# STUDIES ON THE ORGANIC ACID METABOLISM OF A WHEAT SMUT FUNGUS (*TILLETIA CONTRAVERSA*)

by

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# STUDIES ON THE ORGANIC ACID METABOLISM OF A WHEAT SMUT FUNGUS (*TILLETIA CONTRAVERSA*)

## INTRODUCTION

*Tilletia contraversa* Kuhn and other species of Tilletiaceae are of considerable economic importance because of their ability to parasitize and limit production of wheat, other cereals and certain grasses. The principal defense against these organisms is to breed resistant varieties of plants. Unfortunately, at least with wheat, the organisms can apparently hybridize or mutate to produce new physiologic races which then attack the "resistant" wheat. Thus, the control of *Tilletia* species is a difficult and constant problem. Another attack toward solution of the problem is to study the biochemistry of these organisms. Such an approach should yield some insight into the biochemistry of obligate parasitism and host resistance. Then a logical chemical control might be realized through the use of this information.

Actually the biochemistry of obligate parasites is a comparatively untouched field and previous studies have been limited mostly to certain powdery mildews scraped from leaves of infected plants and to wheat rust

uredospores. It seems remarkable that Tilletia species have escaped investigation for so long, for they make an excellent object for a study of this nature. This is because the abundant teliospores of the organism can be separated cleanly from infected wheat.

The principal facts known about the biochemistry of Tilletia species, excluding nutrition studies, can be listed as follows:

1. Identification of trimethylamine in teliospores of T. levis (21, p. 351-358).
2. Studies on the amino acid composition of hydrolysates of teliospores and saprophytic mycelia of T. caries (41, p. 363-366).
3. Identification of the enzymes of the hexosemonophosphate pathway in the saprophytic mycelium of T. caries (45, p. 27-35).
4. Observation that saprophytic mycelium of T. caries oxidized Krebs cycle intermediates very feebly (45, p. 27-35).
5. Tracer studies which indicated that teliospores and saprophytic mycelium of T. caries oxidized glucose quite differently (46, p. 308-311).
6. Demonstration that teliospores of T. contraversa oxidized glucose 67% by means of the hexosemonophosphate pathway and 33% by the Embden-Meyerhof

pathway (47, p. 415-417).

7. Results indicating that the Krebs cycle might not be fully operative in teliospores of T. contraversa (47, p. 415-417).

The above work showed that there was a need for information on the organic acid metabolism in teliospores of T. contraversa. Preliminary studies of these teliospores indicated that they had a comparatively active formic and oxalic acid metabolism. This discovery was considered important enough to warrant the detailed study which is the principal investigation reported in this thesis.

This study is concerned with formate oxidation, oxalate oxidation and metabolic pathways to these compounds in teliospores of T. contraversa.

# REVIEW OF LITERATURE

## Biology of Tilletia Species

There is considerable disagreement about the details of the nuclear cycle in the genus Tilletia. Since it takes expert knowledge to pass judgment on these matters, a version simplified from the literature covered by Fischer and Holton (15, p. 253-258) is given here. Figure 1 is a schematic drawing of the life cycle deduced from this source.

The teliospores germinate putting forth a germ tube (promycelium) which contains a haploid nucleus. The promycelium gives rise to a number of fragile spores called sporidia. As soon as the sporidia are mature they may fuse or they may propagate saprophytically, for example, on artificial media. If the sporidia fuse, the nucleus from one sporidium passes into the other sporidium thereby originating a dikariophase. In order to produce hyphae and subsequently teliospores, the dikariotic sporidium requires a host plant and in this stage of its life cycle it is an obligate parasite. In wheat the fungus hyphae grow forward keeping pace with plant development so that at plant maturity fungal teliospores are formed in place of wheat kernels.

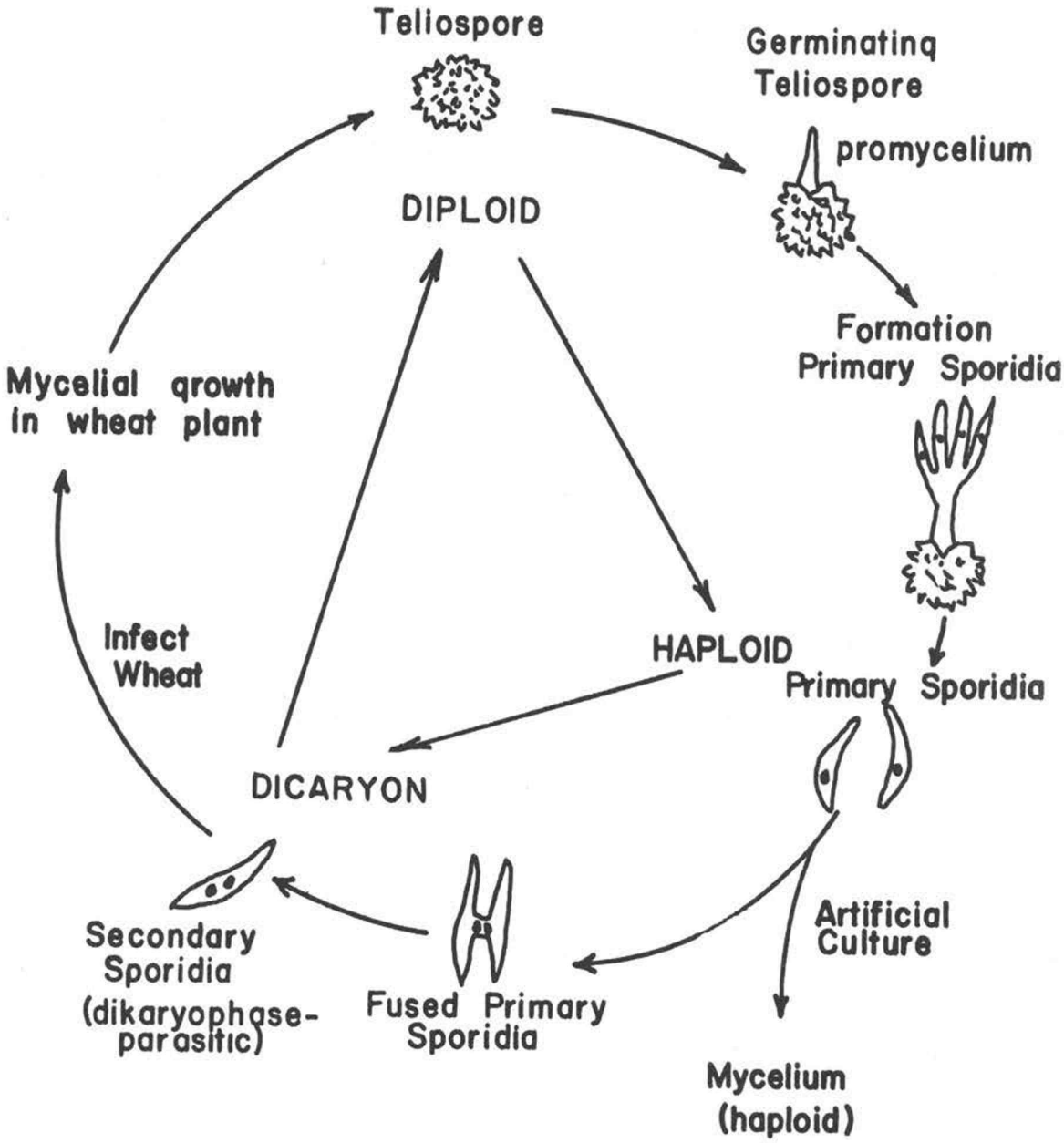



Figure 1. Life cycle of Tilletiaceae

During the period where the dikariotic sporidium eventually gives rise to the haploid nucleus in the promycelium, it is obvious that the two nuclei must fuse to produce a diploid nucleus which in turn must undergo reductive division to form the haploid nucleus in the promycelium. It is on the time of occurrence of these events that confusion exists. However, according to Fischer and Holton (15, p. 253-258), teliospores are binucleate in young spores and diploid in mature spores.

The most prominent symptom of early T. contraversa infection in wheat is the extreme dwarfing of the plant. Other signs of early infection are excessive tillering or shoot production and chlorotic stripping (15, p. 125-134). In mature infected wheat the teliospores replace the entire wheat kernel within the seed coat to form a so-called "smut" or "bunt" ball.

## Pathways to Formate

Formate was known to be produced in Escherichia coli fermentations as early as 1901 (22, p. 610-628), and once even was proposed as a principal intermediate in the complete oxidation of glucose by animals. However, recent knowledge has shown formate to be a very mobile metabolic group which is reversibly transferred

by folic acid coenzymes into a variety of compounds such as purines, histidine, serine, thymine and methionine (28, p. 369-446).

There are also several enzymic systems known to produce formate which are apparently independent of folic acid. Plants (33, p. 130-134; 62, p. 707-718) and animals (37, p. 269-280) have a glycolic acid oxidase which catalyzes the oxidation of glycolate to glyoxalate with the production of hydrogen peroxide. The hydrogen peroxide produced in the reaction then non-enzymically oxidizes glyoxalate to formate and carbon dioxide. Mammalian tissues, however, can form formate and carbon dioxide from glyoxalate enzymically. Nakada and Sund (43, p. 8-13) found that the reaction required glutamate; they postulated N-glyoxyl glutamic acid and N-formyl glutamic acid as intermediates in the reaction. The latter compound is then presumed to undergo hydrolysis to form formate and glutamate. Bacteria (Achromobacter species) can apparently convert glyoxalate to methylene glycol, formaldehyde and formic acid (50, p. 288-298).

From the foregoing it is obvious that compounds which produce glycolate or glyoxalate are potential sources of formate. Among these compounds are glycine (51, p. 119-133; 42, p. 257-270), isocitrate (53, p.

563-569), sarcosine (51, p. 119-133) and acetate (3, p. 372-377). The direct oxidation of acetate to glycolate is of particular interest since the methyl carbon is usually considered to resist oxidation. However, Bolcato and co-workers (3, p. 372-377) have proposed that yeast cells oxidize acetate to formate by the following series of reactions:

acetate → glycolate → glyoxalate → formaldehyde → formate.

Weinhouse (59, p. 282-284) also indicated that Aspergillus niger oxidized acetate to glycolate.

Another mechanism for forming formate is by the cleavage of pyruvate. This reaction has been extensively studied in E. coli and Clostridia species. According to Chantrenne and Lipmann (7, p. 757-767) the carbonyl carbon of pyruvate is activated by a coenzyme A-enzyme complex to liberate formic acid. The reverse of this reaction was studied by Chin et al. (8, p. 127) and was found to be stimulated by tetrahydrofolic acid.

There are two reactions which intimately link formate with oxalate metabolism. The first is the direct decarboxylation of oxalate to formate and carbon dioxide. Shimazono and Hayaishi (54, p. 151-159) purified the enzyme catalyzing this reaction from the mycelium of a

wood destroying fungus (Collyvia veltipes). The decarboxylase was stable and had maximum activity between pH 2.5 to 4.0. The enzyme was peculiar in that catalytic amounts of oxygen were required for the reaction to proceed. No other cofactor requirement could be found. In the second system, exhibited by a soil pseudomonad, decarboxylation of oxalate required coenzyme A and thiamine pyrophosphate. Activity was maximum at pH 6.9 (29, p. 435-446).

Formate is also known to be formed by the oxidation of tryptophan to kynurenine (35, p. 431-438) and from the oxidation of acetone (52, p. 361-371). It is also reasonable to suppose that it could be formed from formyl tetrahydrofolic acid (see reference 28, p. 369-446 for a review of folic acid metabolism).

## Formate Oxidation

One of the fundamental concepts of comparative biochemistry is that there is a basic similarity in the biochemical behavior of living things. It was thus surprising to find that aerobic organisms had evolved at least 4 ways in which to oxidize a simple molecule like formic acid.

