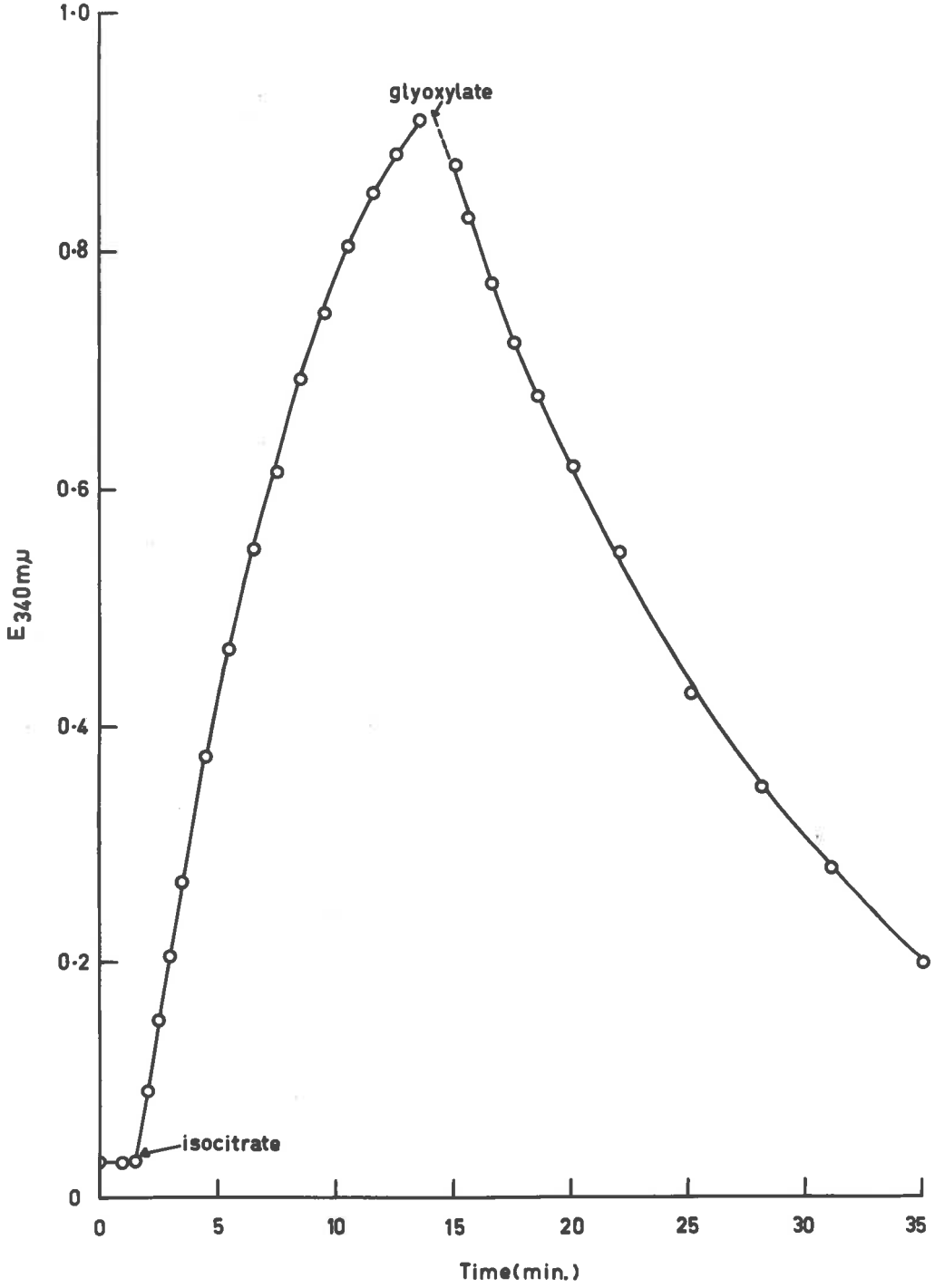
After removal of denatured protein by centrifuging (10,000 g., 5 min.), the protein-free supernatants were incubated with 1.0 ml. of 0.1% 2 : 4-dinitrophenylhydrazine in 2N-HCl for 20 min. at 37°. The keto acid derivatives were separated from excess 2:4-dinitrophenylhydrazine by extraction with ethyl acetate and sodium carbonate as described by El Hawary & Thompson (1953). The 2:4-dinitrophenylhydrazone derivatives of α-oxoglutaric acid and of

Fig. 17. Isocitrate dehydrogenase and glyoxylate reductase activity in extracts of Oxalis shoots.

