METABOLISM OF GLYCOLIC ACID

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Myron Kuczmak

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This is to certify that the

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METABOLISM OF GLYCOLIC ACID

presented by

Myron Kuczmak

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Major professor

N. E. Tolbert

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By

Myron Kuczmak

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University in partial
fulfillment of the requirements for
the degree of

DOCTOR OF PHILOSOPHY

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AN ABSTRACT

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ABSTRACT

The purpose of this investigation was to describe the conditions for activation of glycolic acid oxidase, both *in vitro* and *in vivo* and to study the inhibitory effect of glycolate and glyoxylate on citric acid cycle oxidation.

Glycolic acid oxidase, an enzyme, catalyzing the oxidation of glycolic acid to glyoxylic acid and H_2O_2 and also glyoxylic acid to oxalic acid is a flavoprotein with a prosthetic group of FMN. FAD cannot substitute for FMN when the enzyme is partially purified. Utilization of FAD by crude saps for enzyme activity was due to enzymatic conversion of FAD to FMN.

Glycolic acid oxidase is very active in extracts from leaves of green plants. However, extracts of leaves from etiolated wheat seedlings and sheaths of both green and etiolated plants contain much less activity. Extracts of etiolated leaves contain apoenzyme which could be activated by 10^{-4} M FMN. The amount of apoenzyme was considerably less than the amount of enzyme in green tissue.

The enzyme is predominantly a soluble cytoplasmic enzyme, although some enzymatic activity is associated with chloroplast or mitochondria. During the oxidation of glycolic acid by mitochondria there was a little esterification of orthophosphate.

Exposure of etiolated plants to the light causes the formation of both active enzyme and of more apoenzyme. Etiolated plants contain proportionally more apoenzyme to holoenzyme than plants exposed to light. The ratio of apoenzyme to holoenzyme in etiolated wheat seedlings is 9 to 1 and in green ones about 5 to 1. The amount of the apoenzyme plus holoenzyme from etiolated plants is much less than the amounts in green plants. Thus the increase in this enzyme is associated with greening of the plant.

Green plants contain twice as much FMN as etiolated plants, but this physiological level of FMN was about 10^{-8} M, which is below the 10^{-4} M concentration of FMN necessary to activate the enzyme in vitro.

Glycolic acid oxidase can be activated in vitro at 2°C by the incubation of plant cell-free homogenates with its substrates, glycolate, lactate or α-hydroxybutyrate. This method of enzyme activation cannot be duplicated on a dialyzed ammonium sulfate precipitate of the enzyme. The nature of the in vitro activation is not known.

The enzyme also can be activated in vivo in the dark by feeding the etiolated plants with glycolate, lactate, α-hydroxybutyrate or glyoxylate.

Both glycolate and glyoxylate are the effective inhibitors of the oxidation of citric acid cycle acids by isolated plant mitochondria. Because glycolic acid is

rapidly oxidized by pea mitochondria by associated glycolic acid oxidase, the inhibitory effect of glycolate was attributed to the glyoxylate formed. Glyoxylate was active as an inhibitor of the oxidation by the mitochondria of each acid from the citric acid cycle. The P/O ratio of residual activity in the presence of the inhibitor was not reduced in the inhibited system. Glyoxylate also severely inhibited the action of isolated malic and lactic dehydrogenase.

The inhibitory effect of glyoxylate can be reversed by addition of excess of DPN. This was demonstrated with both malic or lactic dehydrogenase and with mitochondrial oxidation.

The inhibition by glyoxylate of citric acid cycle oxidation is probably due to non-enzymatic formation of a complex with reduced DPNH. This complex was detected on paper chromatograms by its fluorescence and R_f values.

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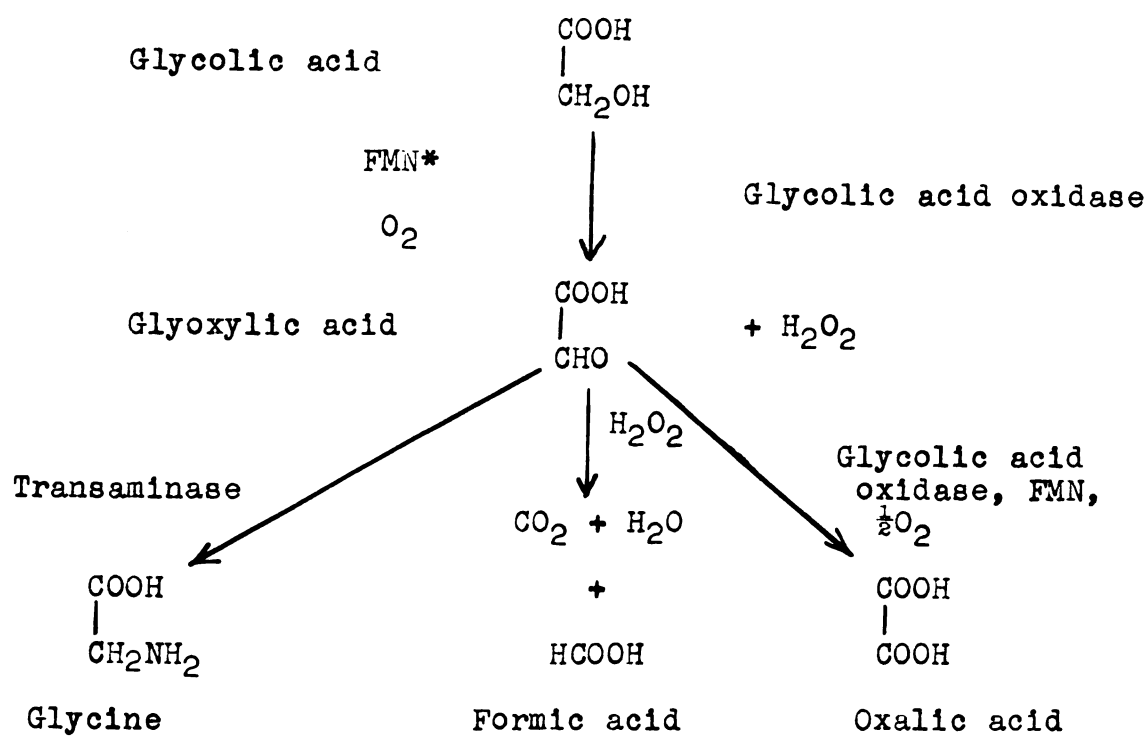
INTRODUCTION

INTRODUCTION

Interest in the metabolism of glycolic acid is a half-century old and a great number of reports have appeared concerning this problem.

Glycolic acid, in the presence of the enzyme glycolic acid oxidase, is metabolized to glyoxylic acid and this, in turn, to glycine or oxalic acid or carbon dioxide and formic acid as represented schematically below:



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* The following abbreviations are used: FMN, riboflavin-5-phosphate; FAD, flavin adenine dinucleotide; DPNH, diphosphopyridine nucleotide (reduced); DPN, diphosphopyridine nucleotide (oxidized); ATP, adenoisine-5'-triphosphate; DOPA, dihydroxyphenylalanine.

Glycolic acid arises as an early product of the photosynthesis (1, 2) and it is an important metabolically intermediate (3) of wide distribution in plant tissues (4). The enzyme glycolic acid oxidase is very widely distributed in plant tissue (4, 5). When combined with glyoxylic acid reductase, which catalyzes the reverse reaction to convert glyoxylic acid into glycolic acid, the system has been considered as a possible terminal oxidase (6, 7). Glycolic acid and glyoxylic acids function as intermediates in the biosynthesis of glycine and serine. On the other hand, glyoxylic acid, as the oxidation product of glycolic acid, inhibits many oxidative reactions, and a number of reports have been devoted to this problem (82, 86, 88).

The present report concerns the activation of glycolic acid oxidase and the effect of glycolate and its oxidation product, glyoxylate, upon the respiration of plant mitochondria.

LITERATURE REVIEW

LITERATURE REVIEW

Glycolic acid seems to be present in microorganisms, microflora, plants, and animals. Also, it has been reported to be in the organic matter of unmanured soil (8).

Glycolic Acid in Microorganisms

In 1932 it was reported that calcium and sodium acetates were converted to glycolic and glyoxylic acids by Aspergillus niger, and that glycolic acid was converted rapidly to glyoxylic acid (9). Glycolic acid as an intermediate product in the oxidation of acetate by E. Coli also has been reported (10). A mutant strain of Pseudomonas saccharophila, capable of adapting D-arabinose as its substrate, produces glycolic acid (11). D-arabinose is oxidized to D-arabono-γ-lactone, the lactone is hydrolyzed to D-arabonic acid which is dehydrated to 2-keto-3-deoxy-D-arabonic acid and finally this acid is hydrogenated and cleaved to yield pyruvic and glycolic acids. The carboxyl group of pyruvic acid is derived from C_1 of arabinose, while the carboxyl group of glycolic acid arises from C_4. Cell-free extracts of Penicillium chrysogenum are able to oxidize glycolate to glyoxylate (12). This oxidation is stimulated by the addition of flavin and pyridine nucleotide cofactors. In crude extracts the glyoxylate disappears rapidly and the only detectable compound produced from glycolate-C^{14}

metabolism is glycine. In the biosynthesis of glycine by Neurospora mutant, glycolic acid is oxidized to glyoxylic acid which, in turn, is transaminated to glycine (13). Cell-free extracts of Pseudomonad synthesize glycine by transamination of alamine, aspartic acid and glutamic acid with glyoxylic acid (14). The non-enzymatic transamination with glyoxylic acid and various amino acids has been reported also (15). Glycine may be converted in the reverse reaction to glyoxylate. Washed cells of Pseudomonas aeruginosa, in the presence of α-ketoglutarate, ATP and pyridoxal, metabolize glycine readily to glyoxylic acid (16). It was suggested that glycine is converted into glyoxylic acid in a transamination reaction. Another source of glyoxylate in microorganisms is the cleavage of isocitrate to succinate and glyoxylate (18, 19, 20). Glyoxylic acid may enter the tricarboxylic acid cycle in microbial metabolism by the condensation with acetate to form malate (17, 18, 19). Cell-free extracts of E. coli convert anaerobically glyoxylic acid to hydroxypyruvate (21, 108). In this process, one mole of CO_2 accumulated per two moles of glyoxylate added and tartronic semialdehyde was identified as the first product which, in turn, was metabolized to hydroxypyruvate.

Glycolic Acid in Animal Tissues

It has been reported that as early as 1919, human tissues were capable of converting glycolic acid into

glyoxylic acid, formaldehyde, and formic acid (22). The oxidative decarboxylation reaction of glyoxylate to formate and carbon dioxide has been demonstrated with rat liver mitochondria (23). Perhaps the first report concerning glycolic acid as a precursor of glycine in intact animals (rabbit) appeared in 1941 (24). Glycolic acid, when administered with benzoic acid, caused an increase in the rate of excretion of hippuric acid. This increase in the rate of excretion of hippuric acid was interpreted as evidence of the ability of the rabbit to convert glycolic acid to glycine. Employing isotopic procedure for the study of glycolic acid metabolism in the intact rat, it was found that this acid was converted to formic acid, and that the carbon of formic acid arose from α-carbon of glycolic acid (25, 26). Similar results were obtained with α-labeled glycine or α-labeled glyoxylate. Glycolic and glyoxylic acids, when injected intraperitoneally into rats, are converted rapidly into glycine (27); whereas, acetate, formate, or oxalate are not. Oxalate was metabolically inert and upon addition of glyoxylate, an accumulation of oxalate was observed in the rat. When rat-liver homogenate is incubated with glycolate or glyoxylate, there was found a trifold increase of glycine content as compared to the endogenous amount of it. Upon the addition of glutamine to the incubation mixture, the content of glycine formed greatly

increases (28). This indicates not only the capability of animal liver homogenates to oxidize glyoxylate, but also the formation of glycine by a transamination process with glutamic acid. Rat-liver specimens are capable of forming glycine from glyoxylic acid as a result of transamination reaction with aspartic acid, asparagine, glutamic acid, and glutamine, both aerobically and anaerobically (29). When glycine was incubated with bovine spermatozoa, glyoxylic acid, formic acid, and carbon dioxide was formed (30). This indicates that glycine was metabolized and that the pathways involved may be similar to those observed in other mammalian tissues.

Glycolic Acid in Plants

When glycolic acid was added to freshly ground barley leaves, there was observed an increased oxygen uptake and an accumulation of glyoxylic acid (31). Glycolic acid is oxidized more rapidly by chloroplast preparations in the light than in the dark. It was suggested that this oxidation is associated with some function of chloroplast in the light (32).

It was observed that oxidation of chlorophyll paralleled the oxidation of glycolic acid by leave homogenates (33, 34). The rate of chlorophyll disappearance was proportional to the concentration of glycolate added to the barley sap (35). It has been assumed that the active

oxidizing agent was the peroxide of glyoxylic acid, but it is more likely that the H_2O_2 produced oxidized the chlorophyll. If the accumulation of peroxides was prevented by the addition of ascorbic acid or phenols, the chlorophyll was not oxidized. On the other hand, in the absence of a carbonyl compound, considerable peroxides could accumulate without the chlorophyll undergoing oxidation (36). The oxidation of chlorophyll takes place in the simultaneous presence of glycolic acid and nitrites (37). Since the oxidation of glycolic acid takes place without nitrites, then this process precedes the oxidation of chlorophyll. In either the light or the dark the amount of nitrates declines when glycolic acid is detectable in the system and the resulting nitrites are rapidly converted into hydroxylamine and other substances in a reaction in which glycolic acid participates.

When detached tobacco leaves were vacuum infiltrated with the solution of glycolic acid, labeling was found in oxalic, succinic, malic, citric, and acetic acids, both in the light and in the dark (38). In the light, malic acid had a specific activity almost four times higher than citric acid; whereas, in the dark, citric acid had a specific activity four times higher than malic acid, although it had a lower total content of labeled carbon. On the basis of this observation, an assumption was made that glycolic acid

can participate also in a tricarboxylic acid cycle. This assumption is supported by the finding that over twice as much labeled carbon from glycolic acid accumulated in glutamic acid in the dark as in the light (39), and by the presumption that a tricarboxylic acid cycle, active in the dark, may be suppressed by light.

Carbons 1 and 2 of ribose are known to be precursors of the α-, and carboxyl carbons of glycine, respectively (40, 41), and glycine, in turn, arises from glycolic acid (42, 43). Therefore, glycolic acid should be synthesized *in vivo* from carbohydrates by a transketolase of transaldolase type of reaction. Recent work, in fact, has shown that glycolic acid is produced from a transketolase attack on fructose-6-phosphate in the presence of excess ferricyanide. When tobacco leaves were infiltrated with ribose-1-C^{14}, the radioactivity appeared in α-carbon of both glycolate and glycine (33).

During the steady-state $C^{14}O_2$ photosynthetic experiments with *Chlorella pyrenoidosa*, much glycolic acid was excreted into the medium (45). This phenomenon was specific for glycolic acid, as it was the only product excreted. This secretion of glycolic acid into a medium was extended to whole chloroplasts during the photosynthesis (46). These results, concerning the formation of glycolate during the photosynthesis, were confirmed on a quantitative

basis with Chlorella (47). From two molecules of carbon dioxide fixed there was found one molecule of glycolic acid. Parallel with this confirmation of glycolate formation during the photosynthesis, another group reported that during photosynthesis by Chlorella, carbon dioxide was converted directly into glycolic acid (48). The only postulated intermediate was a radical, probably (CHO·), which was believed detectable by electron spin resonance. This latter group claimed that they had found a second photosynthetic pathway; however, their results have not been verified.

Glycolic acid is metabolically very active in plants. In tobacco leaf homogenates, glycolate is readily converted into glyoxylate, carbon dioxide, and formate (49). Formic acid is incorporated directly into the β-carbon of serine and into choline (50). The major two products of glycolate metabolism by plants are glycine and serine (42, 43). The carbon atoms of the glycolic acid become the corresponding ones in glycine. The carboxyl carbon of serine arises from carboxyl carbon of glycolic acid and both the α-and β-carbons of serine come from the α-carbon of glycolic acid. Serine synthesis in etiolated plants exposed to the light develops parallel with activation of glycolic acid oxidase and this indicates that the major pathway for serine synthesis during the photosynthesis is via the glycolic acid pathway (51).

By a transamination reaction, glycine arises from glyoxylate which is an oxidation product of glycolate (52, 53, 54). Glyoxylic acid may also enter the citric acid cycle by a reaction catalyzed by malate synthetase and participate in synthesis and energy production in plant material. Malate synthetase, together with other enzymes of the glyoxylate cycle, were first demonstrated in plants of castor beans (55). Operation of glyoxylate cycle in plant metabolism was confirmed for germinating peanuts and castor beans (56) and also for excised cotyledons from etiolated peanut and sunflower seedlings (57). Occurrence of malate synthetase is not limited to the oily, germinating seeds or young seedlings of these seeds. Several other non-fatty tissues, such as, five-day old pea cotyledons, showed also a high malate synthetase activity. However, in mature leaves this enzyme has been detected only in trace amounts (58), and cannot account for glyoxylate metabolism in tissues of leaves.

Since glycolate and glycine are interconvertible in plant metabolism, the α-carbon of glycolic acid may be utilized for methyl group synthesis. This was shown to be the case in the experiments with tobacco plants, where the α-carbon of glycolate or glycine was incorporated into N-methyl group of nicotine (59, 60) and the α-carbon of glycolate into O-methyl groups of lignin (60). The α-carbon

of glycine was incorporated into pectinic acid in radish plant metabolism, and 70% to 80% of the radio-activity of pectinic acid was located in the methyl ester carbon (61).

Glycolic Acid Oxidase

Glycolic acid oxidase catalyzes the oxidation of glycolic acid to glyoxylic acid. The first report concerning the existence of such an enzyme in animal tissue appeared in 1940 (63). Glycolic acid oxidase was found to be widely distributed in green plants (5) and it was described in some detail (64). In the latter publication it was reported that the enzyme appeared to be specific for L-α-hydroxymonocarboxylic acids; that is, glycolic, lactic, and α-hydroxy-n-butyric acid. Glycolic acid oxidase was isolated in crystalline form from spinach leaves (65).

The prosthetic group of the enzyme is riboflavin-5-phosphate (66, 67, 68, 69, 72). A Michaelis' constant 2.4×10^{-3} (64) or 3.8×10^{-4} (69) has been reported which indicates a high affinity of the enzyme for the substrate. The enzyme catalyzes the oxidation with molecular oxygen of glycolic acid to glyoxylic acid, and of lactic acid to pyruvic acid in both plants (37, 49) and animals (70, 71). In cell-free plant homogenates, a byproduct of this oxidation, hydrogen peroxide, non-enzymatically oxidizes glyoxylic acid to carbon dioxide and formic acid (49, 67, 68). However, the enzyme glyoxylic acid oxidase has been

reported in rat liver tissue which is responsible for this oxidative decarboxylation (23). In the presence of excess catalase, the hydrogen peroxide byproduct is destroyed and the glyoxylic acid is not oxidized to formic acid and CO_2 but accumulates (67, 72). Molecular oxygen liberated from the catalase attack on H_2O_2 may be used to oxidize another portion of glycolate. Glyoxylic acid in plants is partially oxidized to oxalic acid. In vitro this oxidation is catalyzed by the same glycolic acid oxidase and riboflavin-5-phosphate that oxidizes glycolic acid (73, 100). The nearly irreversible reaction of reduction of glyoxylate to glycolate is catalyzed by glyoxylic acid reductase at the presence of DPNH (74). This enzyme has been isolated from tobacco leaves (75) and reduces glyoxylate to glycolate or hydroxypyruvate to D-glycerate. Recently Zelitch has discovered another glyoxylic acid reductase which uses TPNH rather than DPNH. The role of these two reductases in plant tissue is not known (62).