A large number of higher plants have a formic dehydrogenase capable of reducing methylene blue. Davison

(12, p. 26-36) cursorily examined 93 species of plant seeds and found that 54 species had this ability. It is of particular interest that wheat seeds were inactive under the conditions of her experiments. Formic dehydrogenase has been purified from bean (13, p. 520-526) and pea (40, p. 667-682) plants and the enzyme from both sources required diphosphopyridine nucleotide for activity.

Escherichia coli oxidizes formate in a different manner. Gale (20, p. 1012-1027) showed that in this bacterium the reaction was independent of pyridine nucleotides and indicated that cytochrome b was the primary hydrogen acceptor. Presumably E. coli and higher plants obtain useful energy from formate oxidation since reduced cytochrome b and the reduced pyridine nucleotide are likely further oxidized by components of the cytochrome system with the concommitant formation of adenosine triphosphate.

In higher animals formate oxidation seems to be mediated by a catalase-hydrogen peroxide complex (49, p. 1661-1665). The ability of catalase to make use of metabolically produced hydrogen peroxide for oxidative purposes was first discovered by Keilin and Hartree (30, p. 141-159). The number of compounds capable of being

oxidized in this fashion is quite large and includes alcohols (30, p. 141-159), pyrogallol (32, p. 310-325), formic acid (4, p. 1341-1369), and nitrite (26, p. 549-556). In this type of "coupled oxidation" the active oxidant is a catalase-hydrogen peroxide complex (4, p. 1341-1369) and the oxidative reaction is analogous to a peroxidase reaction.

Hauge (23, p. 148-155) proposed a unique mechanism to account for formate oxidation in Aspergillus niger. It would seem that this fungus oxidized formate to carbon dioxide and hydrogen peroxide. The enzyme responsible for this reaction required no dialyzable cofactor. In addition to the above mechanism for formate oxidation it has been suggested but never proved that fungi form oxalate through dehydrogenation of 2 moles of formic acid (16, p. 345-346).

## Pathways to Oxalate

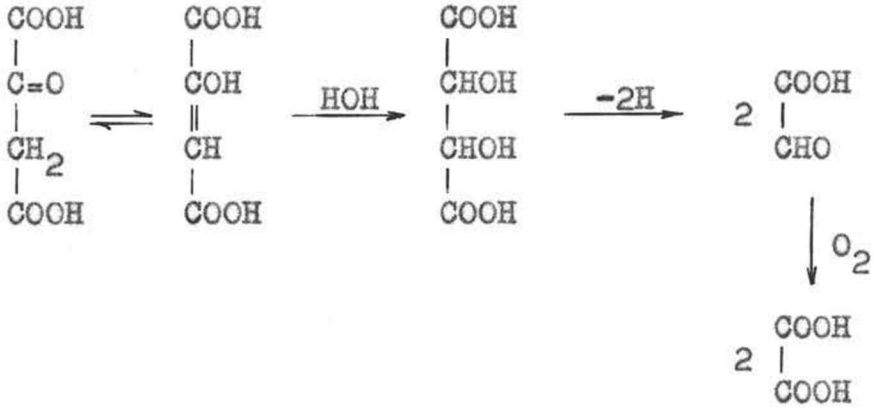
Oxalate is widely distributed in the plant and animal kingdoms. Although plants may contain 20% oxalic acid on a dry weight basis (56, p. 75), wheat contains only 0.11% (44, p. 1040-1043). Humans excrete between 15 and 20 mg of oxalate daily; however, it is generally thought that this oxalate comes largely from

dietary plant materials (24, p. 738-739). Certain fungi produce so much oxalate in cultures that commercial production has been considered (16, p. 333).

Pathways to account for the formation of oxalic acid by fungi are almost as numerous as the number of workers concerned with the problems. According to Foster's extensive review of the literature up to 1949 (16, p. 326-350), no less than 5 mechanisms have been suggested to account for oxalic production in Aspergillus niger alone. These mechanisms include coupling of 2 moles of formate, direct reduction of carbon dioxide, direct oxidation of glyoxalate, and hydrolysis of oxalacetate and oxalosuccinate. This unusual situation has resulted because the data were obtained from nutritional experiments or from the use of preformed mycelial pads.

It would be expected that radioactive tracer studies would clarify the oxalate pathways in A. niger. However, this is not the case. For example, Lewis and Weinhouse (39, p. 2906-2909) implicated oxalacetate as the direct precursor of both oxalate and acetate. On the other hand, Cleland and Johnson (9, p. 595-606) proposed that oxalacetate is oxidatively split into 2 moles of oxalate with tartrate and glyoxalate as the intermediates involved. The latter reactions may be

represented as follows:



Cleland and Johnson (9, p. 595-606) also proposed that glycolaldehyde originating from the hexose monophosphate pathway is oxidized to oxalate.

The complex problem of oxalate metabolism in A. niger has recently been simplified by the use of cell-free extracts. Hayaishi et al. (25, p. 5126) reported that such extracts quantitatively converted oxalacetate into oxalate and acetate. Oxalosuccinate could not substitute for oxaloacetate in this system.

Franke and de Boer (17, p. 70-89) clarified somewhat the reactions of glycolate and glyoxalate. According to these workers the reaction products depended upon the amount of oxygen present. Aerobically, A. niger extracts converted glycolate into formic acid and carbon dioxide in a 1:1 ratio and produced a 10% yield of oxalate. However, when glyoxalate was substituted for

glycolate the principal product could not be identified. Anaerobically, glyoxalate was dismutated to glycolate and oxalate. This latter reaction has also been observed in mammalian tissue (51, p. 119-133).

Higher plants such as tobacco leaves oxidize glyoxalate to produce oxalate and hydrogen peroxide (33, p. 130-134). The enzyme has not been purified to any extent but may be a flavoprotein.

Mammalian tissues as mentioned above have a mutase which converts glyoxalate into oxalate and glycolate. In addition, xanthine aldehyde oxidase from milk and liver forms oxalate from glyoxalate (51, p. 119-133; 42, p. 257-270). The actual substrate for this reaction is probably glyoxylic acid monohydrate.

## Oxalate Oxidation

There are apparently 3 different enzymes which catalyze the decomposition of oxalic acid. As described previously under pathways to formate, there are two enzymes which decarboxylate oxalate to yield formate and carbon dioxide. In addition to these, higher green plants (14, p. 614-624) and mosses (11, p. 602-603) oxidize oxalate to carbon dioxide and hydrogen peroxide. The moss enzyme was remarkably heat stable and was

stimulated by flavin mononucleotide and riboflavin but not by flavinadenine dinucleotide.

Mammalian tissues apparently have little capacity to decompose oxalic acid under physiological conditions (2, p. 144-148).

# MATERIALS AND METHODS

## Harvesting of Spores

Bunt balls were removed from infected wheat plants and winnowed from excess chaff. They were then blended briefly with water in a Waring blendor to release the spores from the bunt balls. The spores were filtered through 8 layers of cheese cloth, collected on filter paper and washed until the wash water was colorless. Spores treated in this way were essentially free of plant material as judged by microscopic examination.

## Enzyme Preparations

### Acetone powders

Spores were blended in a Waring blendor for 10 seconds with 10 volumes of acetone at -10°C. They were then filtered, washed with 2 volumes of acetone, air dried and stored at 3°C.

### Spore extract and spore particles

Acetone powders were ruptured at 3°-8°C by shaking an aqueous suspension with 4 mm glass beads on a Mickle homogenizer. The ratio of spores, water and beads for maximum breakage in a 55 mm x 25 mm diameter Mickle cell

was 1.5 g:5 ml:15 g. Acetone powders contain 22% ether solubles which are released into the medium as the spores are broken. Since this fatty material interfered with the impact of spores and beads, it was generally removed after 5 minutes of shaking. This was accomplished by centrifuging the suspension briefly and skimming off the fat which rose to the surface. Further shaking for 5 minutes resulted in a 80% to 95% rupture of the spores.

After removal and washing of the glass beads, the suspension from a Mickle cell was diluted to 10 ml with water and centrifuged at 17,000 g for 30 minutes. The supernatant fraction, after further removal of fat, was generally used to study formate oxidation and the precipitated fraction to study oxalate oxidation.

Formate oxidase

The spore extract (supernatant fraction) contained 1.5 to 2.0 mg of protein per ml and about 25% of the original formate oxidation activity. Efforts to release more protein from the spores were generally unsatisfactory. However, in some experiments the ruptured spores, prior to centrifugation, were incubated overnight at 32° with 1.5 mg of crystalline trypsin.

The use of trypsin was initiated when it was found

to double formate oxidation activity. Later it was suspected of destroying some of the enzymic activity when experimentation was delayed for a few hours. At present it is felt that trypsin would be useful in increasing activity if optimum conditions for its use were studied. In the experiments employing trypsin, it was presumably inactivated as required by decreasing the pH from 7.0 to 5.1.

Oxalate oxidase

The ability to oxidize oxalate was almost wholly confined to the spore particles (precipitated fraction). Efforts to solubilize activity by sonic oscillation or by incubation of the particles with snake venom, lipase, trypsin, pectinase, cellulase or lysozyme were unsuccessful. However, it was found that 0.2% digitonin in the spore suspension during rupturing extracted some components responsible for 5-10% of the endogenous respiration without affecting oxalate oxidation activity. The dry weight of the digitonin-treated particles used to study oxalate oxidation was between 60-80 mg per ml of aqueous suspension.

Spore homogenate

Spore acetone powders were ruptured as above but were otherwise untreated. Since the fat was not removed these preparations contained 20 to 30% whole spores. Before use they were homogenized with a Dounce homogenizer.

## Analytical Techniques

### Assay of carbon dioxide-$C^{14}$

Since the endogenous respiration of the enzyme preparations was high and the activity of the preparations was low, particularly after dialysis, formate, oxalate and other substrate oxidations or decarboxylations were routinely measured as the radioactivity in carbon dioxide arising from carbon-14 substrates. Unless otherwise mentioned, all experiments were performed in a 50 ml Erlenmeyer flask equipped with a center-well, a rubber serum bottle cap and sometimes a sidearm. The reaction mixtures in the flasks were agitated in a Dubnoff shaker at 30°C and respired carbon dioxide was trapped in the center-well as sodium carbonate. The reaction was stopped as desired by the addition of 1 ml of 20% trichloroacetic acid delivered with a hypodermic

syringe through the serum bottle stopper. The flasks were then agitated at 70°C for 15 minutes to assure that all the carbon dioxide was trapped.

The sodium carbonate was converted and counted as barium carbonate by standard procedures. The errors in all procedures were generally less than 5%.

Manometric measurements

Manometric measurements were made in a Warburg apparatus at 30°C. Carbon dioxide was measured by the "direct method" (58, p. 17-20).

Catalase

Catalase activity of the undialyzed spore supernatant fraction was assayed iodometrically as described by Herbert (27, p. 784-788).

Carbon monoxide inhibition

Carbon monoxide-oxygen mixtures were prepared by displacement of water from a bottle. The reaction flasks with sidearms were filled with the gas mixture by an evacuation procedure. The flasks were protected from light with aluminum foil and the reaction was started by tipping in substrate from the sidearm.

Oxalate-$C^{14}$ determination

Fifteen mg of carrier oxalic acid were added after the reaction mixture was heated to 70°C. After filtering, the oxalic acid was precipitated from solution as the calcium salt at about pH 8. The salt was purified by successive solution in dilute HCl and reprecipitation at pH 10 ($NH_4OH$) until a constant specific activity was reached.