The complete system contained in 3.05 ml., phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (25 μmoles), NADP (0.5 μmoles), Oxalis extract (0.5 ml. at pH 7.4) and isocitric acid (5 μmoles). Increases in extinction at 340 mμ consequent upon the addition of isocitric acid 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 (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 extinction at 340 mμ.

FIG.17


1·0
0·8
0·6
0·4
0·2
0
$E_{340m\mu}$
glyoxylate
isocitrate
0
5
10
15
20
25
30
35
Time(min.)

glyoxylic acid were separated on the same chromatogram as the zero time and 60 min. incubation extracts. The chromatogram was developed in a solvent of butan-1-ol, ethanol, 0.5 $\underline{\underline{N}}$-$NH_4OH$ (70:10:20 by vol.). A photograph of the chromatogram taken under ultra violet light is shown in Fig. 18. This shows a trace amount of α-oxoglutaric acid in the zero time incubation mixture and substantial amounts in the extract from the 60 min. incubation. A trace amount of keto acid derivative was detected in a position corresponding to the 2:4-dinitrophenylhydrazone of glyoxylic acid in the 60 min. incubation extract. The derivative of glyoxylic acid runs as two spots in the solvent used; only the lower one of these was detected in the 60 min. extract.

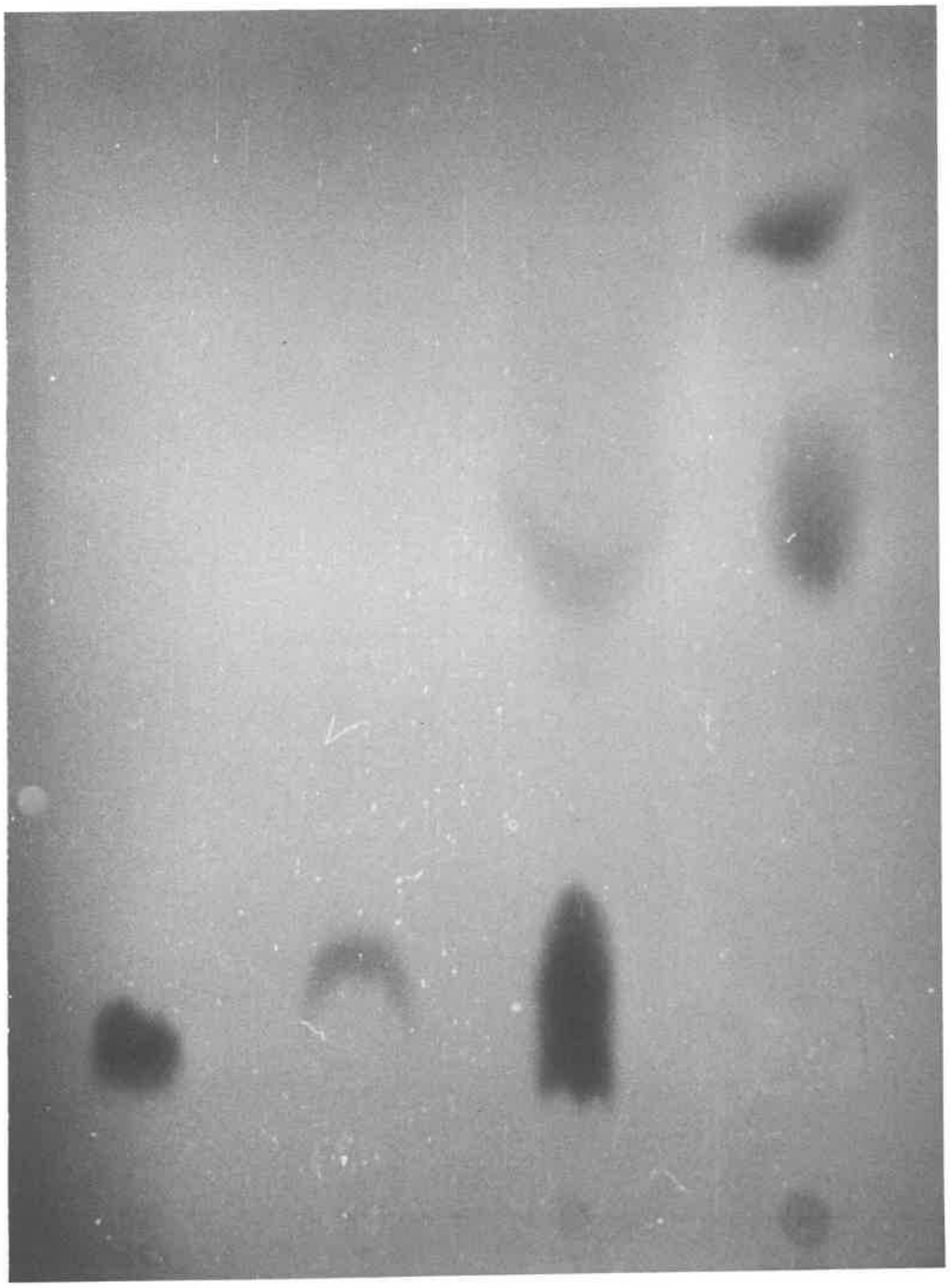
In this assay for isocitrate dehydrogenase activity it was apparent that there was some formation of glyoxylic acid from isocitric acid catalysed by isocitrate lyase in the extract. Substantial amounts of free glyoxylic acid would not be expected since the Oxalis extract also contained glyoxylate reductase.