Glycolic acid oxidase seems to be absent in roots (49, 76), although roots contain small amounts of both glycolic and glyoxylic acids (77). When exposed to the light for a sufficient length of time to develop a green color, root tissue shows glycolic acid oxidase enzymatic activity (76).

The activation of glycolic acid oxidase is of unusual

and particular interest. From the published data we know the activity of the enzyme in plant material, expressed as $Q^N_{O_2}$, varies as shown in Table I. Glycolic acid oxidase is active as isolated from green leaves (5, 64, 69). On the basis of $Q^N_{O_2}$, it is much less active (about one-tenth) as isolated from etiolated leaves (5). However, etiolated leaves are able to convert glycolic-C^{14} acid to glycine (43). The activity of the enzyme can be increased greatly in vivo by exposure of plants to the light for a few hours (5, 34, 78). The activity of the enzyme can also be increased in vivo in the dark by feeding the leaves glycolate (5, 78). From the cell-free homogenate of etiolated leaves, the activity of the enzyme can also be increased in vitro by incubation with glycolate for several hours (5).

Role of Glycolic and Glyoxylic Acid in Plant Respiration

As mentioned in the introduction, there has long been an interest in glycolic acid and glyoxylic acid oxidation and reduction as a possible terminal oxidase system. Zelitch (79) has published data which he interpreted as indicating that a substantial part of the plant respiration could be accounted for by this system. Yet, last year Zelitch (80) showed that glycolic acid oxidation by plant mitochondria produced no energy, for the P/O ratio was zero. These two facts conflict because a major purpose of respiration is the production of energy.

TABLE I

Activity of glycolic acid oxidase from plant tissues (5)

Source of enzyme	$Q^N_{O_2}$
Sap of green leaves	50 to 200
Sap of etiolated leaves	5 to 10
Sap of etiolated leaves which were sprayed with glycolate 24 hours before harvest	100 to 300
Etiolated leaves sap, which was incubated with glycolate for 18 hours at 0°C.	50 to 100

Beevers and Kornberg (55) have shown that glyoxylic acid is a major metabolic constituent of the glyoxylic acid cycle in germinating plant tissue. However, they were not able to demonstrate an active glyoxylic acid cycle in older plant tissue. Therefore, this function seems unlikely in plants during most of their growth.

The main pathway of respiration in higher plants includes the tricarboxylic acid cycle and proceeded by the initial breakdown of carbohydrates and fats to pyruvate and acetyl-CoA, which are then completely oxidized in the citric acid cycle.

Glyoxylic acid is known to inhibit the oxygen uptake of various respiring tissue suspensions. Perhaps the first report concerning this phenomenon appeared in 1943 (81), and this was observed on animal tissues (liver, kidney) where glyoxylic acid exhibited strong inhibitory effects on tissue respiration.

In the course of study of metabolism of phosphorous deficient plants, both citric acid and glyoxylic acid were found to accumulate in plant tissues (82). The accumulation of citric acid in phosphorous deficient plants was ascribed to the enormous formation of glyoxylic acid in this plant followed by the inhibition of citric acid oxidation by glyoxylate. When glyoxylate was incubated with oxaloacetate in rat liver homogenates, it produced a severe

inhibition of citrate oxidation (83, 84).

The inhibitory effect of glyoxylate was also observed on succinate respiration by plant mitochondria preparations (85). In spite of the inhibition of the total oxidation of succinate or citrate by glycolate and glyoxylate in spinach mitochondria, no lowering of the P/O ratio for either of these substrates was observed (80).

The addition of glyoxylate to rat liver homogenates produced an inhibition of the oxidation for all tricarboxylic acid cycle intermediates (86). Simultaneously, with this inhibition in all cases, some increase in the accumulation of citrate was observed. The highest inhibition by glyoxylate and a large accumulation of citrate was observed when oxaloacetate was the substrate. This maximal inhibition of oxidation by glyoxylate when oxaloacetate was used as the substrate and a concurrent, enormous accumulation of citrate was ascribed to the reaction between glyoxylate and oxaloacetate and formation of an inhibitor of citrate metabolism. This inhibition was thought to be oxalomalic acid, which would arise from the condensation of glyoxylate and oxaloacetate.

In a study of the effect of α-ketoglutarate and aspartate on succinoxidase from human mitochondria, it has been found that neither of these acids had a direct inhibitory effect on succinate oxidation (87). The actual

inhibitor was oxaloacetate produced by transamination when both aspartate and α-ketoglutarate were present in the incubation of succinate with mitochondria preparation.

When purified aconitase from pig heart was incubated with cis-aconitate, it has been observed that 100% inhibition of aconitase activity upon addition of both glyoxylate and oxaloacetate to the incubation mixture (88), and the hypothetical oxalomalic acid was assumed to be responsible for this inhibition of aconitase. The chemical nature of this compound is reported to be under investigation, but, as yet, no further report on it has appeared.

Since glycolic and glyoxylic acids are easily interconvertible in plants, inhibitory effect of tricarboxylic acid cycle operation could be exhibited by either one, glycolate or glyoxylate, when tested in vivo (80).

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant Material

Thatcher wheat, Alaska peas, and Sacramento barley were grown in sand with Hoagland solution (89). If green material was needed for the study, the plants were grown in a greenhouse; in order to obtain etiolated plant tissue, the plants were grown in a totally dark room. For experiments with effect of light on glycolic acid oxidase activity during the growth, Thatcher wheat plants were grown in the dark room and before harvest they were exposed to the daylight of "September intensity" (2000-3000 foot candles) for a desired length of time. The age of the plants used for the study was eight to ten days. Swiss chard leaves were obtained from the plants grown under field conditions.

Preparation of Tissue and Enzyme

In order to obtain sap from the plant material for the study, harvested leaves were ground in a cold mortar with some white sand. The sap was squeezed through a double-layer of cheesecloth and the pH of the homogenate was quickly adjusted to about pH 8 with potassium hydroxide, using a Beckman pH meter, Model G. After centrifugation for five minutes at 200 x G in an International Portable Refrigerated Centrifuge, Model PR-2, sediment was discarded and the pH of

the cell-free sap was adjusted to 8.3 when glycolate was used as a substrate, or to pH 7.3 when glyoxylate was used as a substrate. During the preparation of the enzyme extract, the temperature of the sap was maintained between 0 and 2°C.

For preparation of whole chloroplast, cytoplasmic, and protoplasmic fractions of the plant cell, the procedure by Arnon, et al (90), was followed. Throughout this procedure the pH was maintained at 8.3, which is the optimum of glycolic acid oxidase.

Mitochondria-free supernatant was obtained by sedimenting the precipitate at 75,000 x G for one hour in a Spinco Ultracentrifuge, Model L.

An ammonium sulfate precipitate of glycolic acid oxidase was prepared from the cell-free plant sap. A 140 gm. per liter of ammonium sulfate was added to the sap at pH 5.3 and an inactive protein was removed by centrifugation at 4500 x G and discarded (65). The glycolic acid oxidase protein fraction was sedimented by an additional 80 gm. per liter of ammonium sulfate, stirred into the supernatant. After centrifugation at 4500 x G for 90 minutes, the supernatant was discarded, and the precipitate was taken up in 0.1 M phosphate buffer at pH 8.3 and dialyzed against cold water for 4 to 6 hours.

Mitochondria from plant tissues were isolated

essentially after the procedure of Ohmura (91), using one volume of leaves and two volumes of grinding medium of the following composition: 0.45 M sucrose, 0.05 M mannitol, 0.05 M boric acid, 0.03 M potassium citrate, 0.05 M tris (hydroxymethyl) aminomethane chloride (Tris), and 0.01 sodium ethylenediamine tetraacetate (versenate). The pH of the grinding medium was adjusted to 7.6 and this, when used in two volumes with both green or etiolated pea leaves, gave the desired pH 7.2 in the homogenate. Plant debris, chloroplasts, whole cells, et cetera, were removed by centrifuging for seven minutes at 600 x G at 0°C. The supernatant was next centrifuged at 10,000 x G for 20 minutes and the residue of particles was homogenized again with a desired volume of the washing medium. The washing medium was of the following composition: 0.3 M sucrose, 0.05 M tris (hydroxymethyl) aminomethane chloride (Tris) and 2 x 10^{-4} M versenate. The pH of the washing medium was adjusted to 7.5.

Assays and Analyses

The oxygen uptake of both cell-free sap and mitochondria preparations was measured by manometric techniques in 23 ml. Warburg vessels with an atmosphere of air, shaken at 120 oscillations per minute. Temperature was maintained at 30°C. All assays for glycolic acid oxidase activity in plant sap were in 0.033 M final concentration of phosphate

buffer. Each flask contained one ml. of sap. The final volume of reactants was 3 ml. and the pH was 8.3.

The standard reaction components for experiments with isolated mitochondria were (80): sucrose, 300 μmoles; $MgSO_4$, 10 μmoles; potassium phosphate buffer at pH 7.0, 37 μmoles; yeast coenzyme concentrate, 1 mg; ATP, 2 μmoles; mitochondria from 1.5 g. plant tissue and substrate as will be specified. If deviation from this standard assay procedure was made, this will be specified with the corresponding graphs or tables. The reaction mixture was combined at zero time with glucose, 50 μmoles, and yeast hexokinase, 0.2 mg. which had been placed in the side arm. The final volume of reactants was 3.9 ml. and 0.2 ml. of 20% KOH were placed in the center well. Reaction was usually followed for one hour.

For nitrogen determination, the micro Kjeldahl method was employed (92). The μl O_2 taken up per mg. tissue nitrogen per hour is designated as $Q^N_{O_2}$.

Phosphorous was determined colorimetrically by the Fiske-Subbarow method (93). After completion of manometric measurements in the Warburg apparatus, the reaction mixture was deproteinized with 0.5 ml. of 20% trichloroacetic acid, the precipitate was removed by centrifugation and the orthophosphate remaining in the supernatant fluid was determined. The amount of orthophosphate esterified was determined by the difference between the contents of orthophosphate in the

endogenous reaction mixture and in the appropriate substrate reaction. For this determination a Coleman Junior Spectrophotometer, Model 6A, was used.

The flavin content was determined fluorometrically (94, 95) by using an Electronic Fluorometer, Model 12B, of Coleman Instruments, Inc.

Oxaloacetic acid was determined by the oxidative decarboxylation method (96). Malic and lactic dehydrogenase activity was measured spectrophotometrically (97) in the Beckman Spectrophotometer, Model DU. If malate was used as a substrate, its oxidation with DPN in the presence of malic dehydrogenase was followed by the measurement of the DPNH produced at 340 mμ.

Lactic dehydrogenase activity was also measured in the Beckman Spectrophotometer in a similar way, that is, by following lactate utilization by the enzyme as reflected in the optical density changes for the presence of DPN.

For the recording of absorption spectra, a Beckman, Model DK-2, instrument was used.

Source of Chemicals

From the Nutritional Biochemical Corporation, Cleveland, Ohio, was obtained riboflavin-5-phosphate (FMN), flavin adenine dinucleotide (FAD), diphosphopyridine nucleotide reduced (DPNH), L-glutamic acid, L-cysteine

hydrochloride, L-tyrosine, dihydroxyphenylalanine (DOPA), glycine, acetyl phosphate (diAg), sucrose, D-mannitol, and the following organic acids: glyoxylic (sodium salt), citric, cis-aconitic, isocitric (trisodium salt), oxalosuccinic (barium salt 60%), α-ketoglutaric, fumaric, L-malic (active), oxaloacetic, malonic, and ascorbic acid.

Sigma Chemical Company, St. Louis, Missouri, was the source of: Adenosine-5'-triphosphate (ATP)-disodium salt, yeast hexokinase (type II), yeast coenzyme concentrate (Stock No. 202-30), malic dehydrogenase (Stock No. 410-9), cytochrome c (type II, from horse heart), and tris (hydroxymethyl) aminomethane chloride (Tris).

From Eastman Organic Chemicals, Rochester, New York, was obtained glycolic acid (calcium salt), D-glucose, succinic acid, lactic acid (85%), 1-amino-2-naphthol-4-sulfonic acid and trichloroacetic acid (practical).

Diphosphopyridine nucleotide (DPN) was obtained from Pabst Laboratories, Milwaukee, Wisconsin.

Riboflavin was from Merck and Company, Rahway, New Jersey; potassium citrate, Mallinckrodt Chemical Works, New York; boric acid, Baker Chemical Company, Phillipsburg, New Jersey; and sodium ethylenediamine tetra-acetate (Versenate) was obtained from Matheson Company, Norwood, Ohio.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

A. GLYCOLIC ACID OXIDASE IN PLANTS

Organ and Cellular Distribution of Glycolic Acid Oxidase

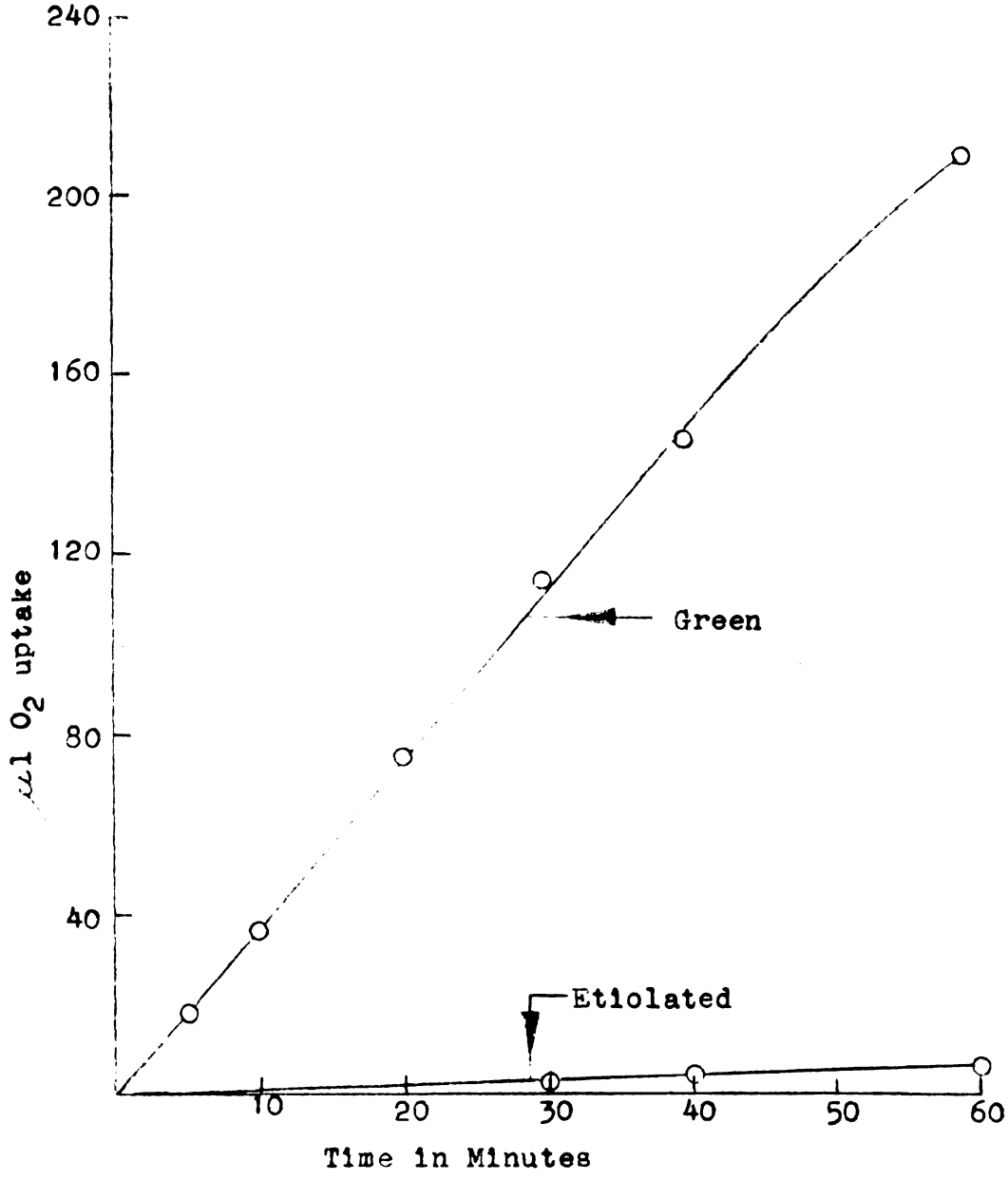
A relative comparison of the glycolic acid oxidase activity as isolated in the sap from green and etiolated leaves of Thatcher wheat is shown in Figure 1. This confirms previous reports of this phenomenon (5, 34, 69).

Most of the work published on glycolic acid oxidase in plant tissue concerns the enzyme as isolated from green leaves and shoots. No enzyme can be extracted from root tissue unless the roots were exposed to the light and until they developed a green color (76). As will be discussed later, the enzyme activity is greatly stimulated from sap of etiolated shoots by both light and by the addition of FMN. Therefore, the effect of FMN upon activation of the enzyme in sap from roots was also studied (Table II). Shoots of green pea seedlings showed high glycolic acid oxidase activity without added FMN, but roots of the same plants showed no enzymatic activity regardless of the addition of FMN. Presumably the roots contain no enzyme or apoenzyme for this oxidation.

Glycolic acid oxidase has been reported as being absent in the sheaths of gramineous plants (98), although

FIGURE 1

Glycolic acid oxidase from green and etiolated Thatcher wheat leaves



Each Warburg flask contained 50 μmoles of glycolate.

TABLE II

Glycolic acid oxidase from shoots and roots of green pea seedlings

Sap and substrate	Time			
	10 min.	25 min.	45 min.	60 min.
	μl O_2 uptake/ml. sap/hr.			
Shoot sap:				
10 μmoles glycolate	64	154	158	158
10 μmoles glycolate+ 1 x 10^{-4} M FMN	75	172	169	181
Root sap:				
10 μmoles glycolate	0	2	3	5
10 μmoles glycolate+ 1 x 10^{-4} M FMN	0	0	0	3

adjacent green leaves showed normal enzymatic activity. Sap from the sheaths and leaves of both green and etiolated wheat seedlings were prepared after careful visual separation of the sheaths. In the sheaths of green plants about 4 per cent as much enzyme activity on a $Q_{O_2}^N$ basis was found as in the leaves of the same plants (Table III). Upon addition of FMN to the saps of both leaves and sheaths, the enzymatic activity doubled for the green leaves but increased about 39 fold for the green sheaths. As a result both tissues of green seedlings had nearly equal enzyme activity on a $Q_{O_2}^N$ basis. The data indicate that a large amount of apoenzyme was present in green sheaths, and in order to reveal it, the addition of coenzyme was needed. These results alter the previous concept that the enzyme was not present in the sheaths (98). Both etiolated tissues, leaves, and sheaths showed very low glycolic acid oxidase activity without the addition of FMN. When FMN was added, the enzymatic activity was greatly increased in both tissues, but the enzymatic activity of etiolated sheaths was only about one-third as great as that of the leaves.

Distribution of the enzyme in isolated cellular fractions from Swiss chard is shown in Table IV and from Thatcher wheat in Table V. Both the chloroplasts from green tissue (Table IV), and mitochondrial particles sedimented between 2,000 and 75,000 x G from etiolated tissue

TABLE III

Glycolic acid oxidase in leaves and sheaths of Thatcher wheat

Kind of tissue	FMN final concentration	$Q_{O_2}^N$
Green leaves	0	55.0
	1×10^{-4} M	107.6
Sheaths from green plants	0	2.2
	1×10^{-4} M	89.4
Etiolated leaves	0	6.6
	1×10^{-4} M	55.2
Sheaths from etiolated plants	0	5.8
	1×10^{-4} M	16.4

Each flask contained 20 μmoles of glycolate.