Acetate-$C^{14}$ and formate-$C^{14}$ determination

The only satisfactory method found for separating low activity acetate-$C^{14}$ and formate-$C^{14}$ from the components of the reaction mixture was by steam distillation followed by column chromatography. The complete reaction mixture, containing 2 mg of carrier acetate and formate, was steam distilled according to the method of Wood and Gest (60, p. 285-292). The distillate at pH 8 was evaporated *in vacuo* to about 3 ml, acidified to pH 2, mixed with sufficient silicic acid so that the mixture had little tendency to adhere to the wall of a glass beaker and placed on a silica gel column for chromatography. The chromatography technique was essentially that described by Corcoran (10, p. 168-171) except that the

glycine stationary phase was buffered at pH 3.0 and the eluant was 10% butanol in 90% chloroform. These modifications permitted a better and faster separation of acetate from formate. The eluate from the column was collected in 5 ml amounts and these fractions titrated under nitrogen with 0.03N NaOH to the phenol red endpoint. After titration, the aqueous layers from the fractions were transferred to metal planchets, dried and their radioactivity determined. The position of various acids fractionated as above on a 50 cm x 1.5 cm diameter column containing 15 g of silicic acid is as follows:

| Acid | Fraction Peak Tube no. |
|---|---|
| X | 3 |
| Acetic | 9 |
| Formic | 17 |
| Pyruvic | >50 |

## Syntheses

### Sodium bicarbonate-$C^{14}$

This was prepared from barium carbonate-$C^{14}$ according to the method described by Aronoff (1, p. 71-72).

Sodium glyoxalate-$C^{14}$

This was prepared by the electrolytic reduction of oxalic acid-$C^{14}$ according to the method described by Krupka and Towers (36, p. 335).

## Chemicals

Pyridine nucleotides, flavins and crude coenzyme A were purchased from Sigma Chemical Co.; folic acid from California Corporation for Biochemical Research; radioactive carbon chemicals (except those synthesized) from Nuclear-Chicago Corp. Folic acid was recrystallized from water before use.

## Abbreviations

The following abbreviations are used throughout this thesis: CoA, coenzyme A; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; FAD, flavinadenine dinucleotide; FMN, flavin mononucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; Tris, tris(hydroxymethyl)aminomethane.

# RESULTS AND DISCUSSION

## PART I. FORMATE OXIDATION

### Effect of Pyridine Nucleotide and Carbon Monoxide

Initially it was anticipated that formate oxidation would be linked to oxygen by one of the pyridine nucleotides and the cytochrome system. However, since neither carbon monoxide containing 10% oxygen nor pyridine nucleotides (see Table I for effect of DPN) had any effect on formate-$C^{14}$ oxidation by spore extracts, it became necessary to examine other mechanisms for formate oxidation.

### Catalase-Hydrogen Peroxide Complex

Another possibility for formate oxidation was a catalase-hydrogen peroxide complex perhaps analogous to the oxidation of ethanol as described by Keilin and Hartree (30, p. 141-159). In their system, this oxidation was coupled to hydrogen peroxide producing systems by means of catalase. Later, other workers showed that formate (4, p. 1341-1369) and nitrite (26, p. 549-556) were oxidized by catalase-hydrogen peroxide (complex I). In view of these observations, if formate oxidation in

TABLE I. EFFECT OF PYRIDINE AND FLAVIN NUCLEOTIDES ON FORMATE OXIDATION*

| Additions | CPM in $CO_2$ (average) | Error (%) | Increase (%) |
|---|---|---|---|
| Control | 3174 | ±3 | |
| FMN | 3870 | ±2 | 22 |
| DPNH | 5774 | ±0 | 82 |
| FMN + DPNH | 9062 | ±2 | 186 |
| FMN + DPN | 3897** | - | 23 |
| FAD + DPNH | 9119** | - | 187 |

* Duplicate reaction mixtures (4.6 ml) contained spore extract dialyzed 18 hours, 1 ml; formate-$C^{14}$, 0.5 µc (0.12 µmole); magnesium chloride, 3 mg; flavins, 0.1 mg as required; pyridine nucleotides, 1.0 mg as required; 0.15M citrate-phosphate buffer at pH 5.1. Incubation time, 1 hour.

**Single experiment.

T. contraversa depended on complex I, it should be competitively inhibited by ethanol and by nitrite. Figures 2 and 3 show that this is so.

Effect of pH

The pH optimum for the coupled oxidation of formate is at 5.1 (Figure 4). The particularly sharp optimum is interpreted as reflecting that only undissociated formate is a substrate for complex I (6, p. 471-481) and

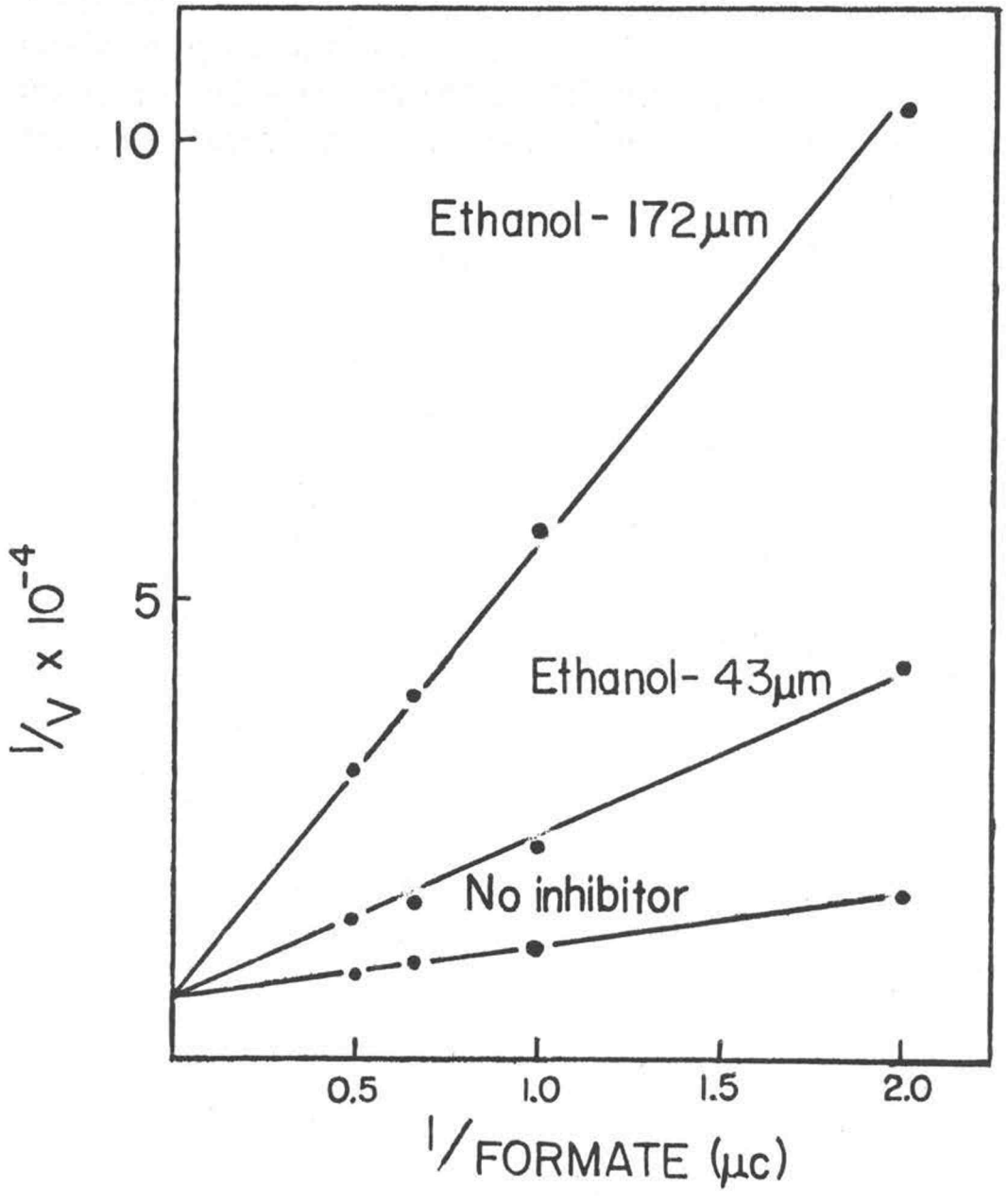



Figure 2. Reciprocal plot of the inhibition of formate oxidation by ethanol. Reaction mixtures (5.4 ml) contained trypsin treated spore extract, 1 ml; formate-$C^{14}$ (4.0 µc/µmole), as indicated; ethanol, as indicated; and 0.15M citrate-phosphate buffer at pH 5.1. Incubation time, 1 hour. Velocity (V) measured as the radioactivity in respired carbon dioxide.

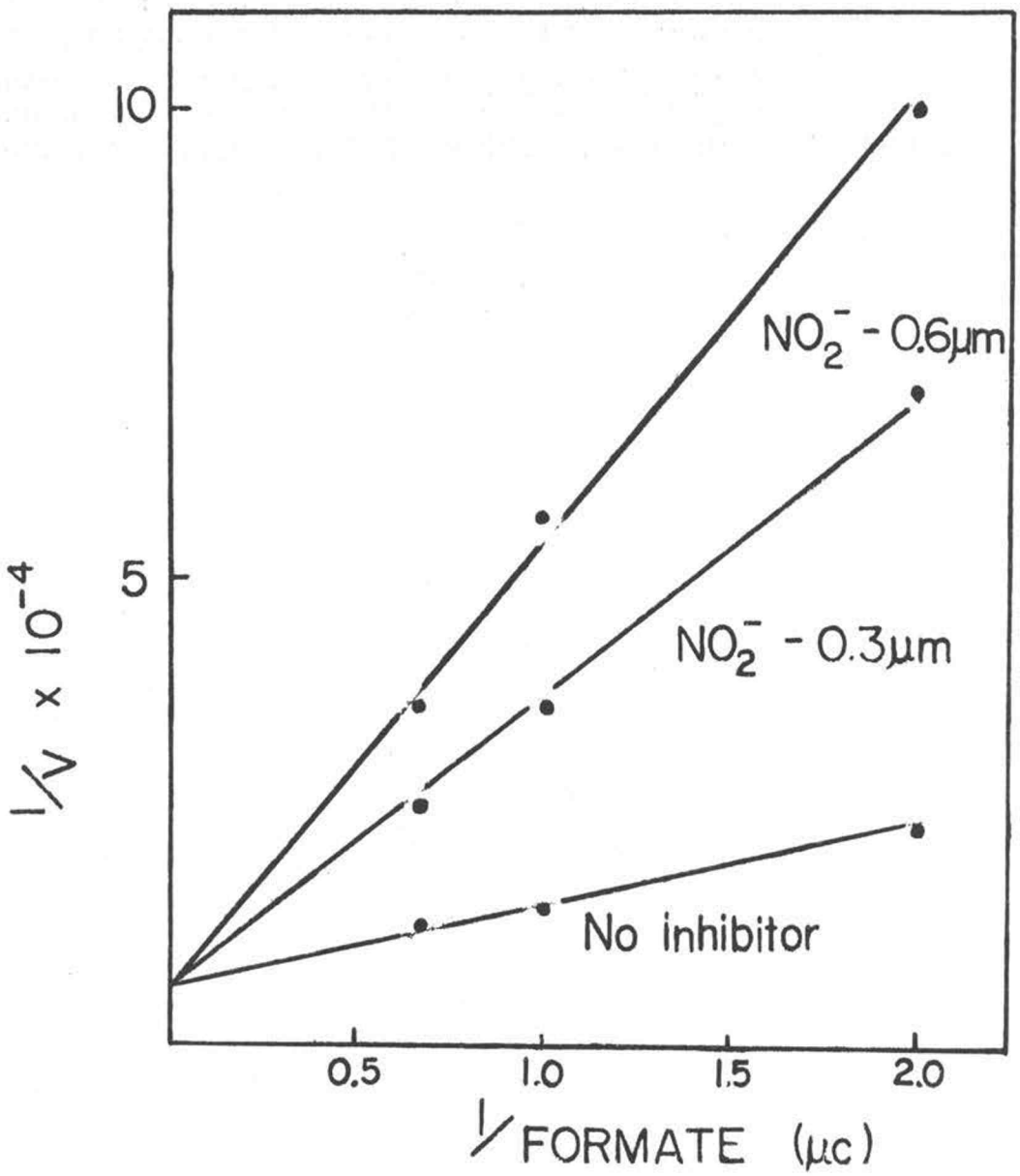



Figure 3. Reciprocal plot of the inhibition of formate oxidation by nitrite. Experimental conditions similar to that shown in Figure 2.