Fig. 18. Separation of 2:4-dinitrophenylhydrazones of keto acids after incubation of isocitric acid with an extract of Oxalis.

The formation of α-oxoglutaric acid from isocitric acid catalysed by isocitrate dehydrogenase of Oxalis extracts was demonstrated by isolation of 2:4-dinitrophenylhydrazone derivatives from the following reaction mixture : phosphate buffer (200 μmoles at pH 7.4), $MgCl_2$ (50 μmoles), NADPH (1 μmole), Oxalis extract (1.0 ml. at pH 7.4) and isocitric acid (20 μmoles at pH 7.0). Reactions were stopped at 0 and 60 min. and keto acid derivatives extracted and chromatographed on paper by the method of El Hawary & Thompsón (1953). The chromatogram was photographed under ultra violet light.

A. 2:4 dinitrophenylhydrazone of α-oxoglutaric acid.
B. Zero time incubation.
C. 60 min. incubation .
D. 2:4 dinitrophenylhydrazone of glyoxylic acid.

FIG.18



A B C D

E. CONCLUSIONS

The demonstration, *in vitro*, of a number of enzymic reactions concerned with oxalic acid synthesis has confirmed and extended previous observations from the *in vivo* studies with *Oxalis* shoots.

In plants, glycollic acid and glyoxylic acid are normally metabolised to glycine and serine (Tolbert & Cohan, 1953b) or converted to glyceric acid and finally to sugars (Rabson, Tolbert & Kearney, 1962). It was apparent from *in vivo* studies in *Oxalis*, that the oxidation of glycollic acid via glyoxylic acid to oxalic acid appeared to be the most significant pathway for the metabolism of these two acids.

Extracts of soluble protein from *Oxalis* shoots showed a low level of oxygen consumption in the presence of glycollic acid, whereas the same type of preparation from green *Oxalis* leaves was very active towards the oxidation of glycollic acid (Fig. 6). This activity was probably due to glycollate oxidase which is widespread in green plants.

The rate of oxygen consumption by soluble protein from *Oxalis* shoots in the presence of glycollic acid or glyoxylic acid was increased by the addition of FMN. For glycollic acid, the presence of FMN resulted in a three-fold increase in the rate of oxidation. With glyoxylic acid as substrate, no oxygen uptake was observed without the addition of FMN, whereas uptakes were observed with FMN present (Fig. 7). For this soluble protein preparation, the rate of oxidation of glycollic acid was approximately five

times that of glyoxylic acid oxidation (Fig. 7). These results are comparable with those obtained for the relative rates of oxidation of these two substrates by glycollate oxidase from photosynthetic tissues (Richardson & Tolbert, 1961a).

When the rate of oxidation of glycollic acid and of glyoxylic acid by soluble protein was compared with rates obtained with extracts prepared from the particulate fraction of Oxalis shoots, it was found that extracts from the particulate fraction were more active (Fig. 8). In addition, the rate of oxidation of the two substrates by these extracts was approximately the same, indicating that the flavin-linked oxidase (or oxidases) was different from glycollate oxidase. Extracts of an acetone powder of the particulate fraction of Oxalis shoots showed the same trends (Fig. 9).