TABLE IV

Location of glycolic acid oxidase in fractions from Swiss chard

Cell constituent	$Q_{O_2}^N$
Chloroplast	62.4
Protoplasm	346.4
Cytoplasm	338.0

Each flask contained 50 μmoles of glycolate.

TABLE V

Location of glycolic acid oxidase in fractions from etiolated Thatcher wheat

Substrate	Cell Constituent*	
	Supernatant	Mitochondria
	μl O_2 uptake/hour	
1 x 10^{-4} M FMN	4	14
10 μmoles glycolate	6	9
FMN + glycolate	139	36

* After 1 hour centrifugation at 75,000 x G, sedimented particles were resuspended in a volume of phosphate buffer equal to the volume of the supernatant.

(Table V), showed lower enzymatic activity on a $Q_{O_2}^N$ basis than the cytoplasmic soluble fraction. These data could be accounted for by absorption of the enzyme on the surface of the chloroplasts or mitochondria. Such a phenomenon of absorption of soluble enzymes is frequently reported (80, 87). On the other hand, much enzyme activity could have been with the particles in vivo and was leached out during the isolation procedure. From such data it is generally considered that the glycolic acid oxidase is predominantly a soluble cytoplasmic enzyme and not associated with chloroplasts as was earlier reported (99). These results confirm an earlier report concerning the distribution of the enzyme (66).

Activation of the Enzyme by Added Excess of FMN

Enzyme activation by the addition of FMN to the cell-free saps is presented in Table VI for wheat and in Table VII for barley. In order to obtain the maximal enzymatic activity in both green and etiolated cell-free homogenates or saps of plant leaves, the addition of FMN was necessary. The apoenzyme could be nearly completely activated by a final FMN concentration of 1×10^{-5} M or greater. The maximum enzyme activity for both green and etiolated leaf extracts was obtained between 1×10^{-4} and 1×10^{-3} M FMN, while a 1×10^{-2} M final FMN concentration was slightly inhibitory. The maximum increase in enzyme activity upon

TABLE VI

FMN activation of glycolic acid oxidase from green and etiolated plants

FMN final concentration	Green sap	Etiolated sap
Molar	$Q_{O_2}^N$	$Q_{O_2}^N$
0	39.9	4.1
1×10^{-7}	38.7	6.9
1×10^{-6}	54.5	11.7
1×10^{-5}	95.0	30.4
1×10^{-4}	105.0	42.1
1×10^{-3}	106.9	40.7
1×10^{-2}	95.0	36.5

Each Warburg flask contained 20 μmoles of glycolate. $Q_{O_2}^N$ for FMN, without glycolate, was 2.1 for green sap, and 1.9 for etiolated sap.

TABLE VII

Oxidation of glycolate and glyoxylate
by etiolated barley sap

Flask additions	μl O_2 uptake/hour
FMN	0
Glycolate	7
Glycolate + FMN	150
Glyoxylate	5
Glyoxylate + FMN	42

Glycolate or glyoxylate 10 μmoles/flask. FMN was at 1 x 10^{-4} M final concentration. Final pH for flasks with glycolate, 8.3, and with glyoxylate, 7.3.

the addition of FMN was higher in etiolated tissue, reaching values of about 10 fold; whereas, in green material the maximum increase in enzymatic activity was less than 2.5 fold. On the other hand, etiolated plant material always showed, on the basis of $Q^{N}_{O_2}$, two to three times less enzymatic activity than green tissue. From either tissue the maximum activity was never obtained, unless excess FMN was added.

In Table VIII are data to demonstrate the stimulation by FMN of the oxidation of glyoxylic acid by wheat sap. It has generally been observed that the oxidation of glyoxylate in the absence of added FMN is extremely slow by cell-free homogenates or mitochondria preparations from both green and etiolated tissues. If glycolic acid was the starting substrate and excess of catalase was omitted, the glyoxylic acid product would be non-enzymatically oxidized by the hydrogen peroxide formed in the reaction to formic acid and carbon dioxide (49, 67, 68). In the present experiments glyoxylic acid was used as the substrate and FMN was added in varying concentrations. A many-fold increase occurred in the oxidation rate of glyoxylate by both etiolated and green tissues. Richardson and Tolbert (100) have shown that glycolic acid oxidase can oxidize glyoxylate as well as glycolate, but that the K_m for the oxidation of glyoxylate is much less than for oxidation of glycolate.

TABLE VIII

Effect of FMN on oxidation of glyoxylic acid by wheat sap

FMN final concentration	Green sap	Etiolated sap
Molar	$Q^N_{O_2}$	$Q^N_{O_2}$
0	2.5	0.0
1×10^{-7}	1.8	0.8
1×10^{-6}	2.4	1.0
1×10^{-5}	6.4	1.0
1×10^{-4}	27.0	3.4
1×10^{-3}	26.7	6.9
1×10^{-2}	30.0	5.4

Each flask contained 20 μmoles of glyoxylate. There was no effect of FMN alone, without glyoxylate.

Effect of Light plus FMN on Enzyme Activity

In order to determine the effect of the length of the light period and the addition of FMN to the cell-free sap on enzyme activity, a series of different experiments were run with wheat plants all of which were 10 days old from time of planting the seed (Table IX). The variable was the duration in hours of exposure to daylight before harvesting of plants. The germinating seedlings appeared above the surface of the sand after five days and, therefore, the longest possible exposure of plants to the light was about four days. The measurable activity of the enzyme without addition of FMN in cell-free homogenates from Thatcher wheat leaves which had been grown in total darkness for 10 days was low ($Q^N_{O_2} = 5.3$). This activity was stimulated 9.0 fold by the addition of FMN. These data suggest that the apoenzyme was present in the dark-grown plants, but that it was not active, perhaps due to insufficient FMN. Exposure of the plants to the light also caused the formation of more active enzyme and of more apoenzyme.

The complexity of these multiple changes is difficult to interpret. However, the ratio of $Q^N_{O_2}$ activity in the presence of added FMN to that without added FMN dropped from 9 for etiolated tissue to 5 or 6 for any tissue exposed to light. Saying this another way, there was proportionally more apoenzyme to holoenzyme in etiolated tissue than in

TABLE IX

Effect of light during growth and addition of FMN to sap upon enzyme activity

Exposure to light	Glycolate $Q_{O_2}^N$	Glycolate + FMN $Q_{O_2}^N$	Ratio of: Glycolate + FMN / Glycolate
0	5.3	47.5	9.0
3 hours	9.8	50.6	5.2
6 hours	10.9	70.2	6.4
12 hours	14.0	92.7	6.6
2 days	19.2	121.8	6.4
3 days	24.6	133.2	5.4
4 days	29.6	158.1	5.3

Each flask contained 10 μ moles of glycolate. FMN final concentration was 1×10^{-4} M. Age of all plants at harvest was 10 days.

tissues exposed to light. However, exposure to light also stimulated the formation of both holoenzyme and apoenzyme which remained, though, in a proportion of five to six times more apoenzyme than holoenzyme. The rapid change in this ratio from 9.0 to 5.2 after only three hours in light occurred before significant new protein synthesis or growth, as evident by the fact that the total enzyme activity in the presence of FMN was still the same in both cases. This suggests that exposure of plants to light had two effects upon glycolic acid oxidase activity. Light immediately was responsible for an increase in holoenzyme from a reservoir of apoenzyme. This would not be caused by protein synthesis, but by an effect upon the coenzyme. Light also stimulated the slow synthesis of new protein or enzyme so that at the end of two to four days the green plants contained much more enzyme than plants of comparable age kept in the dark.

Flavin Content of Wheat Leaves

The total flavin content of Thatcher wheat leaves does not differ significantly between green or etiolated tissue (Table X), but the distribution of the flavins among FAD, FMN, and riboflavin was strikingly different in these two types of leaves. FAD content of etiolated leaves was twice as high as that in green leaves. On the other hand, the FMN content was two times greater in green than in

TABLE X

Flavins content in Thatcher wheat leaves

Flavin	γ/g Dry sample		Calculated molarity*	
	Green	Etiolated	Green	Etiolated
FAD	46	108	4.1×10^{-9}	6.0×10^{-9}
FMN	93	53	1.3×10^{-8}	4.5×10^{-9}
Riboflavin	2	5	4.2×10^{-10}	8.9×10^{-10}
Total	141	166	--	--

* Calculated on the basis of γ of flavin per gram of dry sample and amount of sap obtained from an equivalent quantity of fresh tissue.

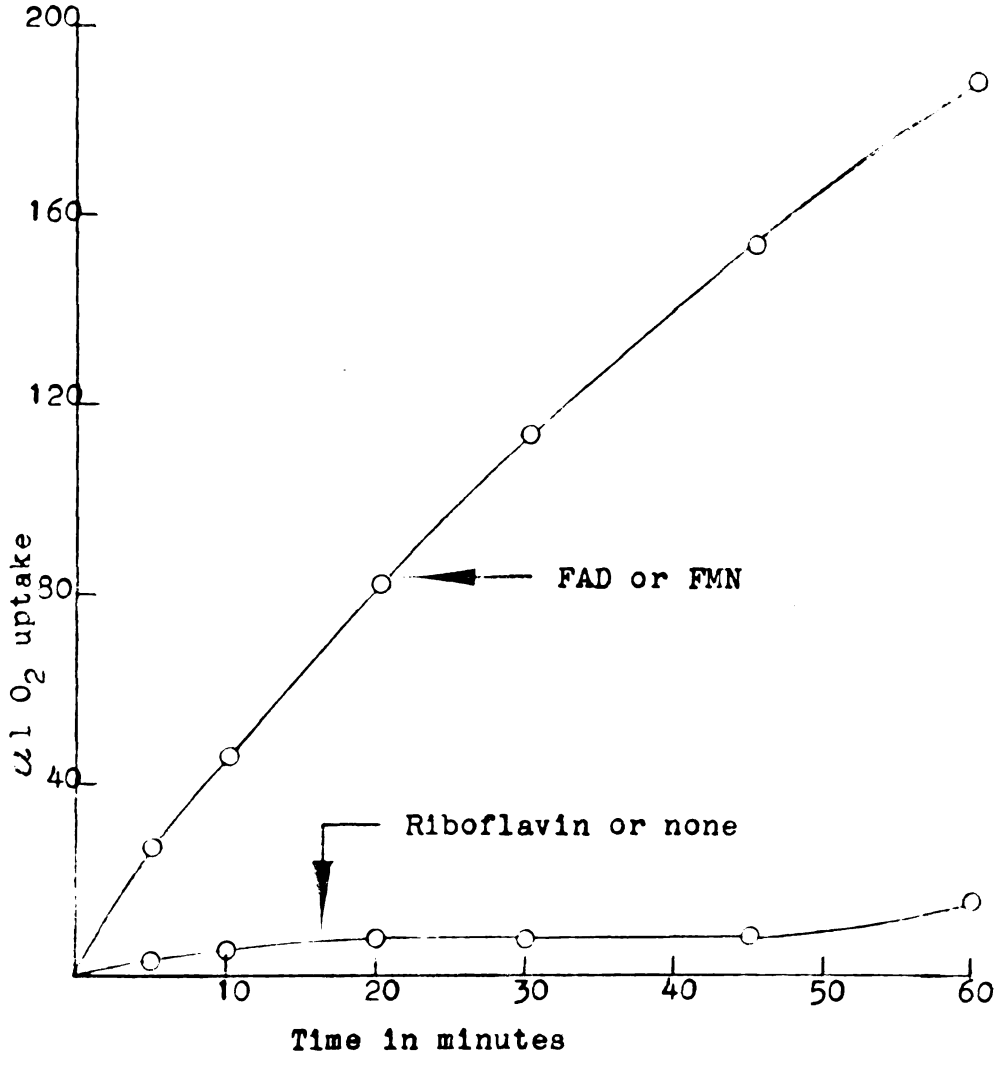
etiolated tissue. These results are consistent with the fact that green tissue has much more of the active FMN requiring glycolic acid oxidase. However, the reduced level of FMN in etiolated tissue is not nearly great enough to account for the very low level of enzyme activity. Ninety-three γ of FMN per gram of dry green leaf tissue is equivalent to about 1.3×10^{-8} M FMN in the sap from this tissue. In Table VI, it is shown that over a 10-fold higher concentration of FMN had no effect upon increasing the *in vitro* activity of the apoenzyme of glycolic acid oxidase. In fact, 10^{-4} FMN was necessary for maximum activity *in vitro* and clearly the leaves *in vivo* never contained anywhere nearly this amount.

Although it has been considered that FMN is the only prosthetic group for glycolic acid oxidase (66, 67, 69), other flavins were also tested for possible effects on enzyme activity. It was found that both FMN and FAD, but not riboflavin, had an equal effect on the activation of the enzyme in etiolated cell-free sap from wheat (Figure 2).

Analyses of the FAD sample showed that it was nearly pure. On the other hand, analyses of an aliquot of sap after incubation with FAD showed that 1 per cent was recovered as FAD, 90 per cent was present as riboflavin, and 9 per cent as FMN (Table XI). Therefore, the FAD had been hydrolyzed by the sap to FMN and riboflavin. Since purified glycolic

FIGURE 2

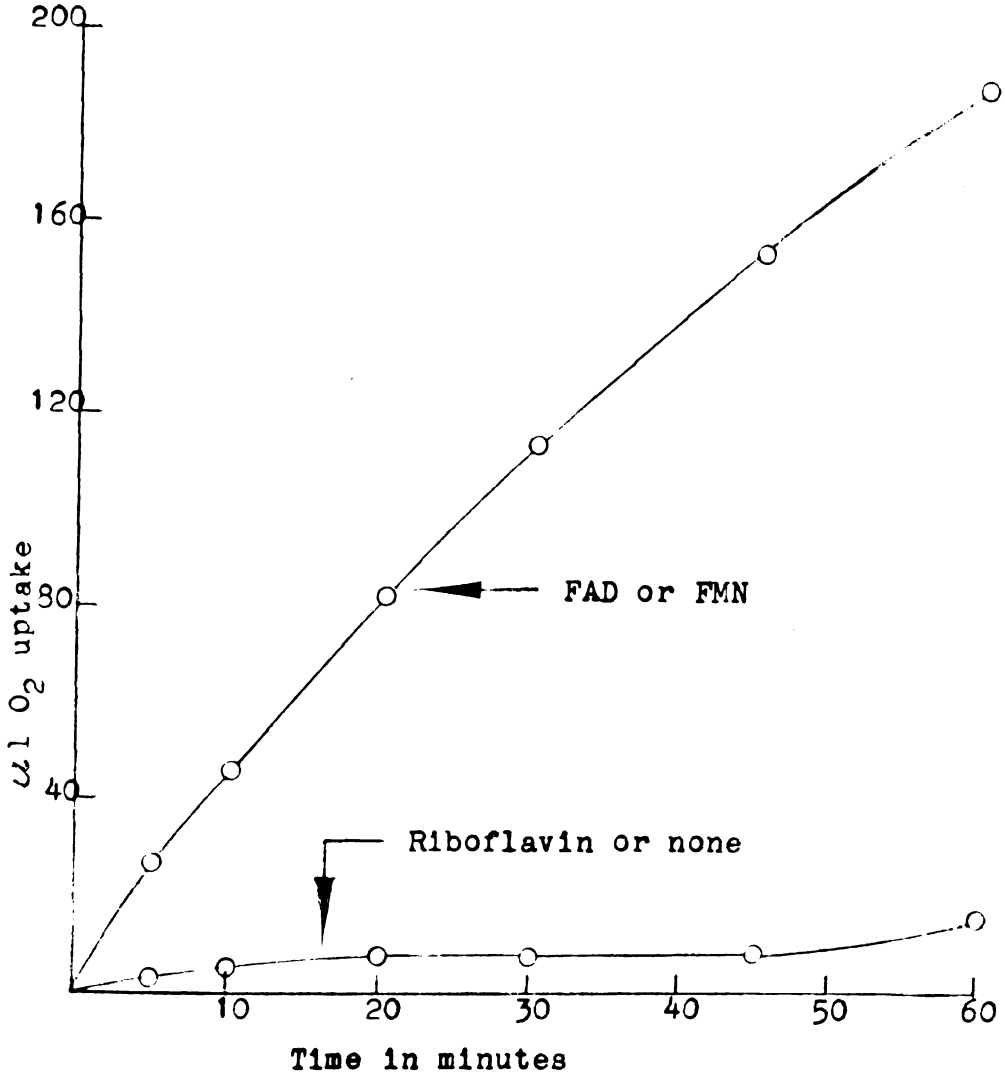
Effect of flavins on enzyme activity of etiolated wheat sap



Glycolate 10 μmoles/flask. Flavins final concentration 1 x 10^{-4} M.

FIGURE 2

Effect of flavins on enzyme activity of etiolated wheat sap



Glycolate 10 μmoles/flask. Flavins final concentration 1 x 10^{-4} M.

TABLE XI

Flavins recovered from 1.6×10^{-7} m moles FAD, after incubation with wheat sap

Flavin	Recovery	
	m moles	% of total
FAD	0.02×10^{-7}	1
FMN	0.18×10^{-7}	9
Riboflavin	1.80×10^{-7}	90
Total recovered	2.00×10^{-7}	

Difference between 2.0×10^{-7} and 1.6×10^{-7} may be accounted for by the added physiological level of flavins in the sap or by an error in analysis.

acid oxidase is not activated by FAD (67), the above effect from FAD on the enzyme in crude sap could be accounted for by its hydrolysis to FMN.

Flavins in tissue undergo a dynamic change which might lead to their synthesis or breakdown. The observation on the breakdown of FAD has been reported by yeast (101), animal tissue (103), and plants (102). The physiological optimal level of FMN necessary to activate the enzyme *in vivo* cannot be determined from our data. However, the order of magnitude of 1×10^{-8} M FMN in green leaves with an active glycolic acid oxidase is in striking contrast to a concentration of 1×10^{-4} M FMN need *in vitro* to activate the apoenzyme. This comparison suggests that *in vivo* the formation of the holoenzyme is also an enzymatic process.

Relative Contents of Apoenzyme in Green and Etiolated Tissues

In Table XII are data concerning the relative levels of apoenzyme in green and etiolated tissues. In order to obtain this answer, a relatively high substrate concentration and an optimal FMN addition at low concentrations of the enzyme was used. It was assumed that the surface of the glycolic enzyme should be saturated with the substrate under these conditions. The data confirm the results of previous experiments; that is, a higher percentage increase of O_2 uptake in etiolated tissue upon the addition of optimal FMN

TABLE XII

Relative levels of apoenzyme in green and etiolated tissues

	$Q_{O_2}^N$ (average of 2 experiments)	
Wheat sap	Glycolate	Glycolate + FMN
Etiolated	21	174
Green	116	370

Glycolate 50 μmoles/flask, FMN final concentration 1.0×10^{-3}, 1/5 enzyme concentration of previous experiments.

concentration than in green material, and the total amount of the apoenzyme, on the basis of $Q_{O_2}^N$, is twofold higher in green material than in etiolated tissue.

Activation of the Enzyme from Ammonium Sulfate Precipitate

When the ammonium sulfate fraction collected between 14 and 22 gms. per 100 ml was dialyzed for six hours, the enzymatic activities in both etiolated and green tissues were low (Table XIII). In order to restore the enzymatic activities in either tissue, an excess of FMN was necessary. These data show that the loss of physiological FMN during the dialysis of these fractions from both etiolated and green tissues resulted in enzymatic inactivity. The green enzyme preparations as a plant extract before precipitation and dialysis showed high enzymatic activity.