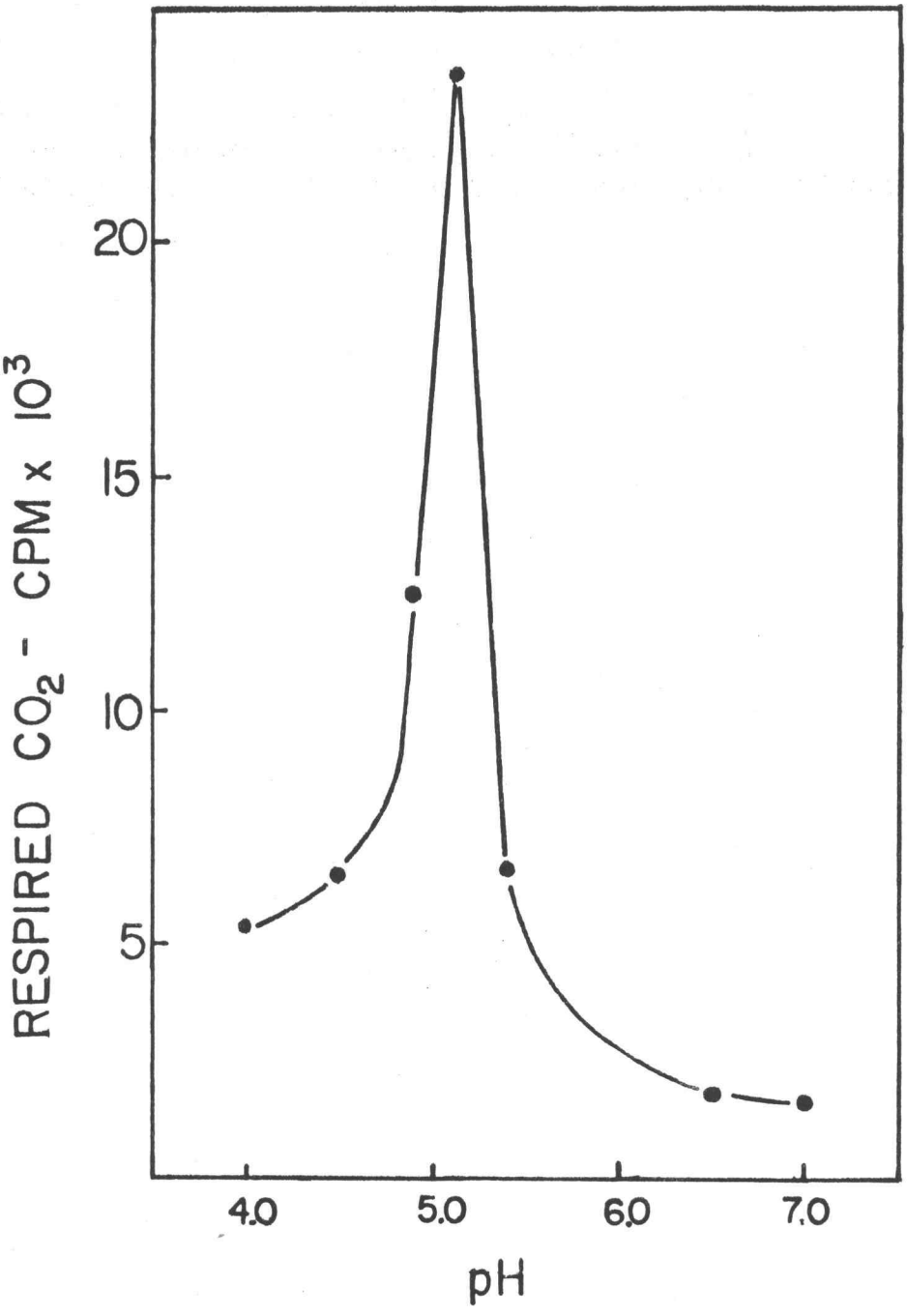



Figure 4. Effect of pH on formate oxidation. Reaction mixtures (5.0 ml) contained spore homogenate, 2 ml; formate-$C^{14}$, 1.0 µc (0.25 µmole); 0.15M citrate-phosphate buffer at the pH indicated. Incubation time, 2 hours.

that the primary hydrogen peroxide producing system is becoming inactivated below pH 5.1.

Catalase activity

The specific catalase activity of these spore extracts is in the order of 0.24 l x $g^{-1}$ x $sec^{-1}$. This value is very low compared to 22.4 for lysed cells of Micrococcus lysodeikticus (27, p. 784-788); however, great metabolic activity cannot be expected of a structure capable of surviving for years without endogenous substrates (15, p. 172).

## Primary Oxidizing System

In the experiments reported above, hydrogen peroxide was supplied by endogenous substrates. Although the primary oxidizing system producing hydrogen peroxide could not be completely identified, it was found that DPNH in conjunction with FMN and FAD stimulated formate oxidation in dialyzed extracts (Table I). It can also be seen that DPN had no effect on formate oxidation, and other experiments (unreported) showed that TPN behaved similarly. TPNH also stimulated formate oxidation, as shown in Table II.

A probable explanation for the above phenomena is

TABLE II. EFFECT OF REDUCED PYRIDINE NUCLEOTIDES AND FOLIC ACID ON FORMATE OXIDATION*

| Additions | CPM in $CO_2$ (average) | Error (%) | Increase (%) |
|---|---|---|---|
| Control | 359 | ±3 | |
| DPNH | 467 | ±11 | 30 |
| TPNH | 544 | ±6 | 51 |
| folate | 473 | ±2 | 32 |
| folate + DPNH | 698 | ±2 | 94 |
| folate + TPNH | 751 | ±5 | 109 |

*Duplicate reaction mixtures (5.2 ml) contained trypsin treated spore extract dialyzed 18 hours, 1 ml; formate-$C^{14}$, 0.5 µc (0.12 µmole); pyridine nucleotides, 16 µg as required; folic acid, 220 µg as required; 0.15M citrate-phosphate buffer at pH 5.1. Incubation time, 1 hour.

that DPNH reduces the flavins which in turn are oxidized by oxygen to produce hydrogen peroxide. Most reduced flavins have some capacity to react with oxygen to form hydrogen peroxide (55, p. 336).

Effect of folic acid

Folic acid stimulates formate oxidation, as shown in Table II. The fact that this stimulation is enhanced by reduced pyridine nucleotides suggests that tetrahydrofolate is involved. On first consideration it did not

seem likely that formyltetrahydrofolate was oxidized by complex I. Chance (4, p. 1341-1369) believed that only small molecules such as ethanol were accessible to the active center of complex I. However, Keilin and Hartree (32, p. 310-325) more recently have shown that molecules as large as catechol and pyrogallol are also oxidized. There is also a report (18, p. 413-421) that extracts from tissues of animals on a diet deficient in folic acid have impaired ability to oxidize formate.

Another explanation for the folate effect may be that formate is transferred to a molecule which in turn is oxidized to hydrogen peroxide or even to $C^{14}O_2$. It would seem that in the experiments employing dialyzed spore extracts that the hydrogen peroxide producing system is most likely to limit formate oxidation.

It is clear that the folate effect needs further investigation and preferably with enzymes more active than those derived from spores of T. contraversa.

## General Discussion

The foregoing experiments extend formate oxidation mediated by a catalase-hydrogen peroxide complex to the fungi, a group of organisms which had not been previously investigated in this respect. It is of interest from the

view of the biochemistry of parasitism that the mechanism of formate oxidation in T. contraversa differs from that expected in the host plant. Although positive reference to formate oxidation in wheat could not be found, higher plants such as the bean (13, p. 520-526) and the pea (40, p. 667-682) use a DPN dependent dehydrogenase to oxidize formate.

There is considerable disagreement about the physiological role of catalase. Lemberg and Legge (38, p. 415-419) are of the opinion that it protects the cell from the deleterious effects of hydrogen peroxide by decomposing it into water and oxygen. Keilin and Hartree (31, p. 293-301) consider this to be a wasteful process and doubt that the above catalatic decomposition occurs to any considerable degree under normal circumstances. They believe that catalase acts peroxidatically to oxidize compounds such as ethanol. Chance (5, p. 185-188), on the other hand, takes an intermediate position and believes that both the catalatic and peroxidatic reactions could occur simultaneously under proper normal conditions.

# RESULTS AND DISCUSSION

## PART II. OXALATE OXIDATION AND THE RELATIVE SIGNIFICANCE OF OXALATE FORMATION

### Properties of Oxalic Acid Oxidase

#### pH optimum and heat stability

The optimum pH for oxalate oxidation by spore particles is approximately 2.6 as shown by Figure 5. This result suggests that the molecule is not esterified and that the undissociated acid ($pK_1$ = 1.19) is the substrate for the enzyme.

It is apparent from Figure 6 that, unlike the moss oxidase (11, p. 602-603), the enzyme is unstable to moderate heat. It is believed that the sharp breaks in the curve are a reflection of the size of the broken spore particles, the smaller particles being inactivated first.

#### Effect of cyanide and fluoride

The data in Figure 7 show that enzyme activity is inhibited 50% by $1.6 \times 10^{-4}$M cyanide and by $2.4 \times 10^{-6}$M fluoride. The data were obtained from experiments performed at different times, hence the differences in reaction rate.