Since it was possible to extract most of the flavin-linked oxidase (or oxidases) from acetone powders in a soluble form, the oxidation of glycollic acid or of glyoxylic acid was also assayed spectrophotometrically. The reduction of 2:6-dichlorophenolindophenol in the presence of Oxalis extract (containing FMN) proceeded at approximately the same rate for glycollic acid or glyoxylic acid (Fig. 10). It was also shown that the presence of oxalic acid at substrate concentrations did not effect the rate of oxidation of glyoxylic acid. Richardson & Tolbert (1961a) found that the oxidation of glyoxylic acid by glycollate oxidase was strongly inhibited by oxalic acid. This inhibition probably accounted for earlier reports that glycollate oxidase was incapable of oxidising glyoxylic acid (viz., Zelitch & Ochoa, 1953; Frigerio & Harbury, 1958). The inhibition provides an explanation for the diversion of glyoxylic acid to glycine in most plants.

Clearly, the flavin-linked oxidase (or oxidases) isolated from the particulate fraction of non-photosynthetic shoots of Oxalis, differs from glycollate oxidase purified from photosynthetic tissues. Since the oxidation of glyoxylic acid is not inhibited by oxalic acid in Oxalis, glyoxylic acid may be readily converted to oxalic acid in this plant.

Oxalic acid itself appears to be a non-functional and undesirable end-product of metabolism in plants. It may be slowly oxidised to carbon dioxide (Finkle & Arnon, 1959) or decarboxylated to formic acid and carbon dioxide (Giovanelli & Tobin, 1961). In Oxalis, oxalic acid is not completely inert. An $NADPH_2$ and CoA-dependent reduction of oxalic acid by Oxalis extracts (Fig. 11) probably involves the intermediate formation of oxalyl-CoA as described for cell-free extracts of P. oxalaticus (Quayle, Keech & Taylor, 1961).

In photosynthetic tissues, glycollic acid synthesis has been associated with the cleavage of hexose and pentose phosphates arising from the photosynthetic carbon dioxide reduction cycle. The administration of $^{14}CO_2$, [$^{14}C_6$] fructose or [1-^{14}C] ribose to Oxalis shoots (see PART 1) did not result in substantial incorporation of isotope into glycollic acid. The origin of glycollic acid in these non-photosynthetic shoots appeared to involve the reduction of glyoxylic acid. A similar situation was observed with endosperm tissue of germinating castor bean (Kornberg & Beevers, 1957).

Glyoxylate reductase from castor bean and from Oxalis shoots was considerably more active with $NADPH_2$ than with $NADH_2$ (Fig. 12). Purified glyoxylate reductase isolated from photosynthetic tissue (Zelitch, 1955; Holzer & Holldorf, 1957) was not active towards the oxidation of $NADPH_2$ in the presence of glyoxylic acid. Recently, however, Zelitch & Gotto (1962) using tobacco and spinach leaves, partially purified a glyoxylate reductase specific for $NADPH_2$. In photosynthetic tissues, the reduction of glyoxylic acid is dependent upon light *in vivo*. Kearney & Tolbert (1962) concluded that this factor controlled the supply of nicotinamide nucleotide co-enzymes necessary for the reduction of glyoxylic acid. Since Oxalis shoots contain isocitrate lyase and isocitrate dehydrogenase, glyoxylic acid derived from isocitric acid may be readily reduced to glycollic acid via $NADPH_2$ from the oxidation of isocitric acid.

Although the flavin-linked oxidase present in Oxalis shoots has properties which differ from glycollate oxidase of green tissue, the presence of this activity in non-photosynthetic shoots may be associated with the production of the substrate (glycollic acid) from glyoxylic acid. Clagett, Tolbert & Burris (1949) and Tolbert & Burris (1950) reported that glycollate oxidase activity was absent in embryos and etiolated tissues. By exposing plants to the light, activity was easily demonstrated. Tolbert & Cohan (1953a) were able to produce the same effect by administering glycollic acid itself to etiolated plants. In most tissues, glycollic acid produced from the intermediates of photosynthesis could induce glycollate

oxidase activity. In Oxalis, glycollic acid synthesised from glyoxylic acid (via isocitric acid) may stimulate the production of an enzyme for the oxidation of glycollic acid.