Activation of the Enzyme in vitro by Incubation with the Substrate

In Figure 3 we have a confirmation of published data that the enzyme of cell-free homogenate of etiolated plants can be activated by incubation in the cold with glycolate (5). There was no activation of the enzyme when the plant sap was incubated under similar conditions with water or with glyoxylic acid or H_2O_2 which are products of glycolate metabolism. H_2O_2 showed even an inhibitory effect on the residual enzyme activity. The activation of the

TABLE XIII

Ammonium sulfate precipitated enzyme from green and etiolated wheat sap

Kind of tissue	μl O_2 Uptake/hour	
	Glycolate	Glycolate + FMN
Green	40	444
Etiolated	20	96

Glycolate 10 μmoles/flask. FMN 1 x 10^{-4} final concentration. O_2 uptake was calculated from respiration rate between 5 and 20 minutes.

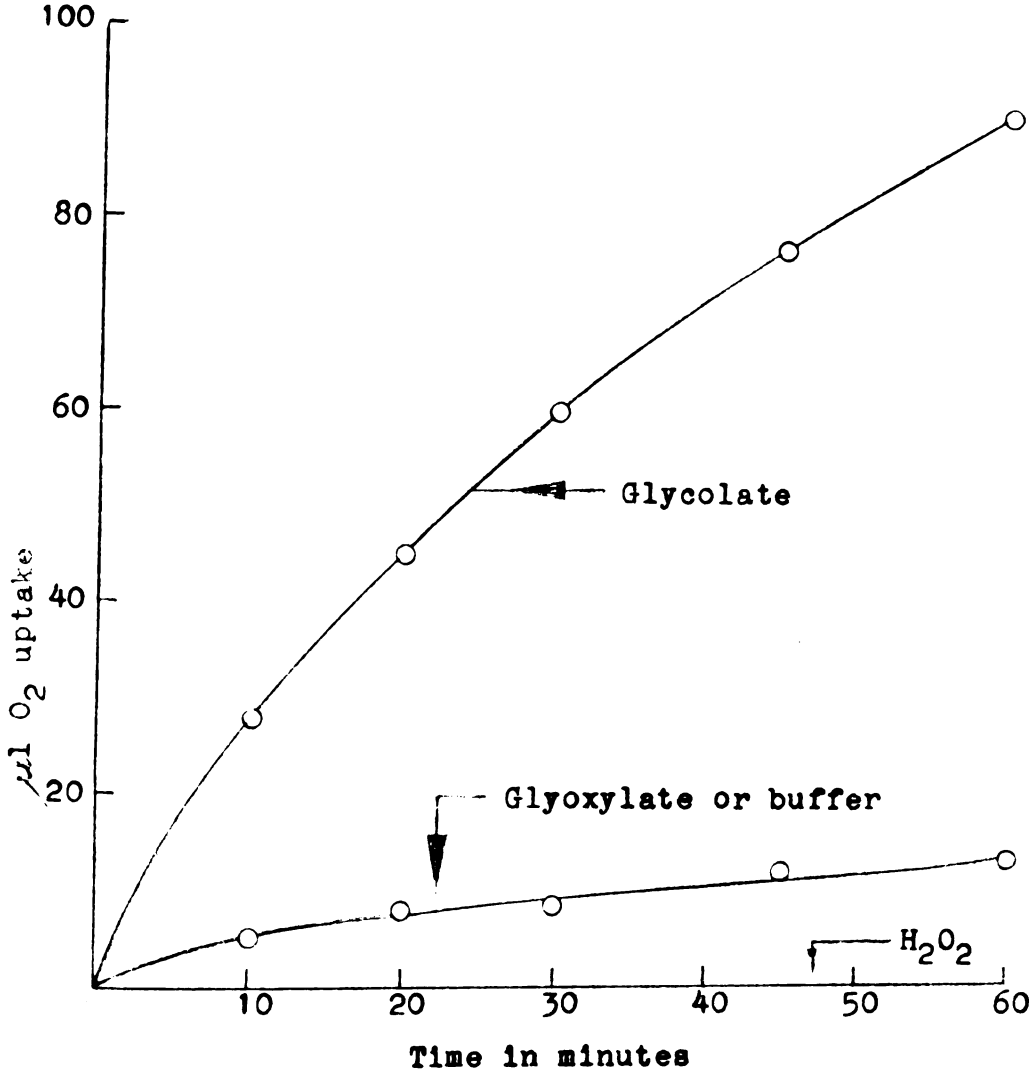
FIGURE 3

Preincubation of etiolated cell-free wheat sap

Cell-free sap was incubated for 18 hours at 2°C. before Warburg determination in 0.033 M. phosphate buffer at pH 8.3. Final concentration of glycolate, glyoxylate or H_2O_2 was 0.008 M, when it was used for incubation. Final concentration of glycolate as a substrate was 0.024 M.

FIGURE 3

Preincubation of etiolated cell free wheat sap



enzyme at room temperature is so slow that inactivation cancels much of the gain and therefore the phenomenon is best demonstrated at about 2°C. for the activation and at 30°C. for measurement of enzyme activity.

The enzyme can also be activated from cell-free sap when incubated with lactate or α-hydroxybutyrate which are natural substrates for the active enzyme (Table XIV). The effect of the incubation with glycolate is also further elaborated in this table. The activity of the enzyme in sap from etiolated leaves which was preincubated with glycolate for 18 hours at 2°C. increased sevenfold ($Q^N_{O_2}$, 10 and 70); and in sap from green leaves only 34 per cent ($Q^N_{O_2}$, 68 and 103). There was no increase in $Q^N_{O_2}$ when FMN was added to the green or etiolated sap which had been preincubated with glycolate. Incubation of etiolated sap with lactate or α-hydroxybutyrate increased three to four-fold enzyme activity ($Q^N_{O_2}$, 10 and 37 or 35). In fact the enzyme activity in sap after 18 hours of preincubation with glycolate, but not with FMN, was equal to that measured immediately in the sap when 1×10^{-4} M FMN, an excess, was added. This important point may be interpreted to mean that all of the apoenzyme had been activated by prolonged incubation with glycolate alone. Presumably this activation involved a combination of apoenzyme with the naturally occurring FMN present in the sap. This natural amount of

TABLE XIV

$Q_{O_2}^N$ of cell-free etiolated sap after 18 hours preincubation

Preincubated with	Warburg addition	Etiolated	Green
Buffer alone	Glycolate	10	68
	Glycolate + FMN	53	106
Glycolate	0	70	103
	Glycolate	58	104
	Glycolate + FMN	69	106
Lactate	0	0	3
	Glycolate	37	113
	Glycolate + FMN	86	146
α-Hydroxy-butyrate	0	0	3
	Glycolate	35	114
	Glycolate + FMN	92	150

Incubation conditions and reagent concentrations for both preincubation and O_2 uptake determination were as specified in Figure 3. FMN final concentration was 1×10^{-4} M.

FMN was insufficient to activate the enzyme quickly during the course of a normal Warburg measurement lasting 30 or 60 minutes. Because of these facts this activation is visualized as a slow enzymatic process requiring glycolate and capable of proceeding at low FMN concentrations.

The addition of FMN to the sap which had been incubated with lactate or α-hydroxybutyrate showed toward the glycolate substrate some further increase of enzyme activity of about twofold. This enzyme activity was even about 20 per cent higher than that fraction of the same sap preincubated with glycolate. Similar results were obtained with sap from green material in that preincubation with lactate or α-hydroxybutyrate gave more active enzyme preparations than glycolate incubation when excess FMN was added to the final assay with glycolate. Without the addition of FMN, differences among the enzyme activities from preincubation with glycolate, lactate or α-hydroxybutyrate were not appreciable. The somewhat better activation of the apoenzyme by lactate and α-hydroxybutyrate than by glycolate is hard to explain. The rate of the active enzyme catalyzed oxidation of lactate or α-hydroxybutyrate is about one-tenth or less the rate of glycolate oxidation (64). Since only one enzyme is supposed to be present for attack on all three of these α-hydroxy acid substrates (64), these differences in the activation phenomenon should

not be caused by activation of other enzymes by lactate and α-hydroxybutyrate. Lactate and α-hydroxybutyrate may serve to activate the glycolic acid oxidase without appreciable concurrent oxidation of these substrates. Therefore, these substrates may remain and be effective longer than glycolate for the activation. Also rapid oxidation of glycolate by the activated enzyme may partially inactivate the enzyme, as for example by the H_2O_2 product of the reaction.

The activation of the enzyme in cell-free homogenates by glycolate cannot be repeated on an ammonium sulfate precipitated and dialyzed protein fraction which contains the active enzyme from sap of green leaves (Figure 4, Table XV). All that is needed for full activity of the ammonium sulfate precipitate is an excess of FMN which can be added at the time of preincubation or at the start of manometric measurement. The $Q^N_{O_2}$ of dialyzed $(NH_4)_2\ SO_4$ fraction, on addition of FMN, increased four or more fold; although this activity was lower by about one-third than that of the freshly prepared enzyme from the green tissue, on the basis of $Q^N_{O_2}$.

Similar, although not identical, results were obtained when the dialyzed ammonium sulfate precipitate from etiolated tissue was incubated with glycolate (Table XVI). The loss of enzyme activity on preincubation for 18 hours in the cold was much higher in etiolated tissue than that in

FIGURE 4

Preincubation of $(NH_4)_2SO_4$ precipitate of green sap

Ammonium sulfate precipitate was prepared as described in Materials and Methods section. Preincubation conditions were as described in Figure 3. FMN final concentration was 1×10^{-4} M and that of glycolate 0.024 M.

For the Warburg assay:

1. Glycolate and 3-glycolate + FMN after preincubation with glycolate.
2. Glycolate and 4-glycolate + FMN after preincubation with 0.033 M phosphate buffer.

FIGURE 4

Preincubation of $(NH_4)_2SO_4$ precipitate of green sap

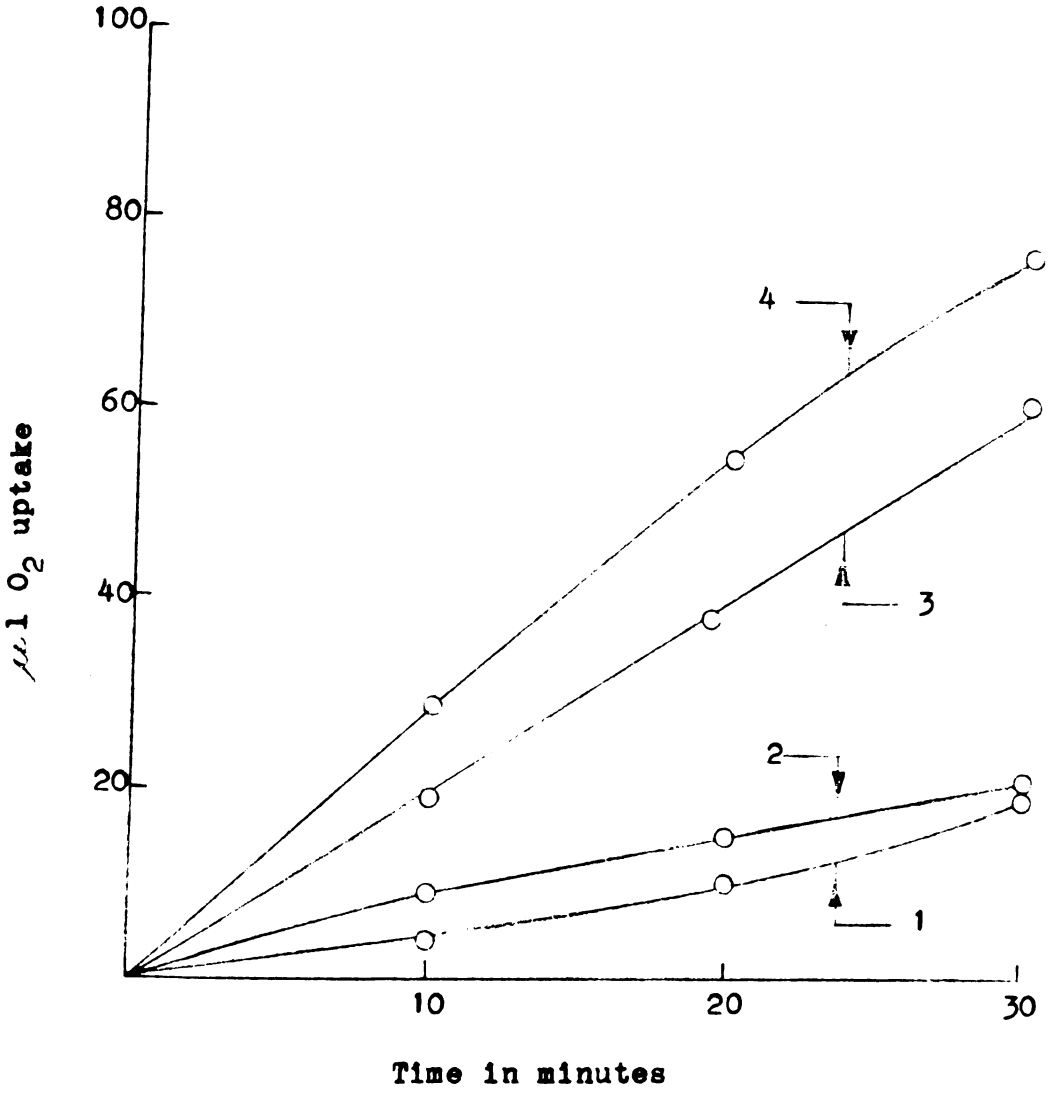


TABLE XV

$Q^N_{O_2}$ of preincubated dialyzed ammonium sulfate precipitate from green wheat

Preincubation solution	Warburg addition		$Q^N_{O_2}$
	Glycolate μmoles	FMN	
None, freshly prepared precipitate	24	0	21
	24	1×10^{-4}	143
Water	24	0	14
	24	1×10^{-8}	14
	24	1×10^{-4}	88
Glycolate 8 μmoles	0	0	20
	16	0	18
	16	1×10^{-4}	78
1×10^{-8} M FMN	0	0	3
	24	0	8
	24	1×10^{-4}	97
1×10^{-4} M FMN	0	0	3
	24	0	108
	24	1×10^{-4}	117
Glycolate 8 μmoles + 1×10^{-4} M FMN	0	0	103
	16	0	83
	16	1×10^{-4}	84

For preincubation details see Figure 4. For Warburg assay glycolate is listed as μmoles/flask and FMN as final concentrations.

TABLE XVI

$Q^N_{O_2}$ of preincubated dialyzed ammonium sulfate precipitate from etiolated wheat

Preincubation solution	Warburg addition		$Q^N_{O_2}$
	Glycolate μmoles/flask	FMN Final conc.	
None, freshly prepared precipitate	24	0	18
	24	1×10^{-4}	94
Water	24	0	5
	24	1×10^{-4}	23
Glycolate 8 μmoles	0	0	5
	16	0	5
	16	1×10^{-4}	18
1×10^{-4} M FMN	0	0	5
	24	0	47
	24	1×10^{-4}	52
Glycolate 8 μmoles + 1×10^{-4} M FMN	0	0	44
	16	0	36
	16	1×10^{-4}	47

For details of enzyme preparation and treatment, see Figure 4. Warburg experiment is the same as for Table XV.

green material. When FMN was added to the preincubation mixture, the loss of enzymatic activity was considerably reduced and was only about 50 per cent. There was no stimulation of enzymatic activity by glycolate incubation of etiolated plant sap and again, in order to restore enzymatic activity, only an excess of added FMN was needed.

The failure to increase the enzyme activity of the dialyzed $(NH_4)_2SO_4$ fraction by incubation with glycolate may be explained by the fact that there was no FMN in the dialyzed protein so activation was impossible. Also if the activation was enzymatically controlled, the protein fraction may have removed an activating enzyme.

Substrate Activation of the Enzyme in vivo

In Table XVII are data on glycolic acid oxidase activity in the sap of etiolated wheat seedlings after the leaves had been sprayed in the dark with glycolate or other substrates one day before harvest. The $Q^N_{O_2}$ of the enzyme from plants sprayed with glycolic acid increased fourfold as compared with plants sprayed with water. These data confirm a previous report on the enzyme activation in vivo by feeding glycolate (5). When plants were sprayed with lactate, α-hydroxybutyrate or glyoxylate it was found that the $Q^N_{O_2}$ of saps prepared from the sprayed plants also increased about threefold over plants sprayed with water. Plants sprayed with acetate did not show appreciable increase

TABLE XVII

Effect of spraying on enzyme activity of etiolated wheat

Spraying solution	$Q_{O_2}^N$	
	Glycolate	Glycolate + FMN
Water	10	88
Glycolate	38	104
Lactate	27	104
α-Hydroxybutyrate	30	116
Glyoxylate	25	97
Acetate	15	94

Each flat of plants was sprayed with 10 mmoles of a compound in 100 ml. Plants were sprayed in total darkness 24 hours before harvest. Age of plants at the harvest was 8 days. Control was sprayed with equal volume of water.

For Warburg assay 10 μmoles of glycolate per flask were used with 1 x 10^{-4} M FMN as specified.

in enzymatic activity.

FMN was added to the cell-free homogenates from the sprayed plants in order to measure the total apo-plus holo-enzyme. There was a many fold increase in total glycolic acid oxidase activity. However, this activity was nearly the same regardless of the prior spray treatment. As in the *in vitro* activation by glycolate, the presence of the enzyme's substrate *in vivo* appears to have resulted in the conversion of all of the apoenzyme to holoenzyme. These results imply a substrate control of active enzyme activity, but not over the apoenzyme content.

B. RESPIRATION OF PLANT MITOCHONDRIA

General Experimental Condition Affecting Respiration

The conditions for reproducible maximum rates of oxidative phosphorylation by pea mitochondria was dependent upon a number of variables which are discussed in this section. The system required added ATP, glucose, hexokinase, and a yeast coenzyme concentrate, but not cytochrome c. Optimum substrate and phosphate concentration and duration of experiments were determined. The order of addition of substrates and enzymes even influence the reproducibility of the results.