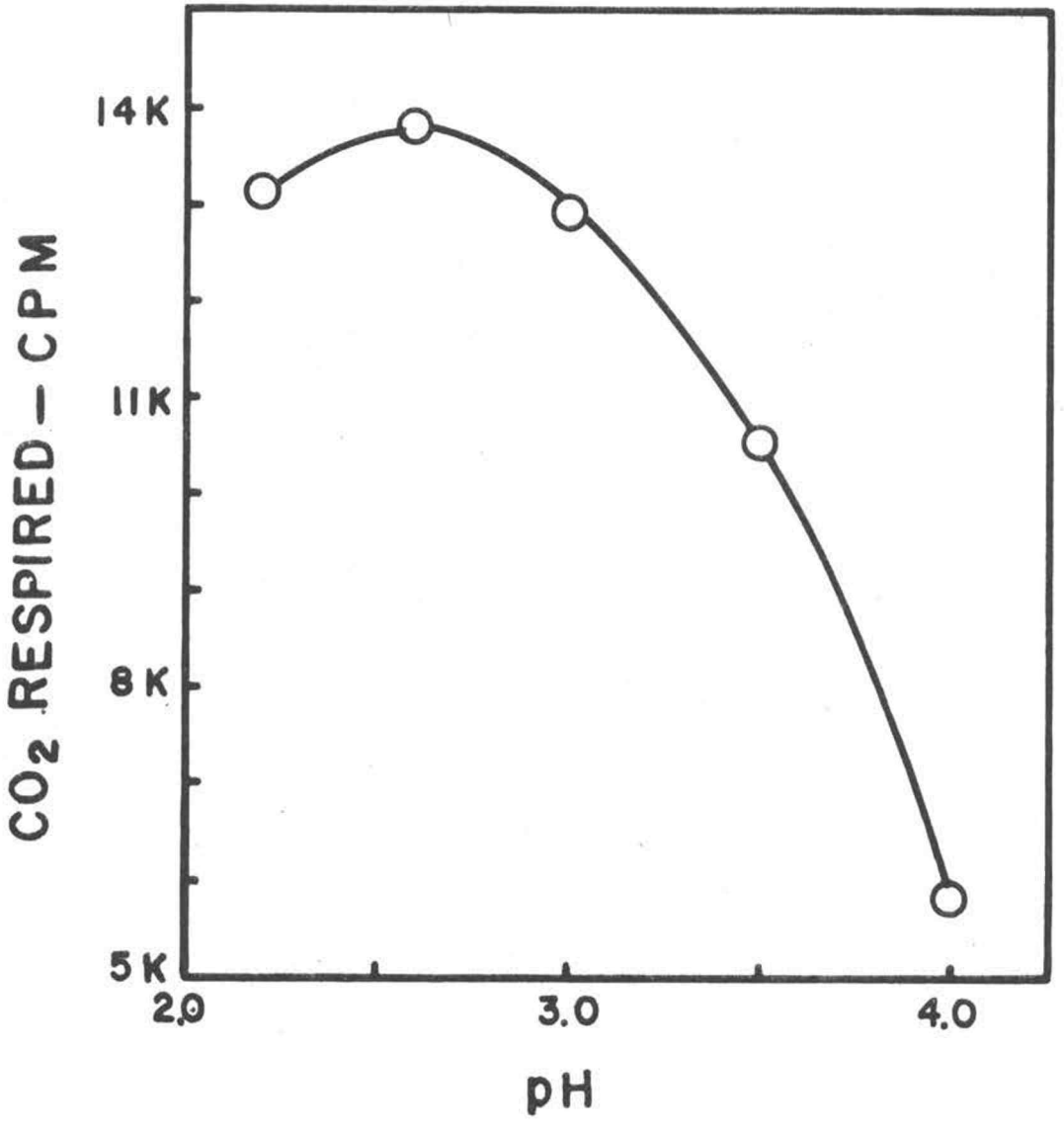



Figure 5. Effect of pH on oxalate oxidation. Reaction mixtures (4.5 ml) contained spore particles, 1 ml; oxalate-$C^{14}$, 0.3 µc (1.9 µmoles); 0.15M citrate-phosphate buffer at the pH indicated. Incubation time, 0.5 hour.

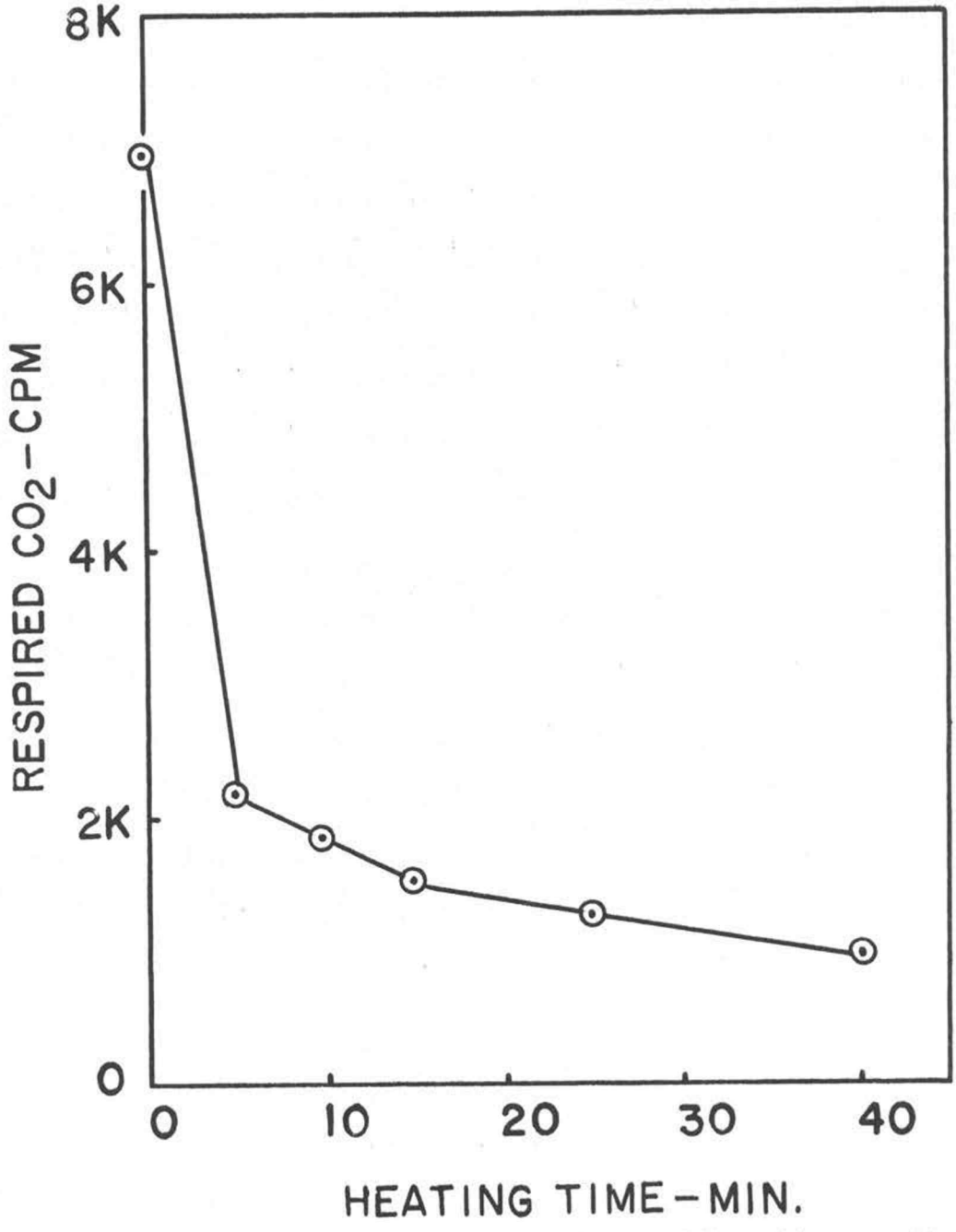



Figure 6. Effect of heat on oxalic acid oxidase. Reaction mixtures (3.5 ml) contained spore particles heated at 70° for the time indicated, 1 ml; oxalate-$C^{14}$, 0.3 uc (1.9 umoles); 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, 0.5 hour.

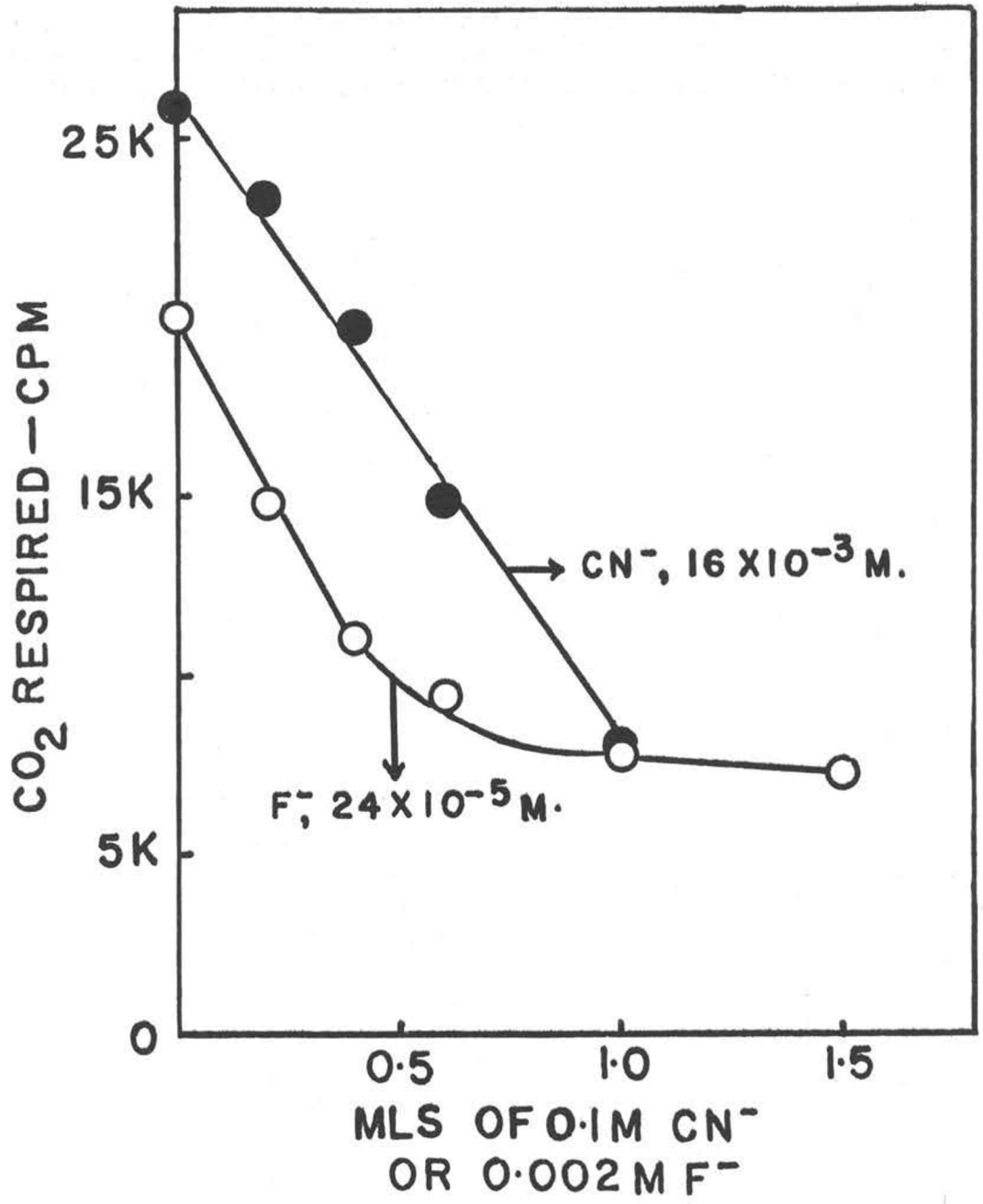



Figure 7. Effect of cyanide and fluoride on oxalate oxidation. Reaction mixtures (4.0 ml) contained spore particles, 1 ml; oxalate-$C^{14}$, 0.5 µc (0.15 µmole); inhibitor as indicated; 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, 0.5 hour.

Oxidation

Figure 8 shows that oxalate catabolism by spore acetone powders is associated with oxygen uptake and is thus an oxidation. The respiratory quotient, corrected for endogenous respiration, suggests that the reaction is the summation (reaction 3) of the following reactions:

1. $(COOH)_2 + O_2 \rightarrow 2\ CO_2 + H_2O_2$ RQ = 2
2. $H_2O_2 \xrightarrow{\text{catalase}} \frac{1}{2}\ O_2 + H_2O$

3. $(COOH)_2 + \frac{1}{2}\ O_2 \rightarrow 2\ CO_2 + H_2O$ RQ = 4

Normally catalase is not expected to be active at the pH values used in this experiment (32, p. 310-325). However, since the cell wall of the acetone powders was intact, there is a possibility that catalase was protected from rapid inactivation which could account for the observed RQ of 3.2.