The production of glyoxylic acid from isocitric acid catalysed by isocitrate lyase has hitherto been considered to be restricted, in plants, to those tissues converting fat to carbohydrate (Carpenter & Beevers, 1959). In these tissues, such as germinating fatty seedlings from fatty seed, glyoxylic acid condenses with acetyl-CoA to form malic acid (Kornberg & Beevers, 1957). These two reactions, constituting the glyoxylate bye-pass, can account for the nett synthesis of cell constituents from acetate. In Oxalis, C-2 units are also rapidly synthesised; however, glyoxylic acid is rapidly oxidised to oxalic acid rather than metabolised to malic acid.

Both isocitrate lyase (Fig. 14) and isocitrate dehydrogenase (Fig. 17) are present in Oxalis. Thus, in vitro, it is difficult to demonstrate the presence of glyoxylic acid in incubation mixtures containing Oxalis extract, isocitric acid, $MgCl_2$ and NADP (Fig. 18); glyoxylic acid formed from isocitric acid catalysed by isocitrate lyase is readily reduced to glycollic acid by an $NADPH_2$-linked glyoxylate reductase. The oxidation of isocitric acid provides sufficient $NADPH_2$ for the continued reduction of glyoxylic acid (Fig. 15). In the absence of added NADP, it was possible to demonstrate the production of glyoxylic acid from isocitric acid (Fig. 14).

Studies with cell-free extracts of Oxalis shoots have established that isocitric acid is a key intermediate in the synthesis of oxalic acid and related compounds in Oxalis. A summary of these reactions is presented in Fig. 19.

A number of plants accumulate one or more organic acids. The most common of these are citric acid, malic acid and tartaric acid. The reason for the accumulation of these acids is not always apparent. In Oxalis, the high content of oxalic acid may be associated with the genetical development of the plant. In those plants in which both isocitrate lyase and malate synthetase occur, glyoxylic acid, formed from isocitric acid, is rapidly metabolised to form malic acid. If isocitrate lyase occurs without malate synthetase, glyoxylic acid so formed would cause inhibition of aconitate hydratase (Ruffo, Adinolfi, Budillon & Capobianco, 1962b). The genetical response of Oxalis and other high oxalate plants to the unfavourable consequences of this inhibition may have resulted in the formation of enzymic systems for the rapid removal of glyoxylic acid. This may be achieved either by oxidation, resulting in the production of oxalic acid, or by reduction of glyoxylic acid catalysed by glyoxylate reductase. Isocitrate dehydrogenase activity would provide $NADPH_2$, necessary for this reduction, near the site of the synthesis of glycollic acid. Although glycollic acid contributes to the synthesis of glycine and serine in Oxalis, the most significant pathway is the oxidation of glycollic acid via glyoxylic acid to oxalic acid. It is likely that this oxidative system is sufficiently removed from the site of aconitate

hydratase so that glyoxylic acid formed from glycollic acid does not inhibit this enzyme.

Oxalic acid accumulation in Oxalis may be an effective method of metabolising glyoxylic acid, which, unless removed, would inhibit aconitate hydratase and thus prevent normal functioning of the tricarboxylic acid cycle.