For active oxidation of citric acid cycle intermediates, ATP, glucose, hexokinase, and yeast coenzyme concentrate containing DPN, TPN, and CoA must be added to

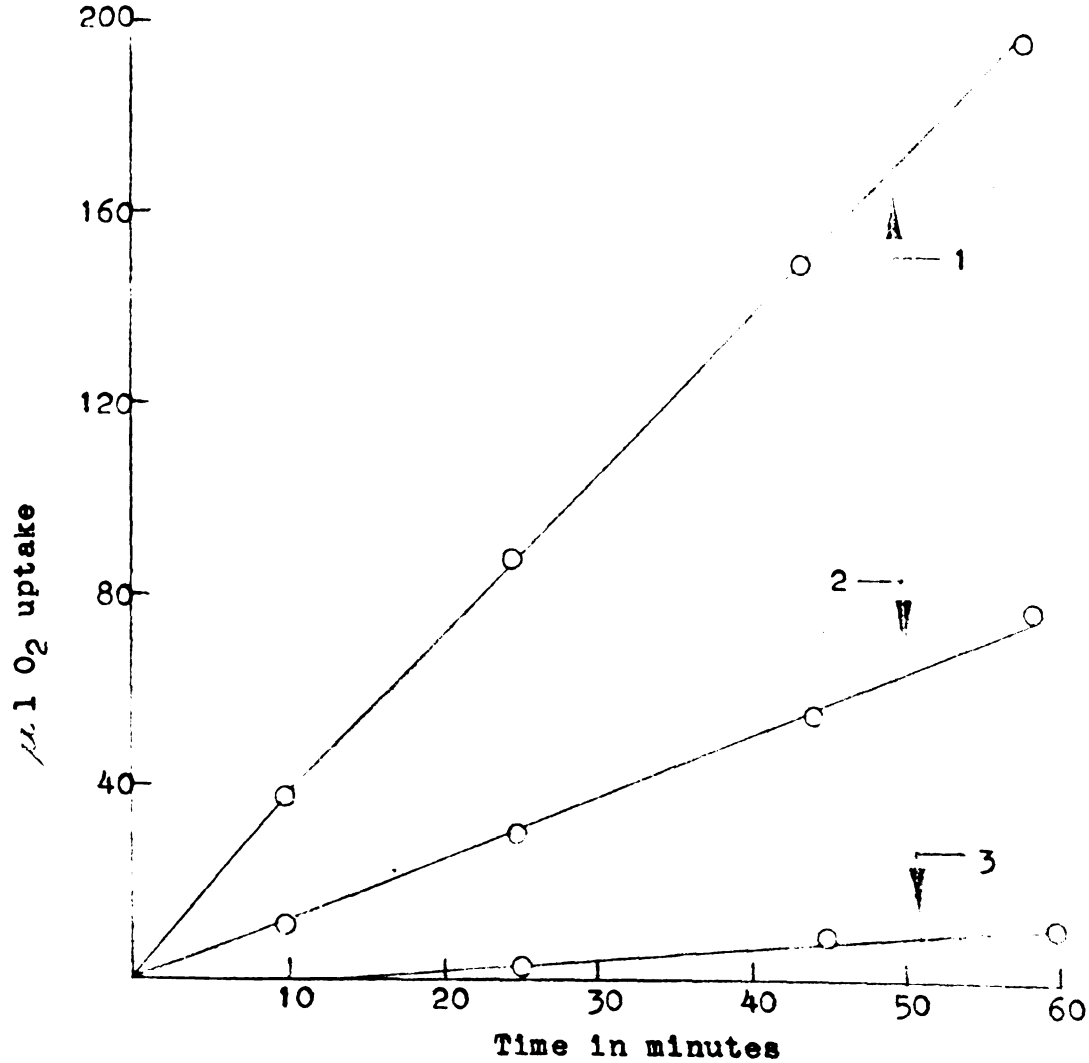
the mitochondria preparation (Figure 5). These requirements are the same components which are characteristic of oxidative phosphorylation by animal mitochondria. It has been reported that P/O ratios, as determined with particles from tobacco leaves, were not stimulated by added yeast coenzyme concentrate (80). However, in the absence of the yeast coenzyme concentrate, pea mitochondria were not capable of catalyzing the oxidation of the components of the citric acid cycle. On the other hand, pea mitochondria preparations contained sufficient cytochrome c that addition of this component to the assay only slightly increased the rate of oxidation of citrate or succinate (Table XVIII).

Optimum substrate concentration was determined for citrate oxidation (Figure 6). A standard amount of mitochondria that was obtained from 1.5 g of fresh tissue was used. The citrate concentration for maximum rate of oxidation was 25 μmoles per 3 ml. of volume in the Warburg vessel. Although it is impossible to predict from this finding the optimum concentration of other substrates, nevertheless, in subsequent studies high substrate concentrations were avoided (104) and usually 10 or 20 μ moles of each substrate per flask were used.

The influence of increasing the phosphate buffer concentration is shown in Figure 7 for citrate oxidation and in Figure 8 for malate oxidation. Both oxygen uptake

FIGURE 5

Effect of ATP-glucose-hexokinase system and yeast coenzyme concentrate on succinate oxidation by green pea mitochondria



1 - Complete, as described in methods section.

2 - ATP-glucose-hexokinase excluded.

3 - Yeast coenzyme concentrate omitted. All vessels contained 10 μmoles of succinate.

TABLE XVIII

Requirements of green pea mitochondria for cytochrome c and effect of glyoxylate on citrate and succinate oxidation

Substrate, 10 μ moles each	Cytochrome c	μl O_2 Uptake/hr.	%
Citrate	0	224	100
Citrate	0.5 mg.	265	118
Citrate + glyoxylate	0	170	76
Citrate + glyoxylate	0.5 mg.	165	62
Succinate	0	190	100
Succinate	0.5 mg.	199	105
Succinate + glyoxylate	0	35	18
Succinate + glyoxylate	0.5 mg.	38	19

FIGURE 6

Substrate inhibition of citrate oxidation.

Each flask contained the standard assay components including the mitochondria which were isolated from 1.5 gm. of fresh tissue.

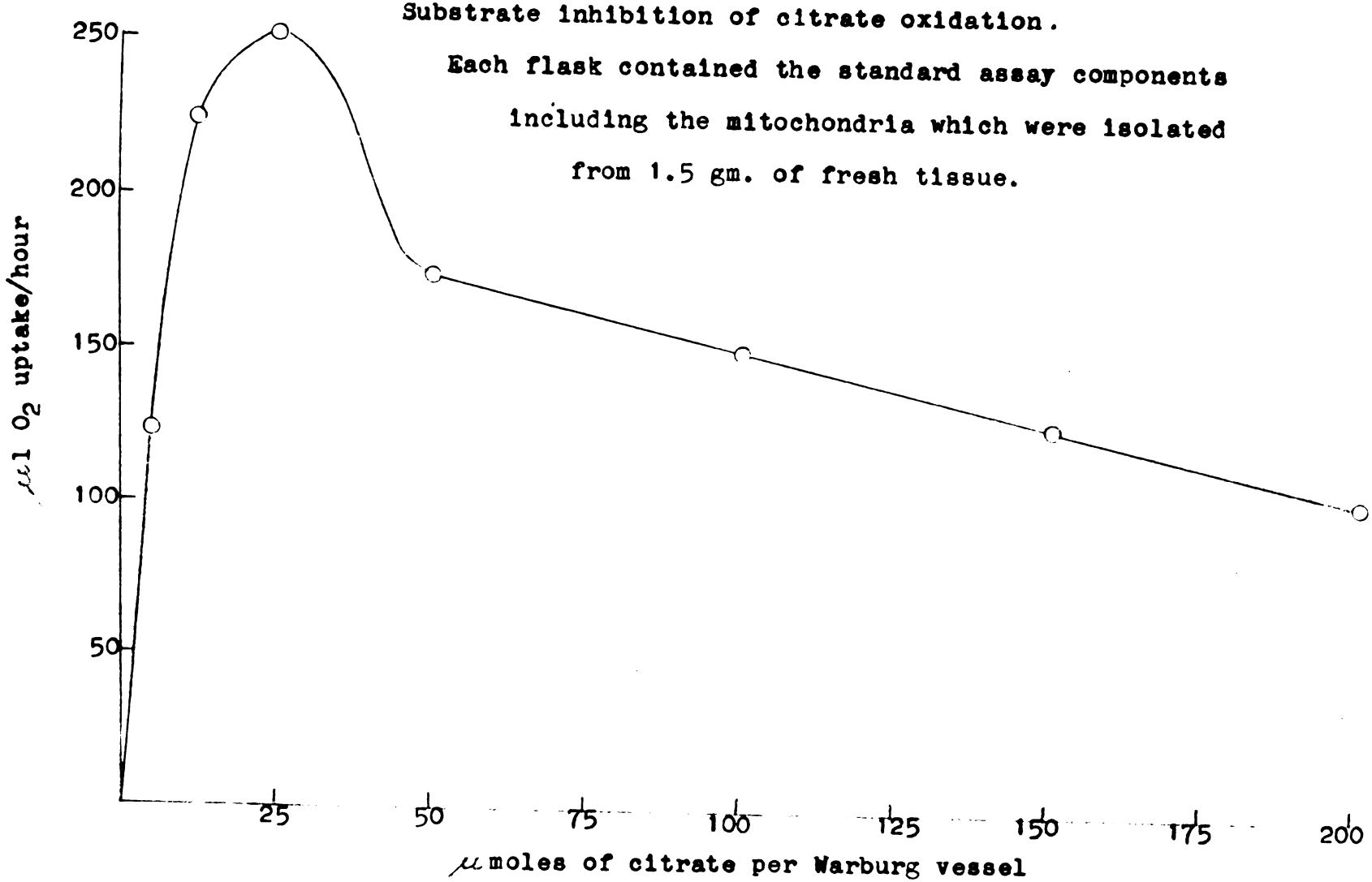
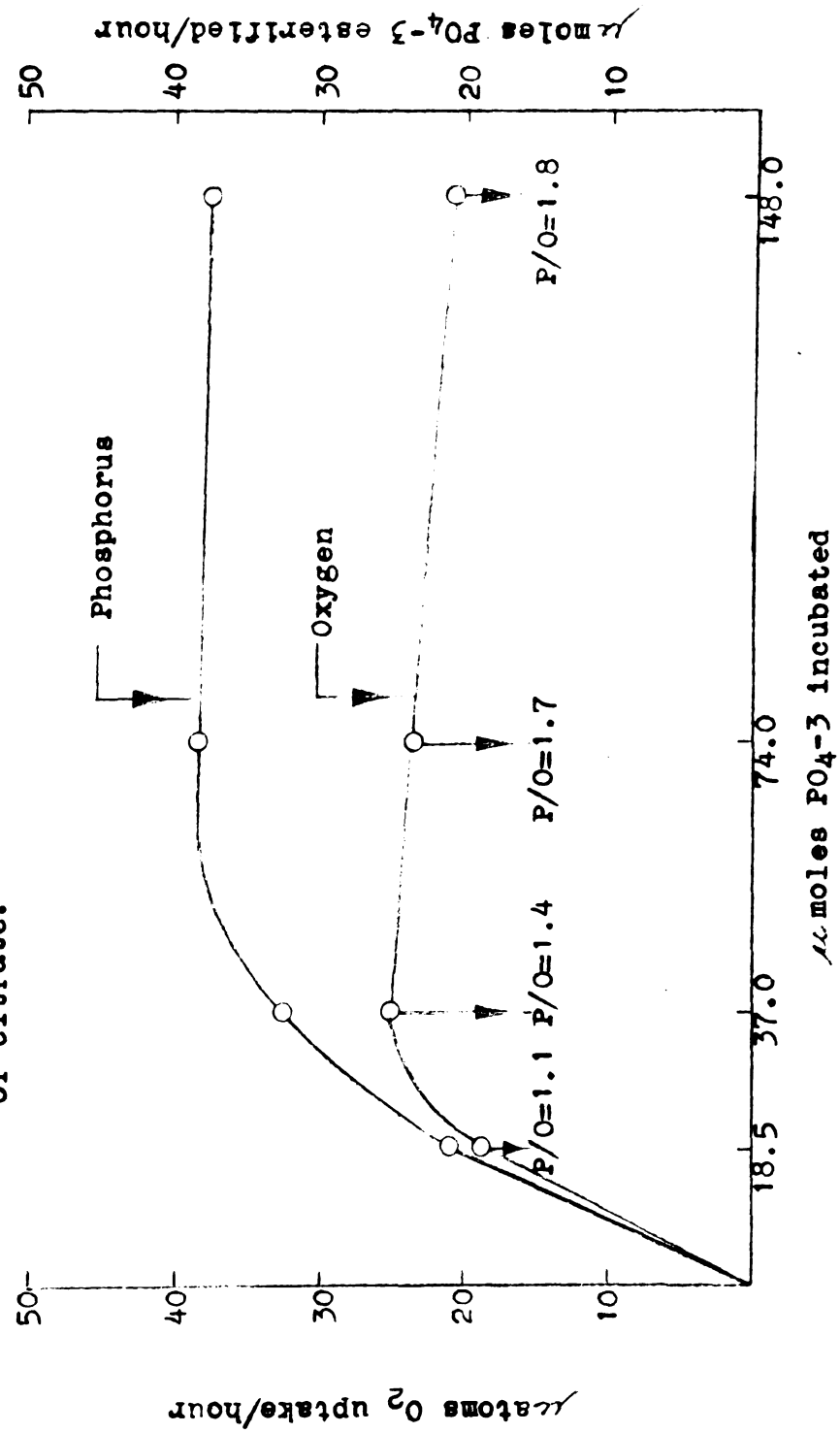


FIGURE 7

Effect of phosphate concentration on oxygen uptake and P/O ratio. Substrate, 20 μ moles of citrate.



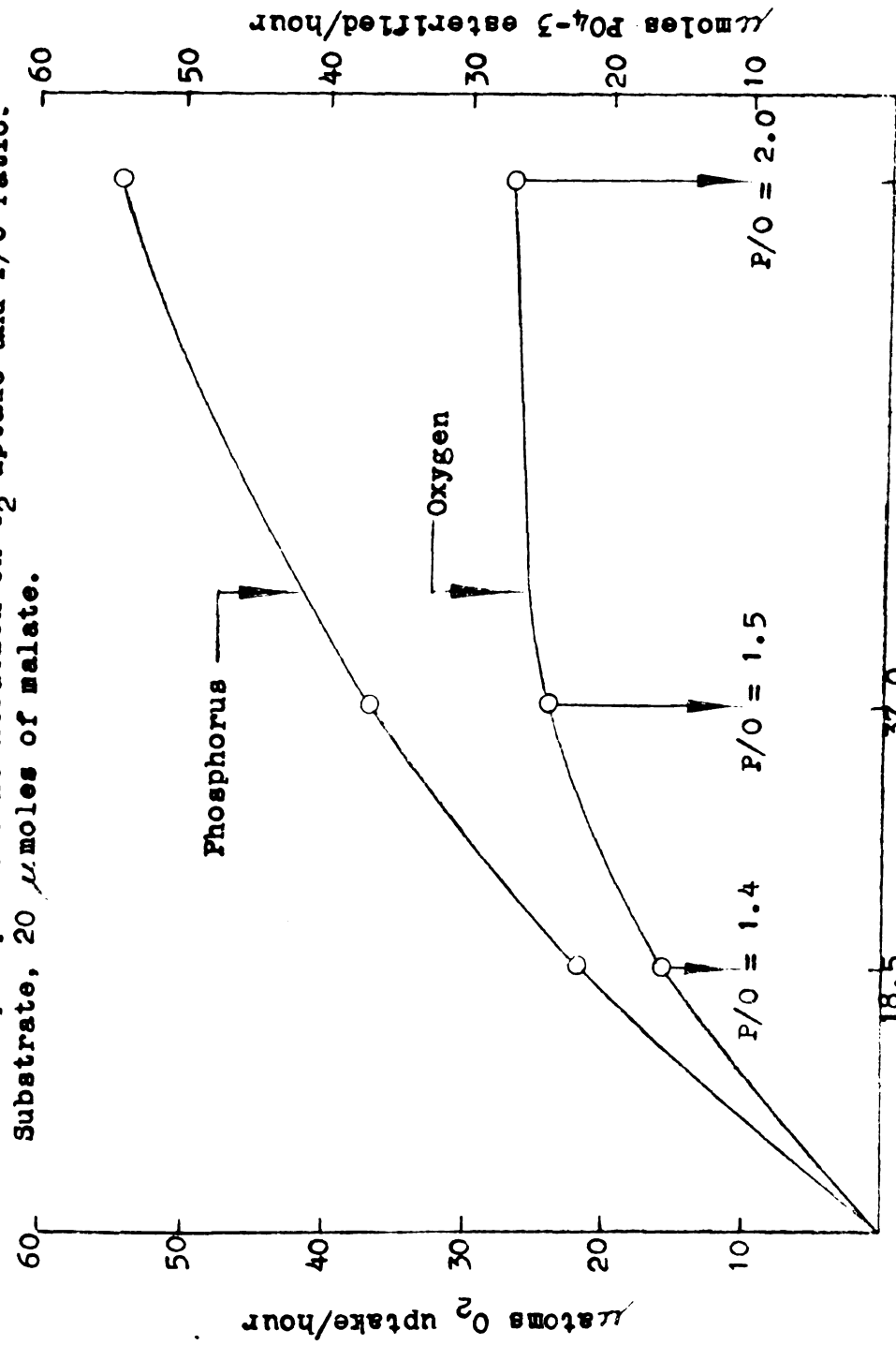


FIGURE 8
Effect of phosphate concentration on O_2 uptake and P/O ratio.
Substrate, 20 μ moles of malate.

and phosphorylation increased with increasing phosphate concentration. At higher phosphate concentration phosphorylation increased more than oxygen utilization so that highest P/O ratios were obtained when an excess of phosphate was present.

In Table XIX is shown the effect of substrate and phosphate concentrations and also the length of the experiment on P/O ratios. Highest P/O ratios when malate was used as a substrate were obtained from short experiment (30 minutes) with a low substrate concentration (10 μmoles) and a rather high phosphate buffer concentration (74 μmoles). The P/O in this case was 2.4. However, a P/O ratio of only 1.3 was obtained when the time of the experiment was doubled and the phosphate concentration halved while retaining the same substrate concentration.

At the beginning of the measured reaction, one of the enzymatic systems, mitochondria enzyme or glucose-hexokinase system, was added from the side arm of the reaction vessel. Contrary to expectation, this sequence of additions greatly altered the rate of oxidation. Addition of the glucose-hexokinase system from the side arm was greatly superior over incubation with the mitochondria in the side arm (Table XX). In the former case the mitochondria was preincubated with a substrate, succinate, for about 45 minutes while the experiment was being set up; 30 minutes at

TABLE XIX

Dependency of P/O ratio upon concentrations of substrate and phosphate and the duration of the experiment

Experiment	μ moles:		P/O
	Phosphate	Malate	
60 min.	37	10	1.3
		20	1.4
		40	1.5
	74	10	1.4
		20	1.8
		40	1.7
30 min.	37	10	1.9
		20	1.7
		40	1.8
	74	10	2.4
		20	2.1
		40	2.0

TABLE XX

Effect of the sequence of addition of reaction components on oxidation of green pea mitochondria

Reactants in:		O_2 uptake μl/hour **
Flask	Separate side arm	
Mitochondria 10 μmoles succinate	Glucose-hexokinase*	179
Mitochondria 10 μmoles succinate + 10 μmoles glyoxylate	Glucose-hexokinase	59
Glucose-hexokinase 10 μmoles succinate	Mitochondria	53
Glucose-hexokinase 10 μmoles succinate + 10 μmoles glyoxylate	Mitochondria	54
Mitochondria	Glucose-hexokinase, 10 μmoles succinate	87
Mitochondria	Glucose-hexokinase, 10 μmoles succinate, 10 μmoles glyoxylate	62

* In one solution.
** Average of two experiments.

0-2° during the flask preparation and then 15 minutes at a temperature up to 30°C. during equilibration and combining of the reactants. Perhaps during this period the substrate stabilized the mitochondrial preparation.

Thus, the enzymatic activity of mitochrondria depends upon many factors and a further important variation was the procedure for mitochondria preparation. Thus, exact duplication of results was difficult. Still another reason for reporting P/O ratios over a range of values for the same substrate was caused by the accuracy of the analytical methods for O_2 uptake by Warburg's method and for esterified phosphate by Fiske-Subbarow colorimetric procedure. These methods are unreliable for low values of O_2 uptake and phosphate esterified. Unfortunately, there are numerous reports in the literature for P/O ratios based on values of one or less μmoles of oxygen or phosphorus (80).

Isolated mitochondria from both green and etiolated pea leaves catalyzed rapid O_2 uptake and orthophosphate esterification during the oxidation of the acids of the citric acid cycle (Table XXI). The P/O ratios obtained confirm similar data now in the literature. Oxaloacetate was the only acid for the citric acid cycle which was not oxidized by my mitochondrial preparations.

TABLE XXI

Oxidative phosphorylation of pea mitochondria

Substrate, 20 μmoles	μatoms O_2/hour*		P/O	
	Green	Etiolated	Green	Etiolated
Citrate	12.6	10.0	2.7	3.2
Cis-aconitate	4.0	--	1.8	--
Isocitrate	11.9	--	1.4	--
Oxalosuccinate	5.1	--	1.7	--
α-Ketoglutarate	31.9	--	2.0	--
Succinate	15.4	7.8	1.7	2.0
Fumarate	5.9	--	2.1	--
Malate	7.2	--	3.1	
Oxaloacetate	0.0	--	0.0	--
Glycolate	8.0	0.0	0.3	0.0
Glyoxylate	0.0	0.0	0.0	0.0
Glyoxylate	2.1**	--	1.3	--

* Mitochondria from 0.66 - 1 gm. tissue.