The Experimental Problem

It was not possible to study the reaction further with acetone powders or spore particles by the usual manometric techniques since increasing the pH of the reaction mixture in an attempt to obtains RQ's closer to the theoretical value simply increased the already high

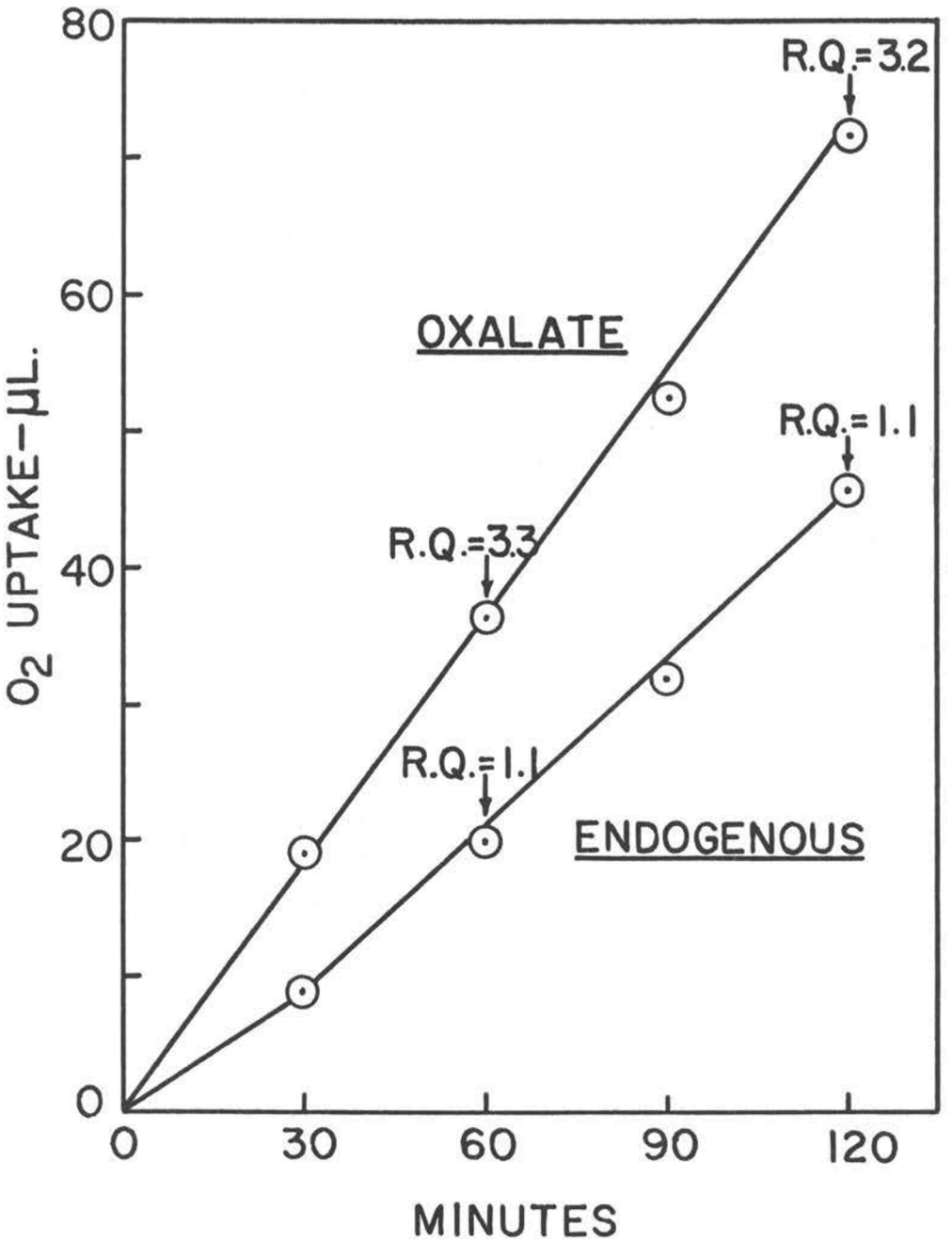



Figure 8. Oxygen consumption during oxalate oxidation by spore acetone powders. Warburg flasks contained spore acetone powders (230 mg) suspended in 2.3 ml of 0.15M citrate-phosphate buffer at pH 2.6; oxalic acid, 0.5 ml (8.0 µmoles); KOH or water in the center well, 0.2 ml.

endogenous respiration. Further, inhibitors of catalase, such as azide, cyanide and fluoride, either evolved gas at low pH or inhibited the oxygen consumption. Even attempts to measure hydrogen peroxide formation failed (vide infra). It thus became necessary to eliminate other alternative oxidative mechanisms from consideration.

The most likely alternative mechanisms appeared to be (1) that pyridine nucleotides and the cytochrome system were involved in oxalate oxidation, or (2) that oxalate was decarboxylated to formate which in turn indirectly stimulated oxygen consumption. Formate stimulation of oxygen consumption would be an indirect effect because formate is oxidized by means of a catalase-hydrogen peroxide complex. The possibility that oxalate was being extensively anabolized and then oxidized was not given serious consideration because of the low pH at which the reaction was studied.

## Products of Oxalic Acid Oxidation

### Non-participation of formate

Figure 9 shows that formate is only slightly oxidized at the pH where oxalate oxidation is almost at a maximum. It is thus clear that if formate were a product of oxalate decarboxylation, it would accumulate in

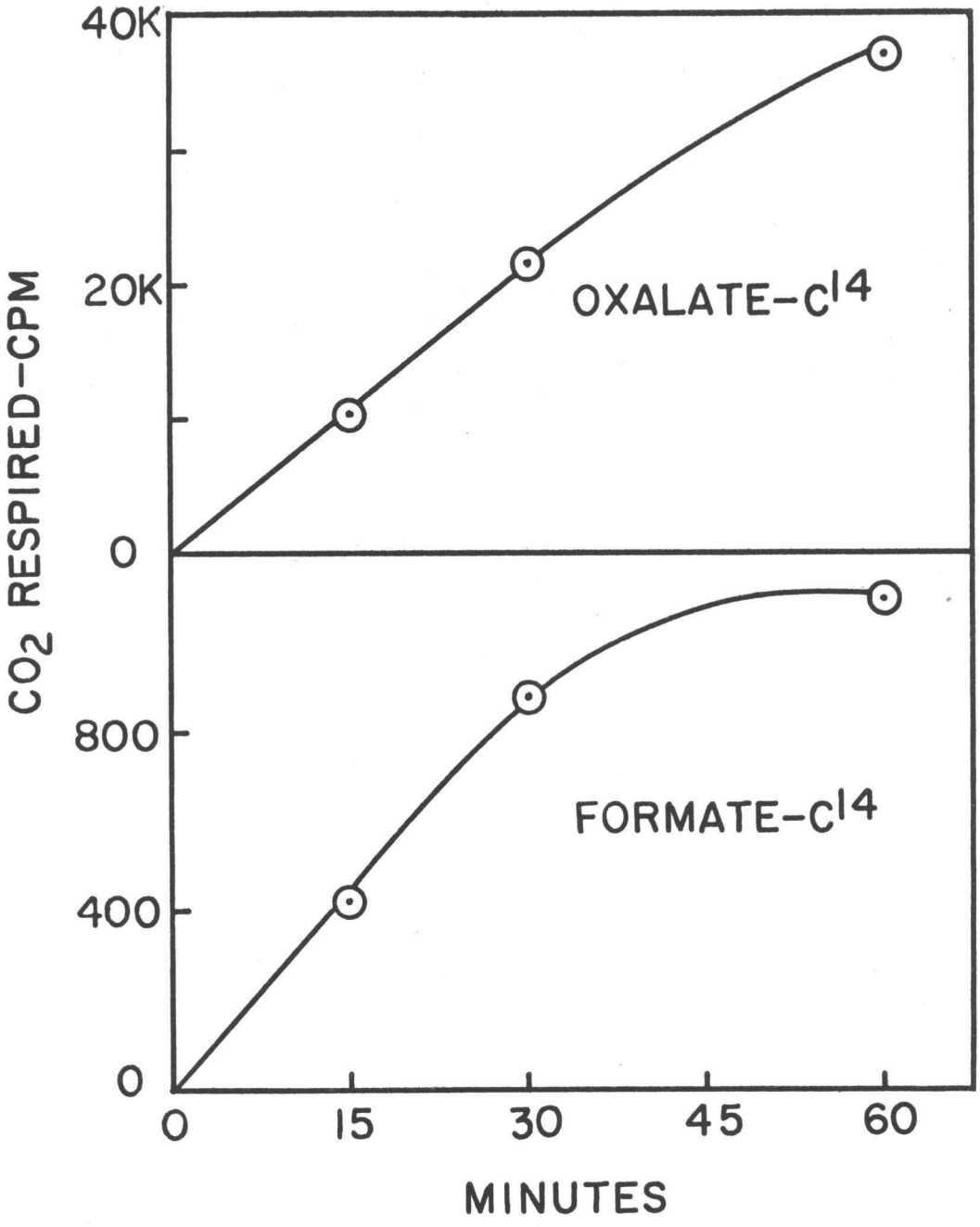



Figure 9. Comparison of formate and oxalate oxidation at pH 3.0. Reaction mixtures (3.5 ml) contained spore particles, 1 ml; oxalate or formate-$C^{14}$, 1 µc (1.9 µmoles); 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, as indicated.

easily detectable amounts. However, formate-$C^{14}$ could not be detected as one of the oxalate oxidation products by the method of steam distillation followed by silica gel chromatography.

Effect of pyridine nucleotides and other cofactors

Table III shows a typical result obtained by the addition of cofactors to the reaction mixture. It should be emphasized that the spore particles were first dialyzed against water containing Dowex 50 to remove pyridine nucleotides. It can be seen that none of the cofactors had a dramatic effect on oxalate oxidation, considering that the experimental methods are subject to an error of about 5%.

The experiment indicated, however, that the enzyme was reasonably stable to dialysis and that cofactors, if any, must be bound to the enzyme.

Evidence for hydrogen peroxide

As mentioned previously, direct evidence for hydrogen peroxide as a product of oxalate oxidation could not be obtained. The methods used to detect hydrogen peroxide were briefly as follows: manometric experiments employing catalase, colorimetric experiments employing

TABLE III. EFFECT OF CO-FACTORS ON OXALATE OXIDATION*

| Additions | CPM in $CO_2$ | Difference % |
|---|---|---|
| Control | 39,100 | |
| FMN, 0.2 mg | 43,700 | +11.8 |
| CoA, 0.2 mg | 35,700 | - 8.7 |
| DPN, 1.0 mg | 38,000 | - 2.8 |
| TPN, 1.0 mg | 40,900 | + 4.6 |

*The reaction mixtures (4.0 ml) contained spore particles dialyzed against Dowex 50 for 40 hours, 1 ml; oxalate-$C^{14}$, 0.5 µc (0.15 µmole); co-factors as indicated; 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, 0.5 hour.

pyrogallol and peroxidase, chemical methods employing iodide or titanium sulphate. However, since the alternative methods for oxalate oxidation were eliminated as described above, it was concluded that hydrogen peroxide was indeed formed but that it might be reacting with undialyzable compounds in the spore particles such as protein or unsaturated fat.