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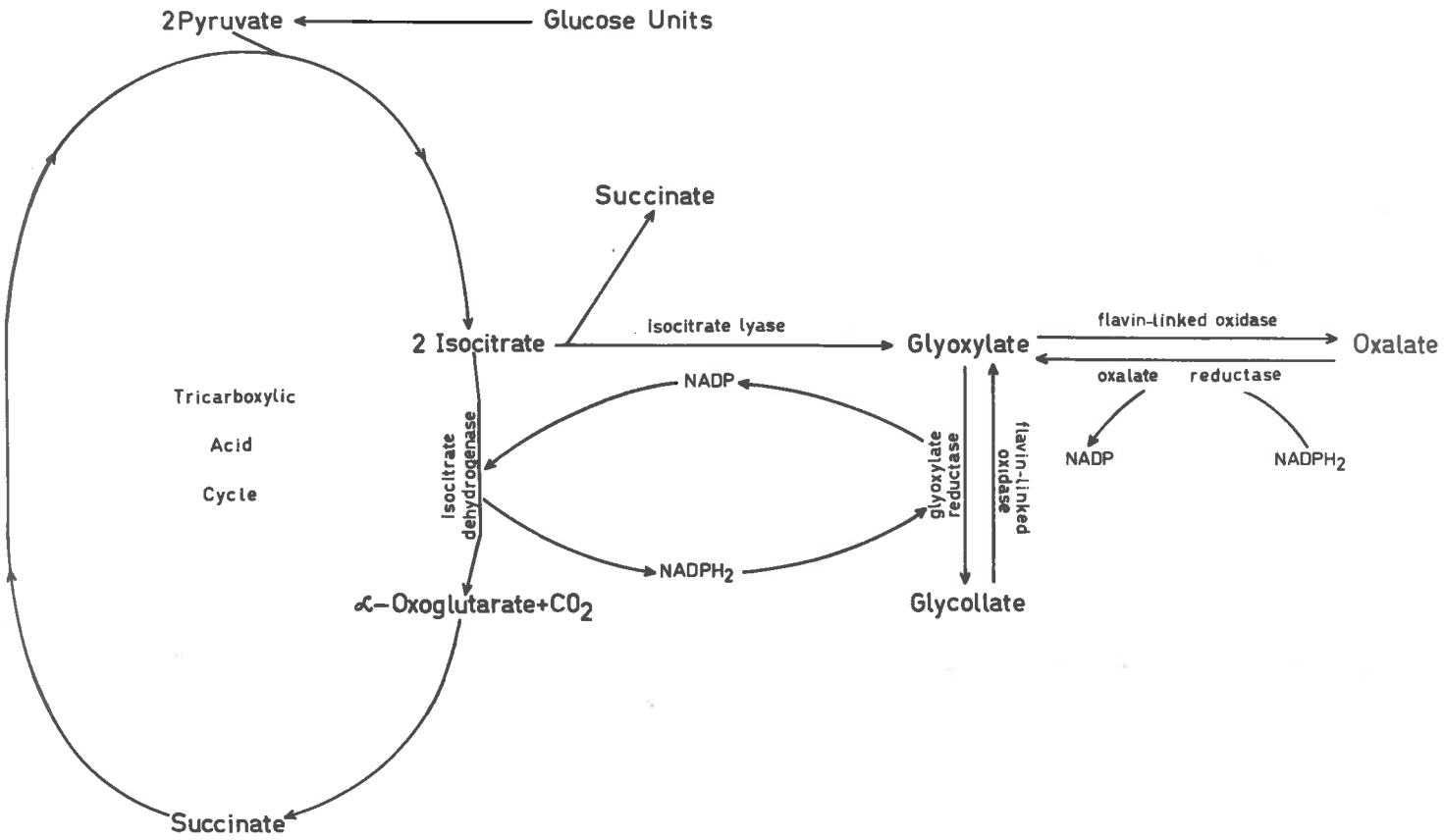
Fig. 19. Reactions involved in the synthesis of oxalic acid and related compounds in *Oxalis*

The accompanying figure illustrates the relationship between a number of compounds concerned with the synthesis of oxalic acid and related compounds in *Oxalis*. The reactions were demonstrated with cell-free extracts of non-photosynthetic *Oxalis* shoots.

FIG.19


2Pyruvate
Glucose Units
Succinate
2 Isocitrate
Isocitrate lyase
Glyoxylate
flavin-linked oxidase
Oxalate
oxalate reductase
NADP
NADPH2
Tricarboxylic
Acid
Cycle
Isocitrate dehydrogenase
NADP
NADPH2
glyoxylate reductase
flavin-linked oxidase
α-Oxoglutarate+CO2
Glycollate
Succinate

SUMMARY

The experiments reported here describe studies to determine the pathway of oxalic acid formation in the white emergent shoots of Oxalis pes-caprae. The synthesis of oxalic acid was studied in the whole shoot, by the administration of a number of ^{14}C compounds considered as precursors of oxalic acid, and with cell-free extracts prepared from the shoots.