** Mitochondria from 2 - 3 gm. tissue, average from 6 experiments.

Oxidation of Glycolate and Glyoxylate by Mitochondria

Glycolic acid was also rapidly oxidized by mitochondrial particulates. Glycolic acid oxidase is considered to be a soluble enzyme of the cytoplasm. Glycolate oxidation could have been catalyzed by this enzyme located in the mitochondria themselves or by the absorption on the mitochondrial surfaces of the cytoplasmic glycolic acid oxidase during their isolation. It is well known that mitochondria possess such absorptive abilitives and even washing of particulates several times does not remove absorbed enzyme (80, 81).

Although glycolic acid was rapidly oxidized, there were only traces of orthophosphate esterified and the P/O ratio was practically zero as Zelitch has also shown (80). It is concluded that the oxidation of glycolic acid by mitochondria is different in character from that involved in the oxidation of the constituents of the citric acid cycle acids. These experiments had been initiated to determine whether the FMN dependent glycolic acid oxidase system could be coupled to the cytochrome electron carriers as is the FAD succinic acid dehydrogenase system. The low P/O ratio of 0.3 indicated that this coupling of glycolate oxidation to oxidative phosphorylation was not feasible.

The oxidation of glyoxylate was extremely slow, and in fact, the oxidation of glyoxylate could only be

demonstrated by increasing the amount of mitochondria three to fivefold. However, there was a reproducible and substantial P/O ratio of greater than one accompanying this oxidation after correction for endogenous effects. The explanation for this phosphorylation has not been elucidated. The most likely explanation is that some malate synthetase was present in the mitochondrial preparations. Malate synthetase has been shown to be very active in mitochondria preparations of five-day old pea cotyledons (58), and it is also present in much lower but measurable amounts in mature leaves of other plants. In our case the mitochondria were prepared from eight to ten-day old pea leaves and, therefore, malate synthetase was very likely still active in such preparations. This enzyme would have condensed glyoxylate with endogenous acetate to produce malate whose subsequent oxidation would account for the P/O values.

Inhibition of Oxidation by Glycolate or Glyoxylate

Both glycolate and glyoxylate inhibited the oxidation of the acids of the citric acid cycle by mitochondrial preparations. The inhibition consisted of a reduction in the rate of oxygen uptake and an uncoupling of oxidative phosphorylation for that part of the oxygen uptake which was not inhibited. Data from the use of glycolate as the inhibitor was difficult to interpret due to the oxidation of

the glycolate itself as discussed in the previous section. This difficulty was surmounted by using glyoxylate which was not oxidized at an appreciable rate or by using mitochondria which had been prepared from etiolated plants since they contained little glycolic acid oxidase.(34).

The effect of glycolate and glyoxylate on the inhibition of mitochondria respiration by each substrate of citric acid cycle has been studied in detail. The effect upon citrate and succinate oxidation by mitochondria from green peas are shown in Tables XVIII, XX, and XXII and Figure 9, the effect upon the oxidation of malate is shown in Figure 10, and for the effect on oxalosuccinate and α-ketoglutarate oxidation see Figure 11. The total oxygen uptake in the presence of glycolate was greater than that for citric acid component alone, but much less than the sum of the oxygen uptake for the citric acid component and glycolate separately. These results were not caused by an insufficiency of oxygen to permit both systems to function at maximum rates. Dilution of the mitochondrial preparations did not alter the ratio of the results. Further glyoxylate also inhibited the oxidation although there was no significant oxygen uptake from the glyoxylate alone. Data on the P/O ratio from glycolate plus a citric acid cycle compound is difficult to interpret because it consisted of two components which could not be differentiated. One

TABLE XXII

Effect of glycolate and glyoxylate on citrate and succinate oxidation by green pea mitochondria

Substrates, 10 μmoles each	Oxygen uptake		Phosphorylation	
	μatoms O_2/hour	%	P/O	%
Experiment A				
Citrate	7.0	100	2.2	100
Glycolate	8.0	114	0.3	14
Glyoxylate	2.1	30	1.3	59
Citrate + glycolate	8.9	127	0.7	32
Citrate + glyoxylate	5.4	77	1.8	82
Experiment B				
Succinate	6.7	100	2.7	100
Glycolate	8.7	130	0.1	4
Glyoxylate	0.6	9	0.0	0
Succinate + glycolate	11.0	164	0.8	30
Succinate + glyoxylate	4.0	60	1.1	41

FIGURE 9

Effect of glyoxylate on citrate and succinate oxidation by green pea mitochondria.

1 - Citrate.

2 - Citrate + glyoxylate.

3 - Succinate.

4 - Succinate + glyoxylate

Substrates, 20 μmoles each. The results with citrate and succinate substrates were determined in two separate experiments.

FIGURE 9

Effect of glyoxylate on citrate and succinate oxidation by green pea mitochondria

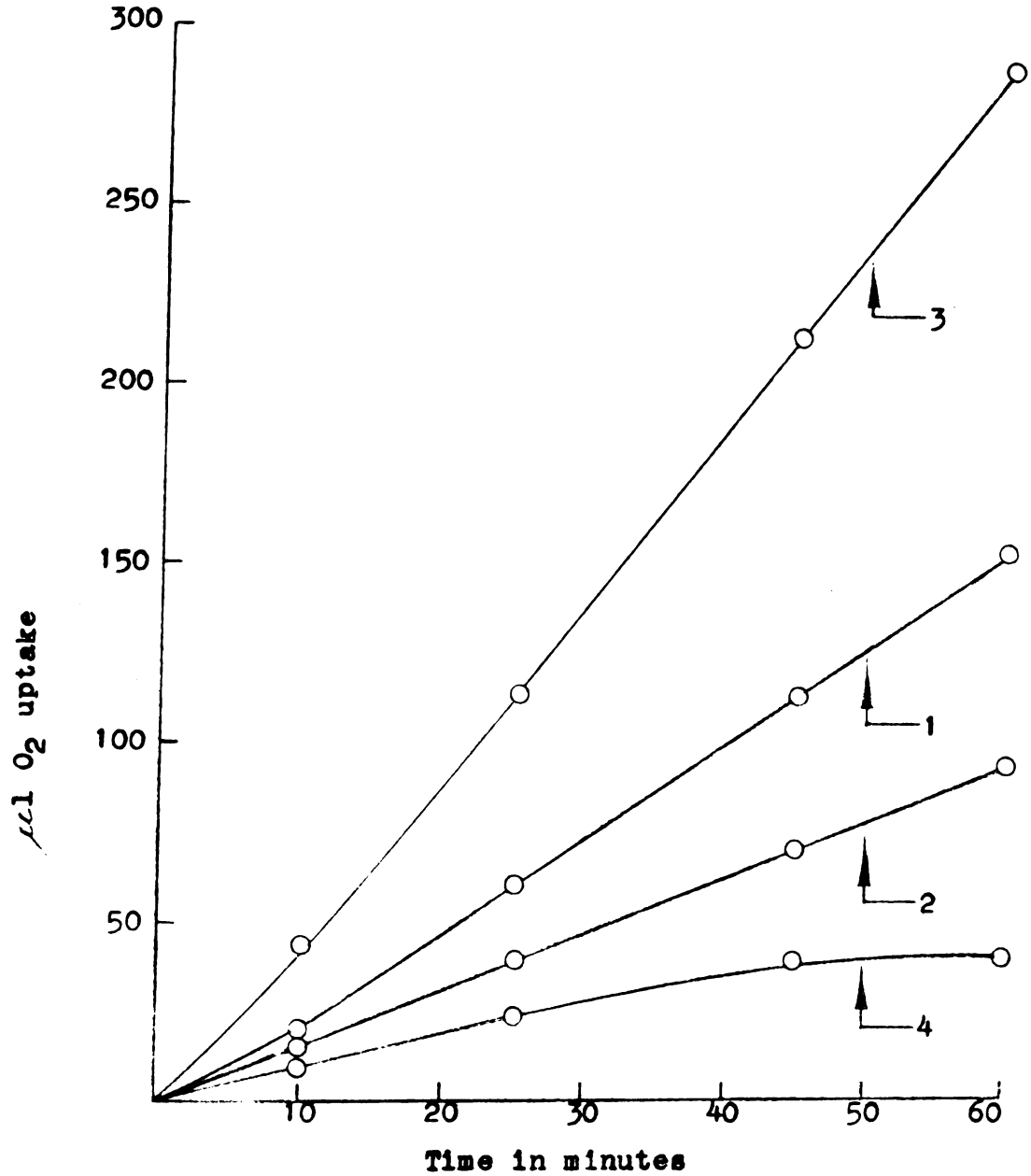
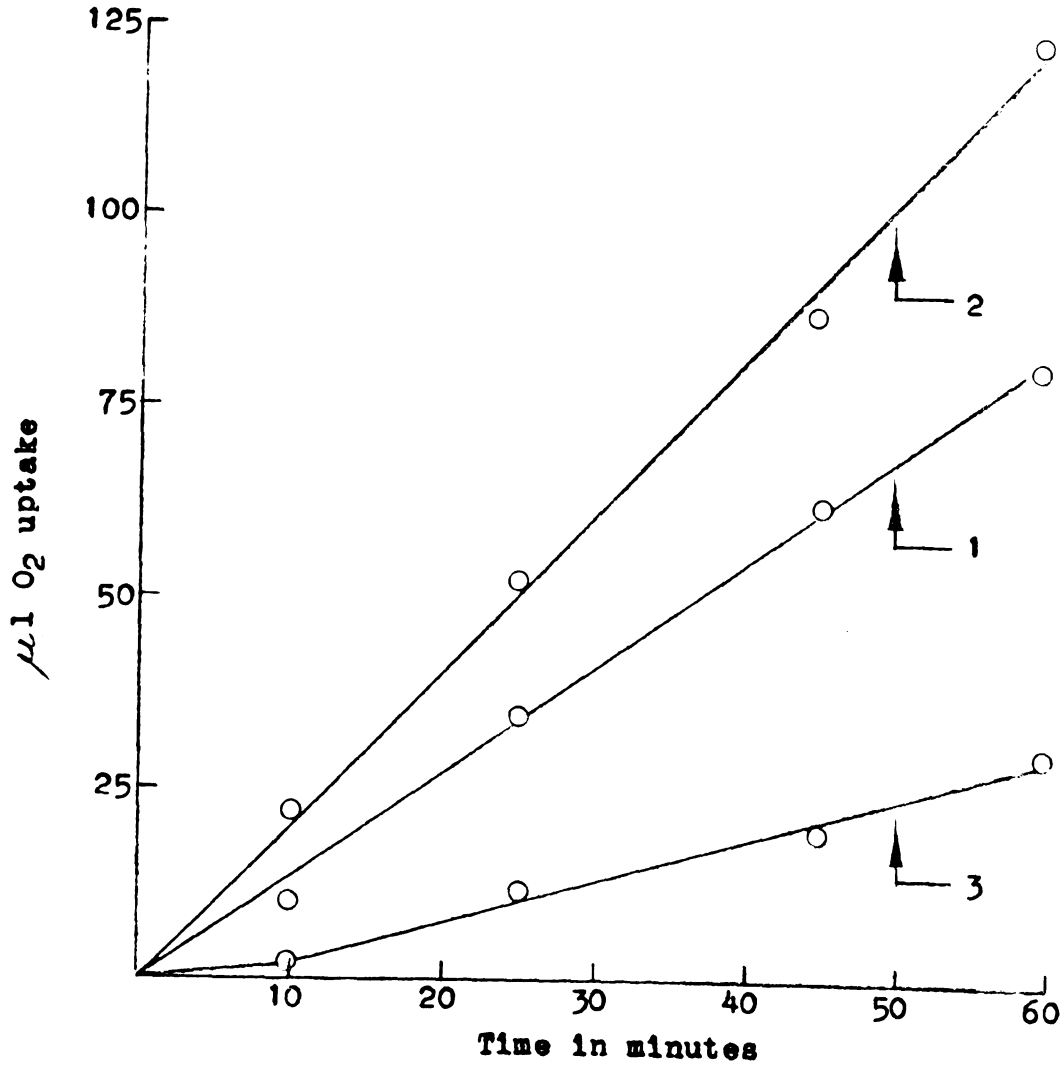


FIGURE 10

Effect of glycolate and glyoxylate on malate oxidation by green pea mitochondria.



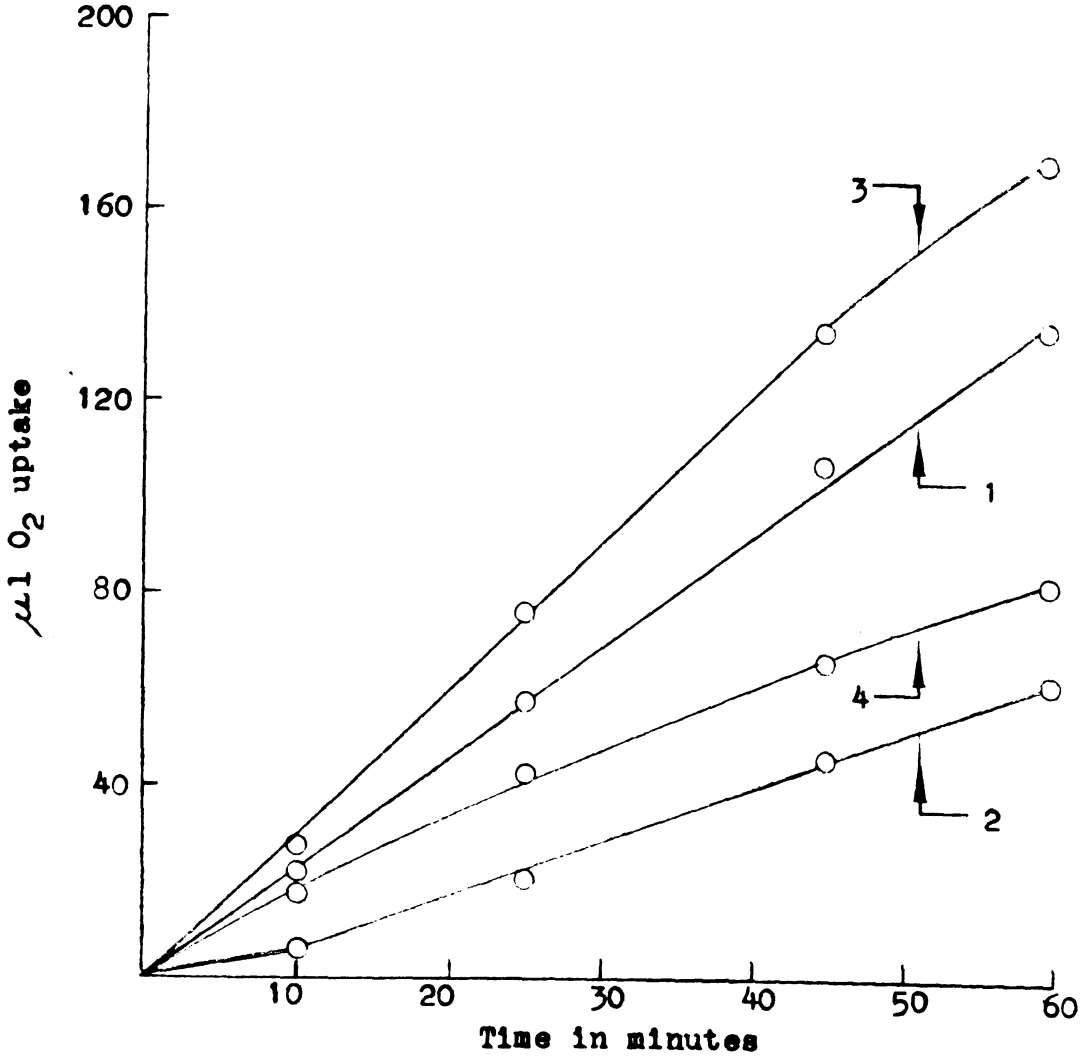
1 - Malate, P/O = 3.1.

2 - Malate + glycolate, P/O = 0.4.

3 - Malate + glyoxylate, P/O = 1.5.
Substrates, 20 μmoles each/flask.

FIGURE 11

Effect of glyoxylate on oxalosuccinate and α-ketoglutarate oxidation by green pea mitochondria.



1 - Oxalosuccinate, 2-oxalosuccinate + glyoxylate. 3 - α-Ketoglutarate, 4 α-ketoglutarate + glyoxylate. Substrates, 10 μmoles each. Two separate experiments were run for each substrate, oxalosuccinate and α-ketoglutarate.

was the oxidation of glycolate itself with a low P/O ratio and the other was the inhibited oxidation of the citric acid substrate. Consequently, very low P/O ratios were obtained from such a system because that portion of the oxidation attributed to glycolate contributed no energy for the phosphorylation.

One way the complexities of the above results are simplified was to use mitochondria that had been isolated from etiolated peas. The activity of glycolic acid oxidase is in the order of tenfold less in etiolated tissue (5, 34), and thus the contribution of glycolate oxidation in the combined system was much less. Glycolate inhibited 50 per cent the rate of oxygen uptake for citrate and succinate oxidation by mitochondria from etiolated peas (Table XXIII). However, for the remaining oxidation, the P/O ratio was not reduced significantly. These results suggested that the effect of glycolate was primarily in inhibition of the initial part of citrate oxidation rather than any effect upon the cytochrome electron transport process.