Evidence that hydrogen peroxide was perhaps reacting with oxalic acid oxidase was obtained by a time-course study of the oxidation. It can be seen from Figure 10 that the reaction slows down after 60 minutes' incubation and practically ceases after 180 minutes. Again the sharp breaks in the curve are thought to be

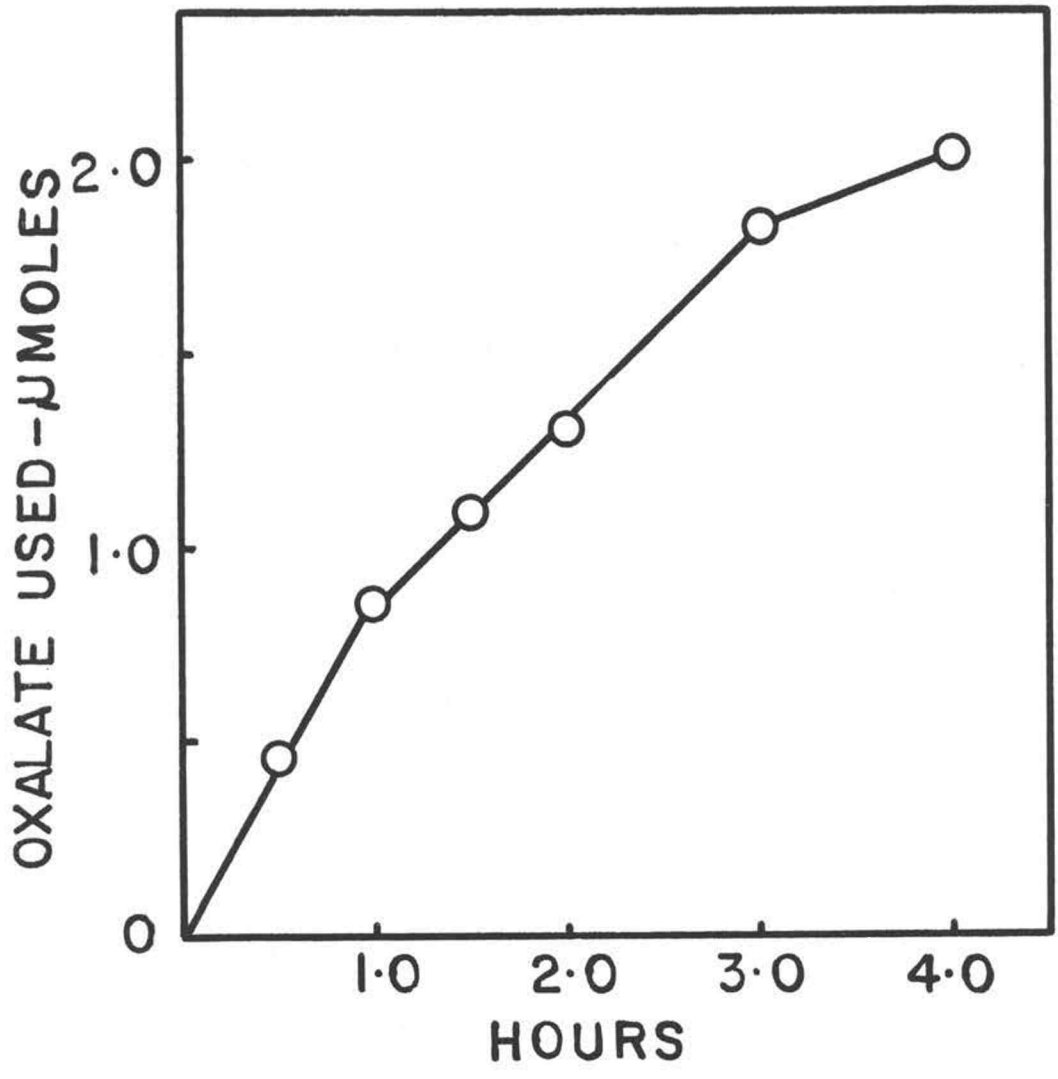



Figure 10. Time-course study of oxalate oxidation. Reaction mixtures (4.0 ml) contained spore particles, 1 ml; oxalate-$C^{14}$, 0.5 μc (30 μmoles); 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, as indicated.

due to the spore particle size. There is no reason to suspect the pH 3.0 buffer for this loss of activity since the enzyme in this buffer is stable for at least 24 hours at 0°C.

The best evidence for the presence of hydrogen peroxide comes from the role of flavins in the oxidation. Table IV shows that riboflavin and FMN (but not FAD) increase oxidation by particles that had been dialyzed against HCl at pH 1.7 for 16 hours at 3-5°C.

TABLE IV. EFFECT OF FLAVINS ON OXALATE OXIDATION*

| Additions | CPM in $CO_2$ | Increase % |
|---|---|---|
| Control | 193 | |
| FAD | 186 | nil |
| FMN | 376 | 190 |
| Riboflavin | 507 | 260 |

*The reaction mixtures (4.2 ml) contained spore particles dialyzed against an HCl solution at pH 1.7 for 16 hours, 1 ml; oxalate-$C^{14}$, 0.25 µc (0.6 µmole); flavins, 0.2 mg; 0.15M citrate-phosphate buffer at pH 3.0. Incubation time, 0.5 hour.

Although this treatment caused excessive destruction of the enzyme, it was the only way in which an unequivocal

flavin effect could be demonstrated.

## Relative Significance of Oxalate Formation

It was desirable to estimate the extent that oxalate participated in the carbon catabolism of T. contraversa. It can be seen from Table V that the ratio of carbon dioxide respired to oxalate formed is quite low

TABLE V. EXTENT OXALATE PARTICIPATES IN CATABOLISM OF PYRUVATE AND ACETATE*

| Substrate | Total CPM | | Ratio |
|---|---|---|---|
| | $CO_2$ | Oxalate | $CO_2$/oxalate |
| Pyruvate-1-$C^{14}$ | 646 | 868 | 0.7 |
| Acetate-1-$C^{14}$ | 738 | 293 | 2.5 |

*The reaction mixtures (5.0 ml) contained spore acetone powders, 0.6 g; pyruvate or acetate-1-$C^{14}$, 1.0 µc (0.20 µmole); 0.15M Tris buffer at pH 7.0. Incubation time, 2.0 hours.

when pyruvate-1-$C^{14}$ and acetate-1-$C^{14}$ are used as substrates. Since pyruvate and acetate are key intermediates in carbohydrate catabolism, the above low ratios probably indicate that oxalate is on the main pathway of carbohydrate catabolism. It was not ncessary

to use an oxalate "trap" in this experiment since oxalate is not detectably oxidized at pH 7.0.

## General Discussion

Since T. contraversa oxalic acid oxidase produces carbon dioxide and probably hydrogen peroxide, it is similar to the higher plant enzymes rather than to the microbial enzymes studied so far. There is a possibility that oxalate could be involved in plant infection symptoms. Oxalate appears to be on the pathogens' main pathway of carbohydrate catabolism and can be expected to accumulate at a pH value near 7. There is thus an opportunity for it to diffuse into the plant. Oxalic acid has several properties which might be of concern in plant pathological processes. It is not only a strong acid, but also a powerful chelator of metal ions. It is also known to competitively inhibit the forward reaction of lactic dehydrogenase (48, p. 1143-1148).

Chelators have received some attention as causative agents of plant disease symptoms. Lycomarasmin, a dipeptide produced by the fungus Fusarium lycopersici, forms water soluble iron complexes. When lycomarasmin is applied to tomato cuttings it carries iron from the stem to the leaves where it releases part of the iron

(34, p. 361). Thus the compound causes iron deficiency in the stem and iron toxicity in the leaves.

Arguments implicating oxalate as a wheat plant toxin are highly speculative in view of the report that wheat seeds contain oxalic acid oxidase (61, p. 139-152) and that healthy plants contain oxalate (44, p. 1040-1043). However, it would be interesting to investigate the possibility that resistant and non-resistant wheat varieties have different levels of the oxidase.

# RESULTS AND DISCUSSION

## PART III. PATHWAYS TO FORMATE AND OXALATE

### Precursors of Oxalate

Tracer studies of the metabolic pathways leading to oxalate and formate in T. contraversa were seriously hampered by the low metabolic activity of the teliospores. Usually it was not feasible to employ the comparatively rapid techniques of paper chromatography for identification of products since the paper absorbed too much of the carbon-14 radiation. The low activity of the teliospores also prevented use of the kinetic approach to determine precursor and product relationships. For these reasons only certain products of the enzymic reactions were examined for radioactivity after incubation of numerous carbon-14 labelled substrates in the presence of T. contraversa spore preparations.

At the outset of these studies it was hypothesized that oxalate was formed from the hydrolysis of oxalacetate. Since oxalacetate-$C^{14}$ was not available commercially it was not possible to test this compound directly. However, in many organisms oxalacetate is formed from pyruvate either (1) by carbon dioxide fixation or (2) by the citric acid cycle enzymes. If

situation (1) were applicable, then oxalate should be labelled similarly if derived from either pyruvate-1-$C^{14}$ or pyruvate-2-$C^{14}$. If situation (2) applied, then C-1 of pyruvate would be rapidly lost as carbon dioxide and oxalate would be more heavily labelled from pyruvate-2-$C^{14}$. The results presented in Experiment 1, Table VI, fail to support either pathways (1) or (2).

Although the experimental data shown in Table VI are only strictly comparable within the individual experiments, the results of Experiment 2 tend to support Experiment 1. This is because pyruvate is decarboxylated to acetate in many biological systems; hence acetate-1-$C^{14}$ is equivalent to pyruvate-2-$C^{14}$.

The oxalacetate hypothesis was tested further with succinate on the assumption that the citric acid cycle was in operation. If the hypothesis were to apply then oxalate should be labelled similarly if derived from either succinate-1,4-$C^{14}$ or succinate-2,3-$C^{14}$. The results presented in Experiment 3, Table VI, again fail to implicate oxalacetate as the direct precursor of oxalate.

Since several previous workers had shown that glycolate and glyoxalate were precursors of oxalate (see p.12-14) glycolate was tested with T. contraversa enzymes. It was not practical to test glyoxalate since

TABLE VI. PRECURSORS OF OXALATE**

| Experiment No. | Carbon-14 Substrate* | Total CPM | | Ratio |
|---|---|---|---|---|
| | | $CO_2$ | Oxalate | $CO_2$/oxalate |
| 1 | pyruvate-1 | | 900 | |
| | pyruvate-2 | | 210 | |
| | pyruvate-3 | | 36 | |
| 2 | pyruvate-1 | 646 | 868 | 0.7 |
| | acetate-1 | 738 | 293 | 2.5 |
| 3 | succinate-1,4 | 2,718 | 10 | 272.0 |
| | succinate-2,3 | 272 | 117 | 2.3 |
| 4 | glycolate-2 | 622 | 81 | 7.7 |
| 5 | formate-1 | 50,000 | 2,290 | 21.8 |
| 6 | $NaHC^{14}O_3$ | | nil | |

*The number indicates the labeled carbon atom

**Experimental conditions. All experiments were brought to the volume indicated with 0.15M Tris buffer at pH 7.0. Otherwise they contained the following:

Experiment 1. Acetone powders, 1.5 g; substrate, 2.0 µc (0.1 µmole); $NaHCO_3$, 1 mg; $CaCl_2$, 5 mg. Volume, 7.0 ml. Incubation time, 1 hour.

Experiment 2. Acetone powders, 0.6 g; substrate, 1.0 µc (0.2 µmole). Volume, 5 ml. Incubation time, 2 hours.

Experiment 3. Acetone powders, 1 g; substrate, 1 µc (0.09 µmole). Volume, 4.5 ml. Incubation time, 1.5 hours.

Experiment 4. Acetone powders, 1.0 g; substrate, 1 µc (1.2 µmoles). Volume, 4.5 ml. Incubation time, 1.5 hours.

Experiment 5. Acetone powders, 0.5 g; substrate, 2 µc (0.3 µmole). Volume, 4.0 ml. Incubation time, 1.5 hours.

Experiment 6. Spore particles, 1 ml; $NaHC^{14}O_3$, 22.4 µc. Volume, 4.5 ml. Incubation time, 1.0 hour.

this compound is oxidized by boiled teliospores and it is thus difficult to prove that an enzyme catalyzed reaction occurs. The results of this experiment are shown in Experiment 4, Table VI, and it is apparent that glycolate is also unsatisfactory to consider as the principal precursor of oxalate.