In the first series of in vivo experiments, the following ^{14}C labelled compounds were injected into Oxalis shoots : [$^{14}C_6$] glucose, [$^{14}C_2$] glyoxylic acid, [1-^{14}C] glycollic acid, [$^{14}C_2$] oxalic acid, [^{14}C] bicarbonate, [$^{14}C_2$] glycine and [$^{14}C_3$] serine. The products which became labelled after injection of these compounds were examined qualitatively by paper chromatography and radioautography, and quantitatively by column chromatography. The results of these experiments indicated that the carbon for oxalic acid synthesis was derived from the sugar pool or derivatives thereof. Although glycine and serine both contributed to oxalic acid synthesis, the low level of incorporation observed suggested that neither of these amino acids was of physiological importance in the synthesis of oxalic acid.

The distribution of ^{14}C among the products from ^{14}C-labelled glucose, glyoxylic acid and glycollic acid indicated that glycollic acid and glyoxylic acid were the immediate precursors of oxalic acid. Although bicarbonate is rapidly incorporated into glycollic acid in photosynthetic tissues, no such pathway was apparent in non-photo-

synthetic Oxalis tissue; glycoxylic acid was an efficient precursor for this synthesis in Oxalis.

To establish the origin of glycollic acid and of glycoxylic acid further in vivo experiments were carried out. ^{14}C-labelled compounds were administered to grooves cut in Oxalis shoots. The products containing radioisotope were separated by paper chromatography and detected by radioautography.

Neither [$^{14}C_6$] fructose, [3-^{14}C] glyceric acid nor [1-^{14}C] ribose contributed significantly to the synthesis of glycollic acid. There was no evidence for the synthesis of this acid other than from the reduction of glycoxylic acid.

The application of [1-^{14}C] glycollic acid and of [1,5-$^{14}C_2$] isocitric acid both resulted in substantial incorporation of isotope into oxalic acid. After the application of these two ^{14}C-labelled compounds to shoots for 1 hr. the amount of ^{14}C incorporated into oxalic acid was approximately the same. In the longer time period (6 hr.) radioisotope from glycollic acid continued to be incorporated into oxalic acid. With isocitric acid, ^{14}C activity accumulated in citric acid. This situation was probably due to the inhibition of aconitate hydratase by oxalomalic acid, a compound formed non-enzymically from glycoxylic acid and oxaloacetic acid. Glycoxylic acid formed from isocitric acid, would be near the active site of aconitate hydratase, whereas glycoxylic acid arising from the oxidation of glycollic acid may not be close enough to this site to cause inhibition.

Enzymic reactions involved in the synthesis of oxalic acid and of related compounds were studied with cell-free extracts of shoots of *Oxalis*. Glycollic acid and glyoxylic acid were oxidised to glyoxylic acid and to oxalic acid respectively, by a flavin-linked oxidase (or oxidases). The enzyme (or enzymes) isolated from acetone powders of the particulate fraction of *Oxalis* shoots was similar to glycollate oxidase of photosynthetic tissues in the requirement for FMN. However, the enzyme isolated from *Oxalis* oxidised glycollic acid and glyoxylic acid at approximately the same rates; furthermore the oxidation of glyoxylic acid was not inhibited by oxalic acid. Glycollate oxidase from photosynthetic tissue is many times more active towards the oxidation of glycollic acid than glyoxylic acid, and the oxidation of glyoxylic acid is strongly inhibited by oxalic acid. Although oxalic acid accumulates in *Oxalis*, it is apparent that it can be metabolised since an $NADPH_2$ and CoA-dependent reduction of oxalic acid was demonstrated.

In confirmation of the results obtained from *in vivo* studies, cell-free extracts of *Oxalis* have been shown to contain isocitrate lyase, isocitrate dehydrogenase and an $NADPH_2$-dependent glyoxylate reductase. The combined action of these three enzymes results in the production from two molecules of isocitric acid, of one molecule each of α-oxoglutaric acid, carbon dioxide, succinic acid and glycollic acid. Glyoxylic acid formed from the cleavage of isocitric acid may be reduced to glycollic acid via an $NADPH_2$-linked glyoxylate reductase. The reduced co-enzyme arises from the oxidation of isocitric

acid catalysed by isocitrate dehydrogenase.

Oxalic acid synthesis in white Oxalis shoots is associated with the oxidation of glyoxylic acid. This acid may arise from the oxidation of glycollic acid or from the cleavage of isocitric acid. Since glyoxylic acid appears to be the immediate precursor of glycollic acid in such tissues, it is probable that isocitric acid is the key intermediate in oxalic acid synthesis in Oxalis.

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