A second way to simplify the studies of the inhibitory effects of glycolate and glyoxylate was to utilize only glyoxylate with sufficient mitochondria to obtain measurable oxygen uptake with the citric acid cycle components but not with glyoxylate. Then the correction for respiration with glyoxylate alone, which was always used for

TABLE XXIII

Effect of glycolate and glyoxylate on citrate and succinate oxidation by etiolated pea mitochondria

Substrates, 20 μ moles each	Oxygen uptake		Phosphorylation	
	μatoms O_2/hour	%	P/O	%
Experiment A				
Citrate	12.0	100	3.0	100
Glycolate	0.0	0	--	--
Glyoxylate	0.6	5	--	--
Citrate + glycolate	5.9	49	2.9	97
Citrate + glyoxylate	6.2	52	2.5	83
Experiment B				
Succinate	22.6	100	1.6	100
Glycolate	0.2	1	--	--
Glyoxylate	0.8	4	--	--
Succinate + glycolate	12.3	54	1.4	88
Succinate + glyoxylate	4.0	19	1.3	81

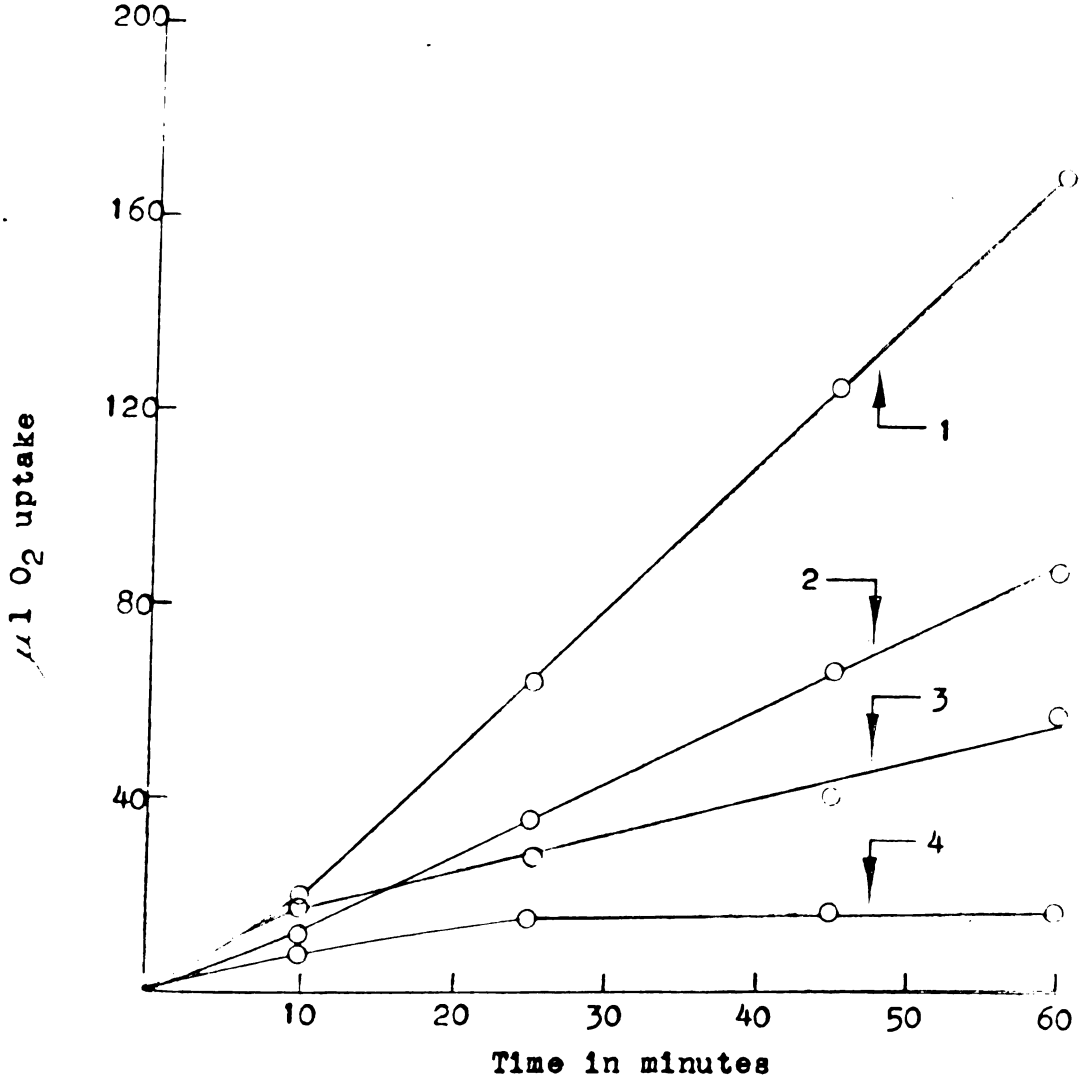
calculating the P/O ratio, was not large and could not introduce great uncertainties. Glyoxylate was at least as potent an inhibitor as glycolate or better. With mitochondria from green peas the glycolate was in fact oxidized to glyoxylate and so it was not possible to know which acid was the true inhibitory, but glyoxylate was a better inhibitor (Table XXIII and Figure 12). Glyoxylate (0.007 M final concentration) inhibited succinate oxidation 80 per cent. With mitochondria from green peas very substantial inhibition was also obtained with glyoxylate (Tables XXII, XXIII, XXIV, Figures 9, 10, 11). In general, the glyoxylate inhibition did not reduce the P/O ratio of the remaining respiration as much as glycolate because the glyoxylate itself was not oxidized. Nevertheless, there was always a very measurable reduction of the P/O ratio in the presence of glycolate. Zelitch and Barber (80) reported no lowering of the P/O ratio from glyoxylate inhibition of succinate and citrate oxidation by mitochondria from spinach. We are not certain about the importance of this difference between plant tissue, because the magnitude of the lowering of P/O ratio in our system with glyoxylate was not always large nor consistent.

Consideration of "Oxalomalate" as Inhibitor

As discussed in the literature review section, Ruffo's group, using liver homogenates, reported on glyoxylate

FIGURE 12

Effect of glyoxylate on citrate and succinate oxidation by etiolated pea mitochondria.



1 citrate, 2 citrate + glyoxylate, 3 succinate, 4 succinate + glyoxylate. Substrates, 20 μmoles each. Two separate experiments were run for each substrate.

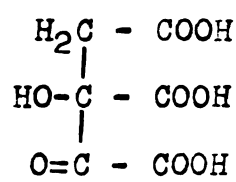
TABLE XXIV

Oxidation of oxaloacetate by green pea mitochondria

Substrate, 10 μmoles each	μ l O_2 uptake			
	10 min.	25 min.	45 min.	60 min.
Oxaloacetate	0	0	0	0
Glyoxylate	3	11	13	18
Succinate	27	68	136	188
Glycoxylate + succinate	15	33	43	52
Glyoxylate + oxaloacetate	1	1	9	13

After respirometric measurements, it was found that 73% of oxaloacetate unreacted when it was incubated alone, and 63% of it remained when it was incubated together with glyoxylate.

inhibition of oxidation of the citric acid cycle components. This inhibition was particularly severe when both glyoxylate and oxaloacetate were added to the incubation mixture and simultaneous accumulation of citrate had been observed. That group postulated that the inhibition was caused by the hypothetical compound "oxalomalic acid"



which could have been formed by a condensation of glyoxylic acid and oxaloacetic acid. Since oxalomalate has a configuration similar to citrate, it could have inhibited citrate oxidation as a competitive inhibitor for aconitase.

Our results differ from the observations from Ruffo's group and consequently we do not find their explanation of glyoxylate inhibition satisfactory for plant mitochondria. For mitochondria from peas glyoxylate inhibition was not limited to citrate oxidation, but it was also severely effective on the other substrates of the citric acid cycle. For α-ketoglutaric acid oxidation, for instance, there was no lag in the rate of this inhibition at the beginning of the experiment (Figure 11). It is unlikely that a specific inhibitor of aconitase could instantaneously affect, at a constant rate, the oxidation of a large excess

of α-ketoglutaric acid, succinate, or malate.

Oxaloacetic Acid and α-Ketoglutaric Acid Oxidation

Added oxaloacetate was not oxidized by our pea mitochondria even in the presence of acetyl phosphate, coenzyme A and transacetylase (Table XXIV). Analysis for oxaloacetate from incubation for an hour with the mitochondria, revealed that, despite this compound's known instability, 73 per cent of it was still present in the system.

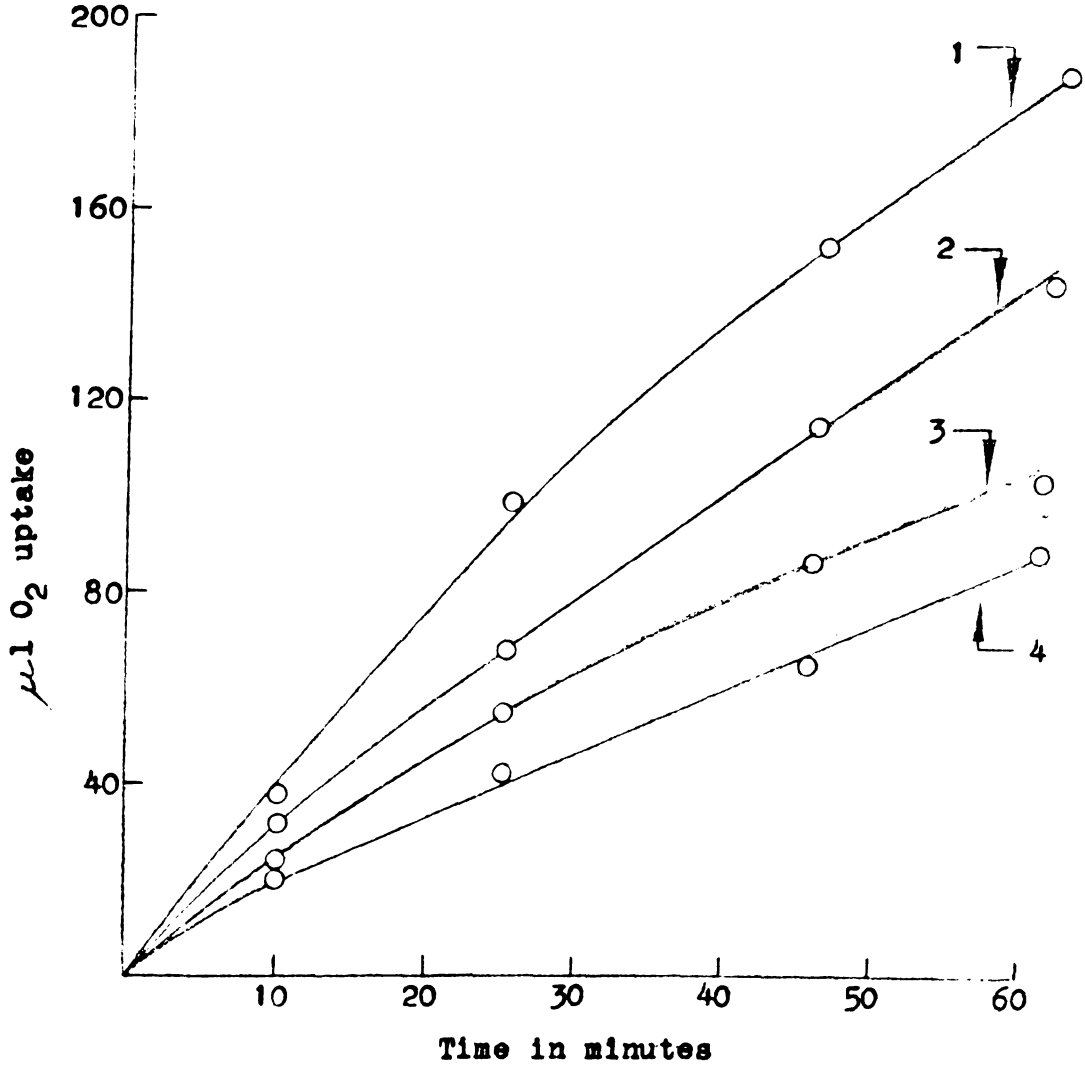
When malate-C^{14} was oxidized by pea mitochondria only traces of C^{14} appeared in citrate during the periods of time normally used to determine P/O ratios. Apparently the observable oxidation was mainly accounted for by steps before citrate formation.

These data in oxaloacetate oxidation need further investigation. Apparently the condensing enzyme system was severely restricted in the mitochondria as isolated. The data supports the contention that the glyoxylate inhibition of malic and succinate oxidation cannot be explained on the basis of an effect only on aconitase by "oxalomalate."

The inhibition by glyoxylate of α-ketoglutarate oxidation was not as severe as the inhibition by malonate (Figure 13). However, the inhibitoy effect from both glyoxylate and malonate was nearly additive. Since malonate

FIGURE 13

Single step oxidation of α-ketoglutarate



1 α-ketoglutarate, 2 α-ketoglutarate + glyoxylate, 3 α-ketoglutarate + malonate, 4 α-ketoglutarate + malonate + glyoxylate. Malonate alone was not oxidized. Substrates, 10 μmoles each/flask.

is known to block succinate oxidation, the inhibition by glyoxylate would have to be accounted for by an inhibition of the steps before malate formation. This argument would support our contention that glyoxylate inhibition is at least not all accountable for by an effect on aconitase.

Oxaloacetate was an inhibitor of mitochondrial oxidation of other components of the citric acid cycle (Table XXV). Oxaloacetate inhibited citrate oxidation by 65 per cent and α-ketoglutarate oxidation by 25 per cent. The inhibition from oxaloacetate and glyoxylate combined was additive and consequently very severe.

Stimulation of Succinate Oxidation by Glyoxylate and and Inhibition by Thioglycolate

Whereas, glyoxylate in equimolar concentrations with succinate inhibited succinate oxidation, low concentrations of glyoxylate produced as sustained stimulation of succinate oxidation (Figure 14). Careful measurements of oxygen uptake when 10 μ moles of succinate and 10 μ moles of glyoxylate were present showed an initial stimulation of respiration followed by severe inhibition. After one hour succinate oxidation was inhibited by 48 per cent. When the glyoxylate concentration was only one μ mole, however, the initial stimulation of succinate oxidation was maintained for at least an hour during which time there was recorded a

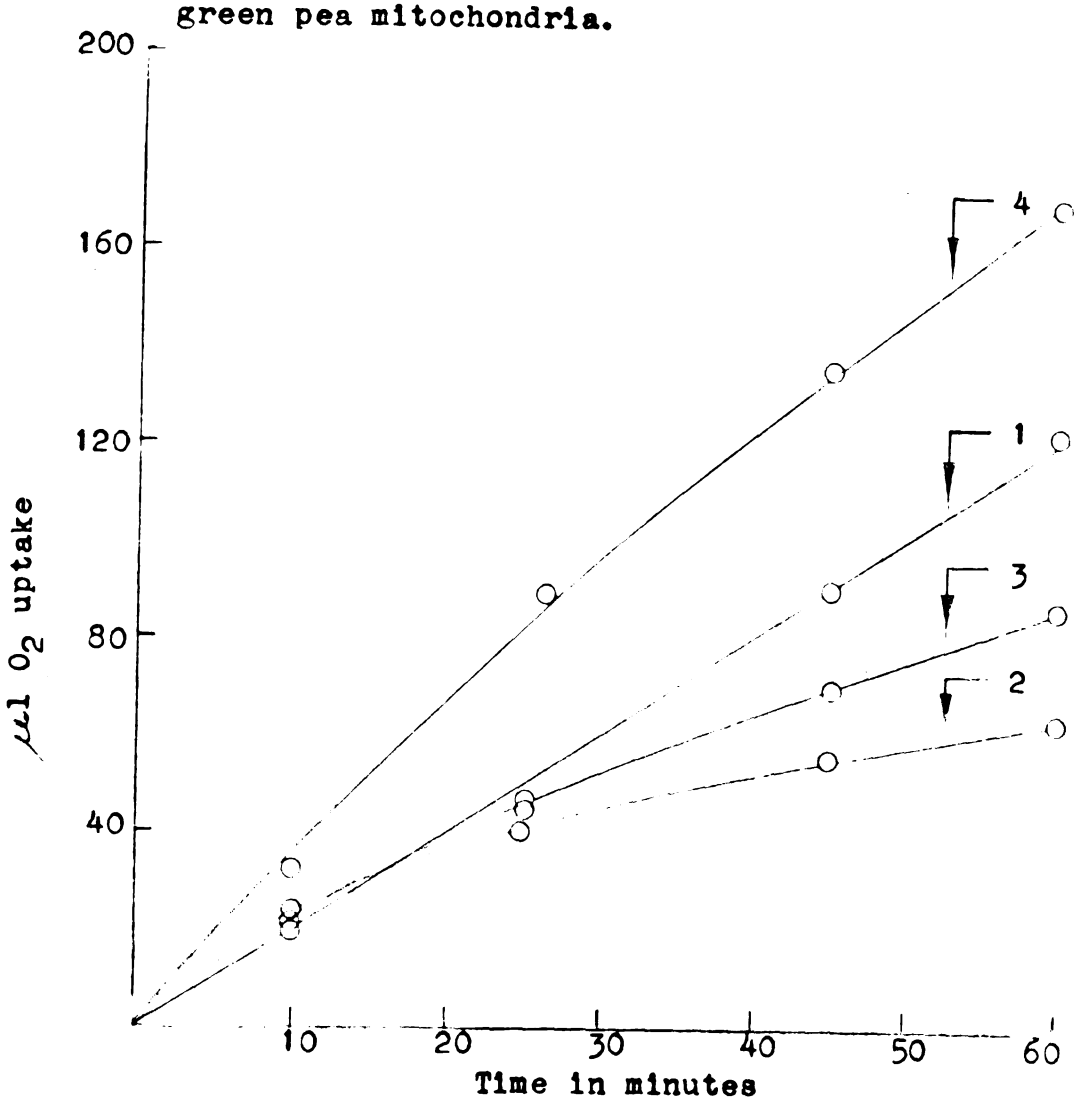
TABLE XXV

Effect of glyoxylate and oxaloacetate on citrate and α-ketoglutarate oxidation by green mitochondria

Exp.	Substrate, 20 μ moles each	Oxygen uptake							
		10 min.		25 min.		45 min.		60 min.	
		μl	%	μl	%	μl	%	μl	%
1	Citrate	25	100	72	100	145	100	184	100
	Oxaloacetate (OAA)	2		6		5		3	
	Glyoxylate	5		16		18		19	
	OAA + glyoxylate	4		8		12		10	
	Citrate + glyoxylate	23	92	65	90	123	85	148	80
	Citrate + OAA	0	0	14	19	50	34	65	35
	Citrate + glyoxylate + OAA	3	12	11	15	21	14	21	11
2	α-Ketoglutarate	56	100	151	100	277	100	352	100
	OAA	0		0		0		0	
	Glyoxylate	5		2		15		15	
	OAA + glyoxylate	0		0		4		6	
	α-Ketoglutarate + OAA	30	54	85	56	195	70	265	75
	α-Ketoglutarate + glyoxylate	30	54	68	45	141	51	177	50
	α-Ketoglutarate + glyoxylate + OAA	8	14	18	12	65	23	86	24

FIGURE 14

Effect of various glyoxylate concentrations on oxidation of succinate by green pea mitochondria.



1 - Succinate 10 μm.

2 - Succinate 10 μm + glyoxylate 10 μm.

3 - Succinate 10 μm + glyoxylate 5 μm.

4 - Succinate 10 μm + glyoxylate 1 μm.

total of 39 per cent stimulation of respiration by glyoxylate. Thioglycolate has often been used to protect enzymatic systems because of its -SH component, but its use with the plant mitochondria was most inhibitory (Table XXVI).

Stimulation of Glyoxylate and Succinate Oxidation by FMN

Addition of FMN to green pea mitochondria stimulated several fold either glyoxylate or succinate oxidation (Table XXVII). The reason for either of these stimulations is not clear. Glyoxylate reduction by glyoxylic acid reductase would require DPNH (75) and FMN could only indirectly affect the system by stimulating the reoxidation of glycolate by glycolic acid oxidase.

Glyoxylate utilization by isocitrase should not be stimulated by FMN. Glyoxylate oxidation to oxalate by glycolic acid oxidase (73) would be stimulated by added FMN. 1×10^{-2} molar FMN stimulated succinate oxidation 100 per cent. Although this is a high concentration it is not indicative of the concentration inside the mitochondria. Succinic dehydrogenase from yeast bacteria and animals have FAD as a prosthetic group. The nature of flavin in succinic dehydrogenase from plant tissue has not been established. Though data suggests the possibility that succinic dehydrogenase might be an FMN linked system in plants, other explanations for the data are possible.

TABLE XXVI

Effect of thioglycolate on succinate oxidation by green pea mitochondria

Substrate	O_2 uptake	
	µl/hr.	%
10 µ moles succinate	76	100
10 µ moles glyoxylate	18	
50 µ moles thioglycolate	30	
50 µ moles thioglycolate + 10 µ moles glyoxylate	23	
10 µ moles succinate + 10 µ moles glyoxylate	54	71
10 µ moles succinate + 50 µ moles thioglycolate	36	47
10 µ moles succinate + 10 µ moles glyoxylate + 50 µ moles thioglycolate	26	34

TABLE XXVII

Effect of glyoxylate and FMN on succinate oxidation by green pea mitochondria

Substrate, 10 μmoles each	FMN Final Conc.	μl O_2 Uptake/hr.
Glyoxylate	0	4
	1×10^{-2}	28
	1×10^{-3}	32
	1×10^{-4}	29
Succinate	0	79
	1×10^{-2}	160
	1×10^{-3}	132
	1×10^{-4}	93
Succinate + glyoxylate	0	46
	1×10^{-2}	73
	1×10^{-3}	66
	1×10^{-4}	41

There was no oxygen uptake with FMN alone.