Since all of the experiments discussed so far failed to implicate a two carbon fragment as a precursor of oxalate, a new hypothesis was formulated which assumed that oxalate was formed by condensation of two moles of formate. When this hypothesis was tested, it was found that formate was indeed an excellent precursor of oxalate (Experiment 5, Table VI). The possibility that the carbon dioxide evolved in formate oxidation was involved in oxalate formation was ruled out on the basis of the results shown for Experiment 6, Table VI.

In an attempt to obtain better evidence for formate as a direct precursor of oxalate, an experiment was designed in which the catalase-induced formate oxidation was inhibited by cyanide. It was anticipated that this would make more formate available for oxalate synthesis. Table VII shows the results obtained from this experiment. It can be seen that cyanide inhibits formate oxidation but that oxalate synthesis is not significantly affected. Although the last result was unexpected it

TABLE VII. EFFECT OF CYANIDE ON OXALATE FORMATION FROM FORMATE-$C^{14}$*

| NaCN (mg) | Total CPM | |
|---|---|---|
| | $CO_2$ | Oxalate |
| 0 | 62,126 | 1,208 |
| 0.2 | 24,420 | 1,224 |
| 0.5 | 13,805 | 1,164 |
| 1.0 | 9,391 | 1,181 |

*The reaction mixtures (5.0 ml) contained acetone powders, 0.5 g; formate-$C^{14}$, 2.0 µc (0.3 µmole); sodium cyanide, as indicated; 0.15M Tris buffer at pH 7.0. Incubation time, 1 hour.

might be explained on the basis of the amount of oxalate synthetase present. Very small amounts of this enzyme would be saturated by small amounts of formate; thus an excess of formate would not change the amount of oxalate formed in a given time.

## General Discussion of Oxalate Precursors

The possibility that formate synthesizes oxalate by self condensation is of interest in connection with formate oxidation by the catalase-hydrogen peroxide complex. It will be recalled (p. 29-30) that the enzyme system producing the hydrogen peroxide necessary

to react with catalase could not be completely identified. It would indeed be ironic if formate itself were supplying some of the hydrogen peroxide to oxidize itself, for example, through the following reactions:

(1) $2\ HCOOH \rightarrow (COOH)_2 + 2\ (H)$

(2) $2\ (H) + O_2 \rightarrow H_2O_2$

(3) $HCOOH + H_2O_2 \xrightarrow{\text{catalase}} CO_2 + 2\ H_2O$

---

Sum: $3\ HCOOH + O_2 \rightarrow (COOH)_2 + CO_2 + 2\ H_2O$

This, however, cannot be the only source of hydrogen peroxide because a summation of these reactions indicates that the radioactivity in oxalate should be twice that of carbon dioxide. That this is not true is shown by the data in Experiment 5, Table VI.

In addition to the above conjectures there are some facts that were revealed by the studies on the pathway of oxalate formation. It is apparent that formate can enter into synthetic reactions even though it is rapidly oxidized by a catalase-hydrogen peroxide complex. Further, the enzyme or enzyme system catalyzing oxalate synthesis from formate is resistant to cyanide poisoning.

## Precursors of Formate

Experiments designed to account for the pathways to formate in T. contraversa were especially time consuming because the small amounts of formate involved could only be isolated by techniques involving steam distillation and column chromatography.

The results of an experiment employing pyruvate as a substrate are shown in Experiment 1, Table VIII. These results suggest that pyruvate is at least partially degraded to acetate. This being true then the methyl carbon of acetate, equivalent to carbon-3 of pyruvate, was implicated as a possible precursor of formate. The reason that acetate was not similarly labelled from pyruvate-2-$C^{14}$ and -3-$C^{14}$ was not investigated but it may be due to recycling of carbon atoms.

The possibility that the methyl carbon of acetate was a precursor of formate was confirmed by Experiment 2, Table VIII. The carboxyl carbon was not so implicated by this experiment.

A route for the conversion of the methyl carbon of acetate to formate was described by Bolcato et al. (3, p. 372-377). It involved the following sequence of events:

TABLE VIII. PRECURSORS OF FORMATE AND ACETATE**

| Experiment No. | Carbon-14 Substrates* | Total CPM | | |
|---|---|---|---|---|
| | | $CO_2$ | Acetate | Formate |
| 1 | Pyruvate-1 | 9,750 | 40 | 99 |
| | Pyruvate-2 | 3,600 | 893 | 56 |
| | Pyruvate-3 | 2,560 | 9,397 | 560 |
| 2 | Acetate-1 | | | nil |
| | Acetate-2 | | | 1,665 |
| 3 | Acetate-1 (control) | 29,500 | | |
| | Acetate-1 + glycolate | 30,700 | | |
| | Acetate-1 + glyoxalate | 27,100 | | |

* The numbers indicate the labelled carbon atom.

**Experiment conditions. All experiments were brought to the volume indicated with 0.2M phosphate buffer at pH 7.0. Otherwise they contained the following:

Experiment 1. Washed spores, 5.0 g (wet weight); pyruvate-$C^{14}$, 2.0 µc (2.3 µmoles); sodium formate, 2.0 mg. Volume, 10.0 ml. Incubation time, 2 hours.

Experiment 2. Spore homogenate, 1.5 g; acetate-$C^{14}$, 2.5 µc (0.5 µmole); sodium formate, 0.5 mg. Volume, 6.0 ml. Incubation time, 1 hour.

Experiment 3. Acetone powders, 1.0 g; acetate-1-$C^{14}$, 2.0 µc (0.39 µmole); sodium glycolate or glyoxalate, 26 µmoles as required. Volume, 7.0 ml. Incubation time, 1.5 hours.

$$\begin{array}{l}\text{COOH}\\ |\\ \text{CH}_3\end{array} \xrightarrow{\frac{1}{2}O_2} \begin{array}{l}\text{COOH}\\ |\\ \text{CH}_2\text{OH}\end{array} \xrightarrow{-2H} \begin{array}{l}\text{COOH}\\ |\\ \text{CHO}\end{array} \xrightarrow{\frac{1}{2}O_2} CO_2 + HCOOH$$

If these reactions were applicable in T. contraversa then the addition of the non-radioactive intermediate should dilute the specific activity of the radioactive intermediate formed metabolically and consequently that of the respired carbon dioxide. The results presented in Experiment 3, Table VIII, show, however, that neither glycolate nor glyoxalate significantly alter the radioactivity of the respiratory carbon dioxide. It thus appears unlikely that acetate is oxidized to formate by Bolcato's mechanism, in T. contraversa.

Similar results perhaps could have been obtained if the actual intermediate was a derivative of glycolate or glyoxalate, as for example glycolyl coenzyme A. However, since glycolate-1-$C^{14}$ and -2-$C^{14}$ as well as glyoxalate-$C^{14}$ are readily converted to carbon dioxide by the same acetone powders, there is no reason to suspect that any necessary derivative could not have been formed.

In connection with glyoxalate oxidation or decarboxylation, it should be mentioned again that the reaction also occurs with boiled acetone powders. It is, therefore, difficult to determine whether or not any

enzymatic mechanism exists for its degradation.

## General Discussion of Formate Precursors

Since the above experiments failed to implicate glycolate and glyoxalate as intermediates in acetate oxidation to formate, it was necessary to formulate a new hypothesis to account for the experimental results. A survey of Fruton and Simmonds (19, p. 605-606) and Thimann (57, p. 474-475) indicated that the following reactions were biologically feasible and gave the correct radioactive labelling:

(1) $2\ C^*H_3.COOH \rightarrow C^*H_3.CO.C^*H_2.COOH \rightarrow C^*H_3.CO.C^*H_3 + CO_2$

(2) $C^*H_3.CO.C^*H_3 \rightarrow C^*H_3.CHOH.C^*H_2OH \rightarrow C^*H_3COOH + HC^*OOH$

It should be noted that these reactions regenerate acetate and thus can explain the differences in the labelling of acetate from pyruvate-2-$C^{14}$ and -3-$C^{14}$ (Experiment 1, Table VIII). It can be seen from the equations that the carboxyl group of acetate is readily lost as carbon dioxide whereas the methyl group tends to be conserved.

There is yet another possibility for the formation of formate. It will be recalled that pyruvate-1-$C^{14}$ was a comparatively reasonable precursor of oxalate

(Experiment 1, Table VI) and that formate was implicated in oxalate formation (Experiment 5, Table VI). Thus it may be that pyruvate is split between carbon-1 and carbon-2 to yield formate and acetate. Although little formate could be isolated from pyruvate-1-$C^{14}$ in Experiment 1, Table VIII, this could result if a formyl derivative were the actual product of a pyruvate split. In that event the formate trap used in the experiment would perhaps be ineffective and formyl-X could be rapidly converted to oxalate.

Although this section of the thesis on pathways to formate and oxalate is inconclusive, it is believed that the results of these experiments provide a logical premise for future work.

## SUMMARY

A study was made of the metabolism of formic and oxalic acids in teliospores of the wheat smut fungus, Tilletia contraversa. The high endogenous respiration and low activity of teliospore enzyme preparations made it necessary to depend largely on radioactive carbon compounds for information on metabolism.

It was found that formate decarboxylation by teliospore extracts was competitively inhibited by ethanol and nitrite and unaffected by carbon monoxide or oxidized pyridine nucleotides. These results indicated that formate was oxidized by a catalase-hydrogen peroxidase complex, a reaction hitherto not described for the plant kingdom. The pH optimum for this oxidation was at 5.1.

The enzyme system or systems producing the hydrogen peroxide necessary to form the catalase-hydrogen peroxide complex could not be completely identified. However, formate oxidation was stimulated by reduced diphosphopyridine nucleotide in conjunction with flavin nucleotides. These reactions are well known for their ability to produce hydrogen peroxide.

Folic acid in conjunction with reduced diphosphopyridine nucleotide or reduced triphosphopyridine

nucleotide also stimulated formate oxidation. It thus seemed likely that tetrahydrofolate was the stimulating factor. Beyond this, the folic acid effect was not investigated.

Teliospores also oxidized oxalic acid. The enzyme concerned with this oxidation could not be solubilized from broken teliospore particles. These particles oxidized oxalic acid optimally at a pH near 2.6, which indicated that only undissociated oxalic acid was a substrate for the enzyme. The oxidation was inhibited 50% by $2.4 \times 10^{-6}$M fluoride, by $1.6 \times 10^{-4}$M cyanide and by heating at 70° for 4 minutes.

The products of oxalic acid oxidation are carbon dioxide and probably hydrogen peroxide. Although hydrogen peroxide could not be determined directly, it was shown that formate was not a product and that pyridine nucleotides were not involved in the oxidation. The best evidence for hydrogen peroxide was that the oxidation could be stimulated by riboflavin and flavin mononucleotide, but not by flavin adenine dinucleotide.

The low ratio of carbon dioxide to oxalate formed from key intermediates of carbohydrate metabolism such as pyruvate-1-$C^{14}$ (ratio 0.7) and acetate-1-$C^{14}$ (ratio 2.5), was considered to be evidence that oxalate was on

the main pathway of carbohydrate metabolism. Since oxalate is apparently metabolically inert at pH 7.0, there is an opportunity for it to diffuse from the pathogen into the host plant. Thus, oxalate by virtue of its properties as a strong acid, chelator, and enzyme inhibitor could be responsible for some of the symptoms of plant disease.

An attempt was made to find the substrate directly responsible for oxalate and formate synthesis. The evidence indicated that oxalate might be formed by condensation of two moles of formate. The participation of oxalacetate, acetate, glycolate or other two carbon compounds in oxalate synthesis appeared most unlikely.

The pathway to formate synthesis is still in doubt. However, there were reasons for considering that pyruvate might be cleaved into formate and acetate. The experimental evidence also was in accord with a hypothesis which involved the methyl carbon of acetone, originating from acetoacetate, as a precursor of formate.

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