Interreactions Between Succinate, Glyoxylate and Glutamate

For considerations about any regulatory control of citric acid cycle oxidations by glyoxylate, the effect of associated reactions of glyoxylate which control the availability of glyoxylate would be pertinent. One such set of reactions of glyoxylate is its conversion to glycine in the presence of the glyoxylate-glutamic acid transaminase system. In our present investigations glutamate was oxidized by the pea mitochondria, presumably via α-ketoglutarate (Table XXVIII). The oxidation of succinate and glutamate was additive as expected and glyoxylate inhibited succinate oxidation as previously observed. Glyoxylate stimulated glutamate oxidation probably because it accelerated its conversion to α-ketoglutaric acid by the transaminase reaction. Addition of glutamate to the glyoxylate inhibited succinate system stimulated the respiration fourfold. Though an exact interpretation of so complex a system as the latter is not possible, the results indicate that the expected interactions among the substrates were occurring. Addition of glutamic acid resulted in stimulated respiration due to formation of α-ketoglutarate and probably to removal of the inhibitory glyoxylate.

Effect of Glyoxylate and Cysteine on Mitochondrial Respiration

One way in which glyoxylate might inhibit the

TABLE XXVIII

Effect of glyoxylate and glutamate on succinate oxidation

Substrate, 10 μmoles each	μl O_2 uptake				
	5 min.	10 min.	25 min.	45 min.	60 min.
Succinate	25	44	92	115	194
Glutamate	9	14	32	58	75
Glyoxylate	5	6	8	15	14
Succinate + glutamate	32	54	120	210	255
Succinate + glyoxylate	14	21	36	52	57
Glutamate + glyoxylate	19	38	86	151	190
Succinate + glutamate + glyoxylate	25	47	105	181	231

mitochondrial oxidation of the acids of the citric acid cycle was through a reaction of its addehyde group with an -SH site which was necessary for enzymatic activity. We might, therefore, expect -SH compounds to protect against the inhibitory action of glyoxylate. Such was not the case with thioglycolate (see previous section) (Table XXVI). However, a fivefold excess of cysteine did prevent glyoxylate inhibition of succinate oxidation (Table XXIX), but not of malate oxidation. Cysteine by itself, like thioglycolate, was inhibitory to mitochondrial oxidation of succinate. The failure of cysteine to prevent glyoxylate inhibition of malate oxidation excludes the possibility of a cysteine and glyoxylate interaction and in this way the removal of glyoxylate.

Effect of Tyrosinase and Ascorbic Acid Oxidase

Since glycolate and glyoxylate are the substrate and product of a terminal oxidase, it was conceivable that other terminal oxidase systems might inhibit mitochondrial respiration. Tyrosinase was absent in the pea mitochondrial preparations (Table XXX) and ascorbic acid oxidase activity was low even in comparison to the activity of the glycolic acid oxidase. Substantial amounts of all three of these oxidases were present in the cytoplasm. The oxidation of succinate in the presence of either tyrosine and DOPA or

TABLE XXIX

Reversal of glyoxylate inhibition of succinate oxidation by cysteine

Substrate	O_2 uptake	
	µl/hr.	%
10 µmoles succinate	139	100
10 µmoles glyoxylate	14	
50 µmoles cysteine	20	
50 µmoles cysteine + 10 µmoles glyoxylate	7	
10 µmoles succinate + 10 µmoles glyoxylate	54	39
10 µmoles succinate + 50 µmoles cysteine	92	66
10 µmoles succinate + 10 µmoles glyoxylate + 50 µmoles cysteine	134	96

TABLE XXX

Effect of glyoxylate, tyrosine, 3,4-di-hydroxyphenylalanine (DOPA), and ascorbic acid on oxidation of succinate

Substrate, 10 μ moles each	μl O_2 uptake			
	10 min.	25 min.	45 min.	60 min.
Experiment 1				
Glyoxylate	2	6	11	11
Tyrosine	0	0	2	0
DOPA	0	0	5	5
Succinate	26	68	130	166
Succinate + glyoxylate	23	46	70	84
Succinate + tyrosine	31	73	121	150
Succinate + DOPA	30	70	110	135
Experiment 2				
Glyoxylate	0	3	11	13
Ascorbate	12	17	34	41
Succinate	37	74	136	174
Succinate + glyoxylate	31	48	76	88
Succinate + ascorbate	40	75	134	176

ascorbate was not substantially suppressed. These results indicate that the effect of the glycolate-glyoxylate terminal oxidase system on inhibiting mitochondrial respiration was different from the other terminal oxidases.

In Vivo Inhibition of Respiration by Glyoxylate

If glyoxylate were inhibiting mitochondrial respiration, then feeding plant tissue glyoxylate should result in an inhibiting of respiration in vivo (Table XXXI). Normally the amount of glyoxylate present in leaves is extremely small. Though much carbon from the photosynthetic carbon cycle moves through a pathway going from glycolate to glyoxylate, to glycine, to serine, to glyceric and then to sugars, the pool size of the glyoxylate is so extremely small that seldom is any glyoxylate-C^{14} detected on chromatograms. The significant inhibition of respiration in vivo by glyoxylate suggests that if it were to accumulate in leaves it would be inhibitory.

Nature of the Glyoxylate Inhibition

The Lineweaver-Burk plot for the effect of glyoxylate inhibition of succinate oxidation by green pea mitochondria indicates that the glyoxylate inhibition was noncompetitive (Figure 15). The fact that glyoxylate inhibited the oxidation of all the components of the citric acid cycle is an additional reason for believing that this glyoxylate effect was not caused by substrate similarity with the other acids.

TABLE XXXI

Effect of glyoxylate on citrate and succinate oxidation *in vivo*

Substrate, 20 μmoles each	O_2 uptake	
	μl/hr.	%
0	156	100
Glyoxylate	146	94
Citrate	137	88
Succinate	131	84
Citrate + glyoxylate	95	61
Succinate + glyoxylate	122	78

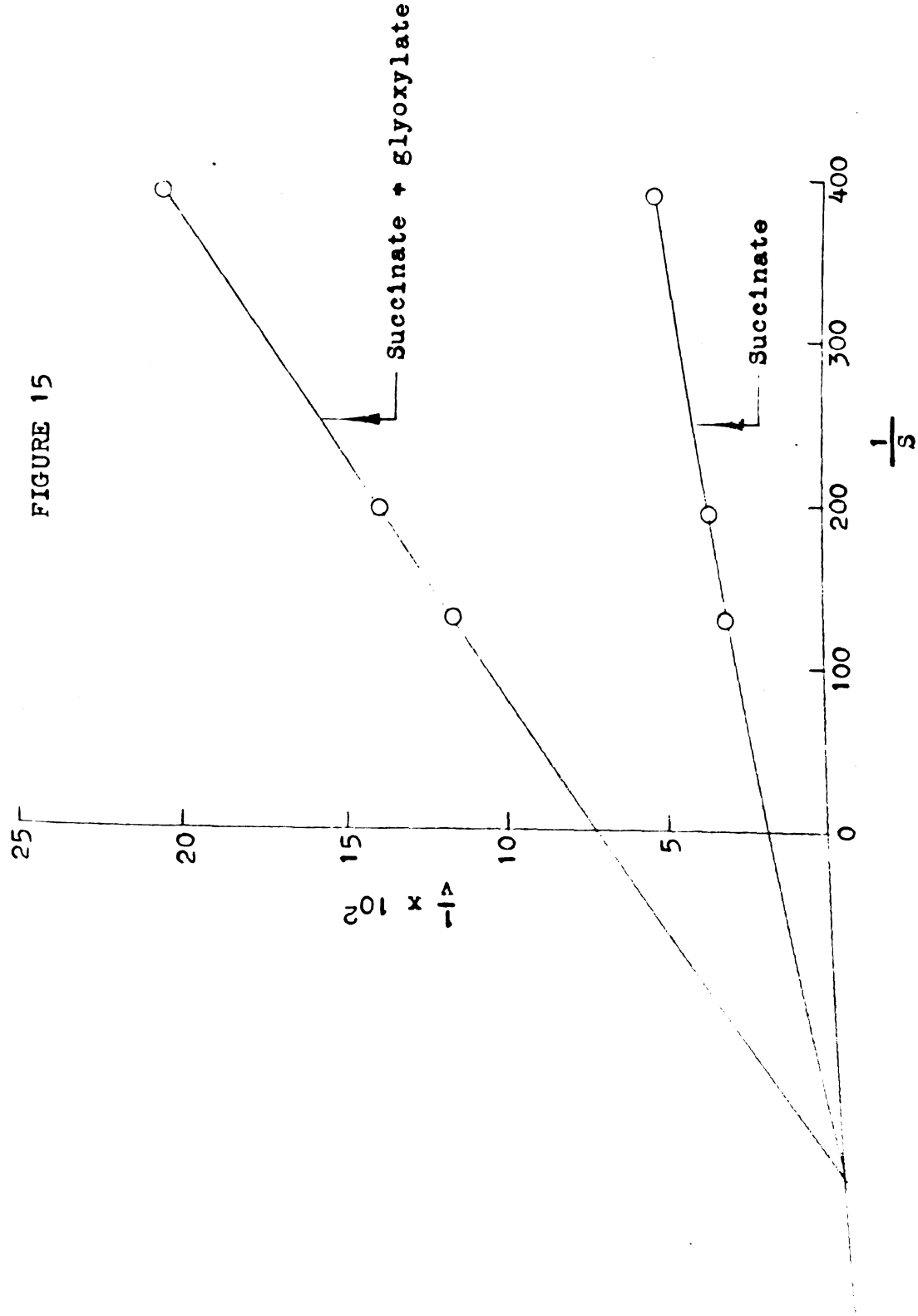
0.5 g. of 1 cm. long segments of the uppermost part of 10-day old green pea seedlings were placed in each Warburg vessel containing 37 μmoles of phosphate buffer at pH 7.3, total volume of reactants was 3 ml.

FIGURE 15

Inhibition of various levels of succinate oxidation by glyoxylate

Enzyme source was prepared from green pea mitochondria as described in the Materials and Methods Section. Standard manometric assay was used. Three levels of succinate, 10, 20, and 30 μ moles were used as a substrate and 20 μ moles per flask of glyoxylate as an inhibitor. S represents molarity of succinate used and v μatoms of O_2 taken up per hour.

FIGURE 15


Succinate + glyoxylate
Succinate
$\frac{1}{v} \times 10^2$
$\frac{1}{S}$
25
20
15
10
5
0
100
200
300
400

Reversal of the Glyoxylate Inhibition by DPN

In the study of a citric acid cycle intermediate oxidation by mitochondria preparations, the first product formed is usually also further metabolized and the results recorded as O_2 uptake are not necessarily due entirely to the oxidation of the added substrate. In order to eliminate this complexity pure malic dehydrogenase was used and the inhibitory effect of glyoxylate on malate oxidation was studied.

In Figure 16 are data representing the effect of glyoxylate on the malic dehydrogenase system. The results were recorded as the optical density increase at 340 mμ in a Beckman DU spectrophotometer due to a reduction of the added DPN. Optimum final concentration of DPN was 0.003 M and 0.001 M DPN was also nearly an optimum concentration for 25 units of the enzyme. When 0.05 M final concentration of glyoxylate was added to the cuvette containing 0.003 M DPN, there occurred a 39 per cent inhibition of malate oxidation and in the presence of 0.001 M DPN this inhibition was 56 per cent. Upon the addition of DPN in excess (0.02 M), no inhibition of malate oxidation by glyoxylate was obtained.

Similar effects upon the inhibition of lactic acid dehydrogenase by glyoxylate were obtained and again excess of DPN prevented the inhibition (Figure 17). Because the equilibrium constant is very unfavorable for the reaction lactate $\longrightarrow$ pyruvate (109), a high concentration of

FIGURE 16

Glyoxylate inhibition of malic dehydrogenase

1 - DPN, 0.001 M

2 - DPN, 0.003 M

3 - DPN, 0.001 M + glyoxylate, 0.05 M

4 - DPN, 0.003 M + glyoxylate, 0.05 M

5 - DPN, 0.02 M + glyoxylate, 0.05 M

All cuvettes contained: glycine, 0.1 M; malate, 0.01 M; and malic dehydrogenase, 25 units. Final pH was 10.5. Reagents are listed as final concentrations.

FIGURE 16

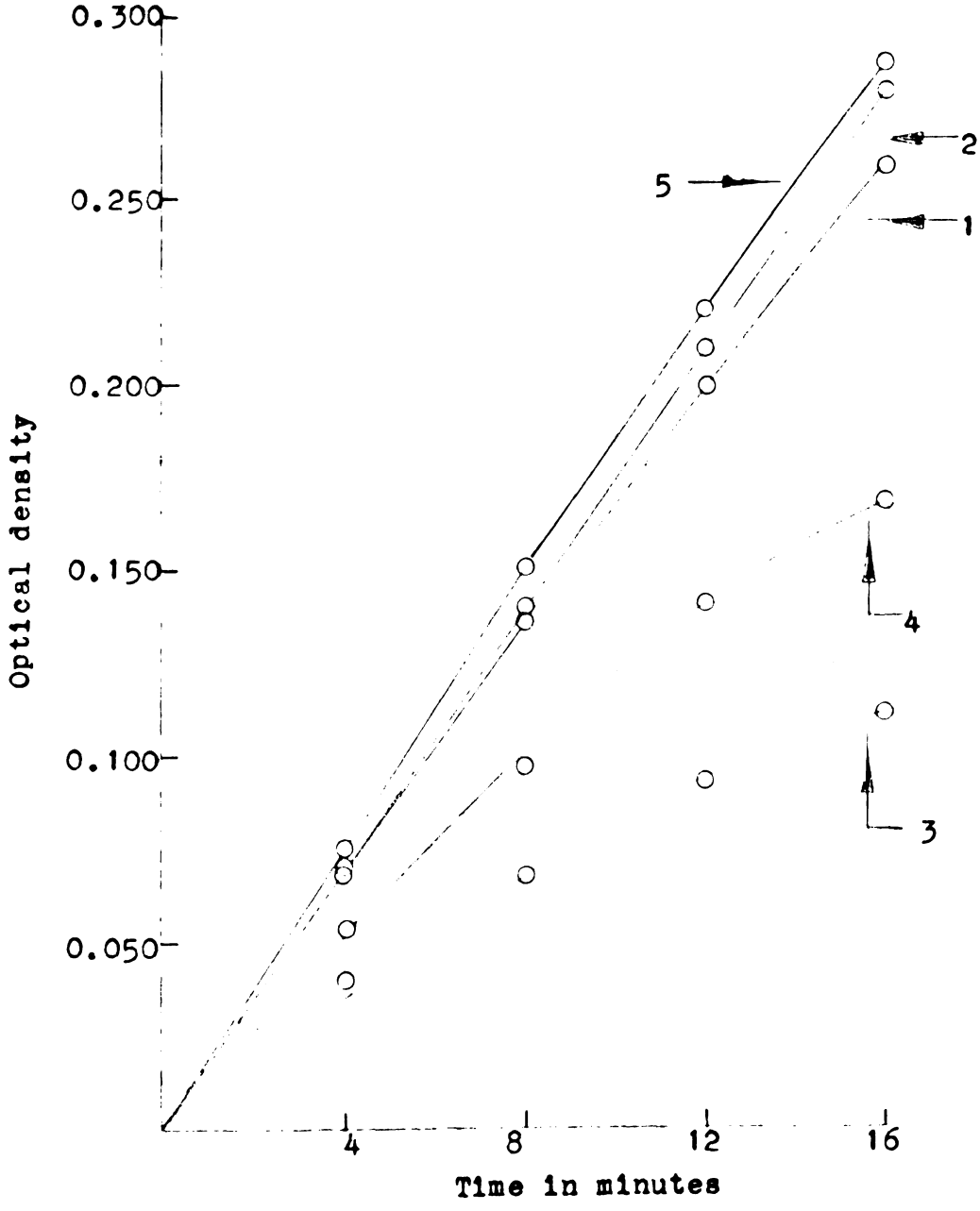
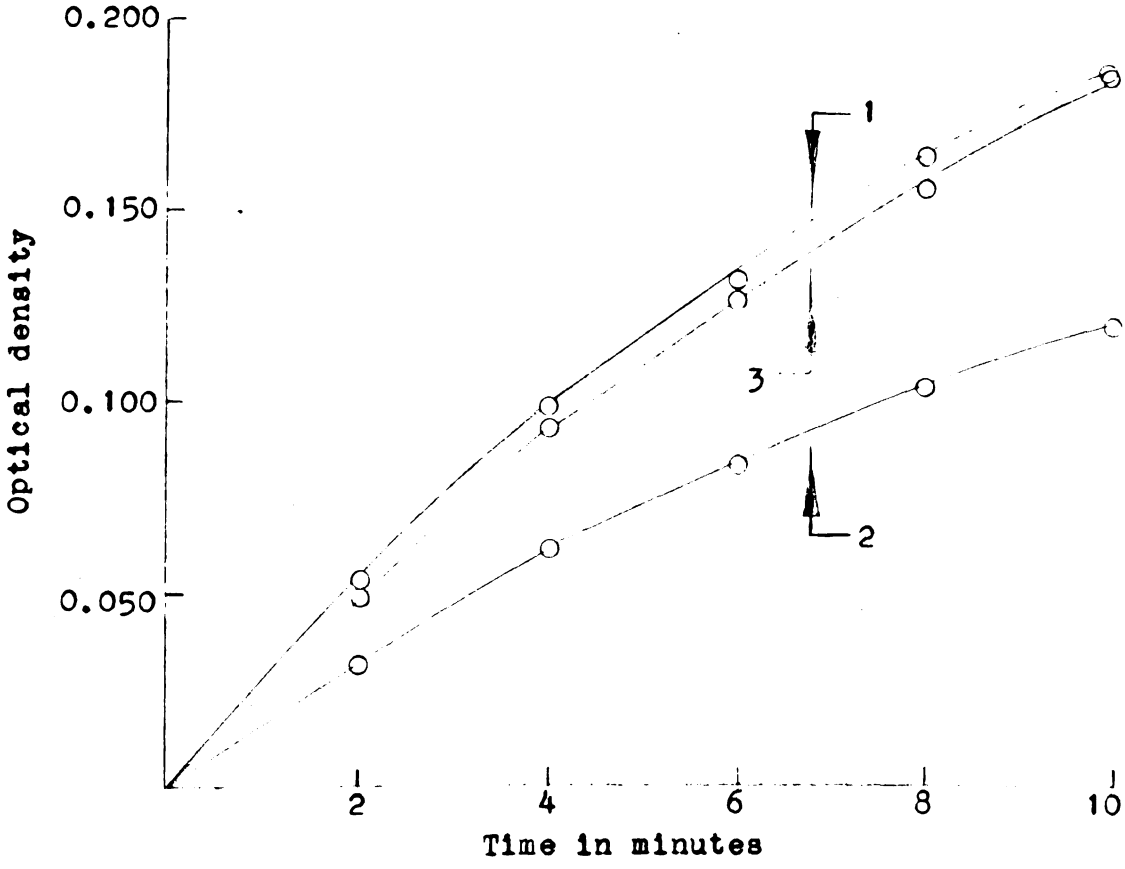

0.300
0.250
0.200
0.150
0.100
0.050
Optical density
4
8
12
16
Time in minutes
5
2
1
4
3

FIGURE 17

Glyoxylate inhibition of lactic acid dehydrogenase.



1 - 0.003 M DPN (optimal).

2 - 0.003 M DPN + 0.15 M glyoxylate.

3 - 0.05 M DPN + 0.15 M glyoxylate.

All curettes contained: Lactic dehydrogenase, glycine 0.1 M, and lactate 0.1 M. Final pH was 9.9. All reagents are listed as final concentrations.

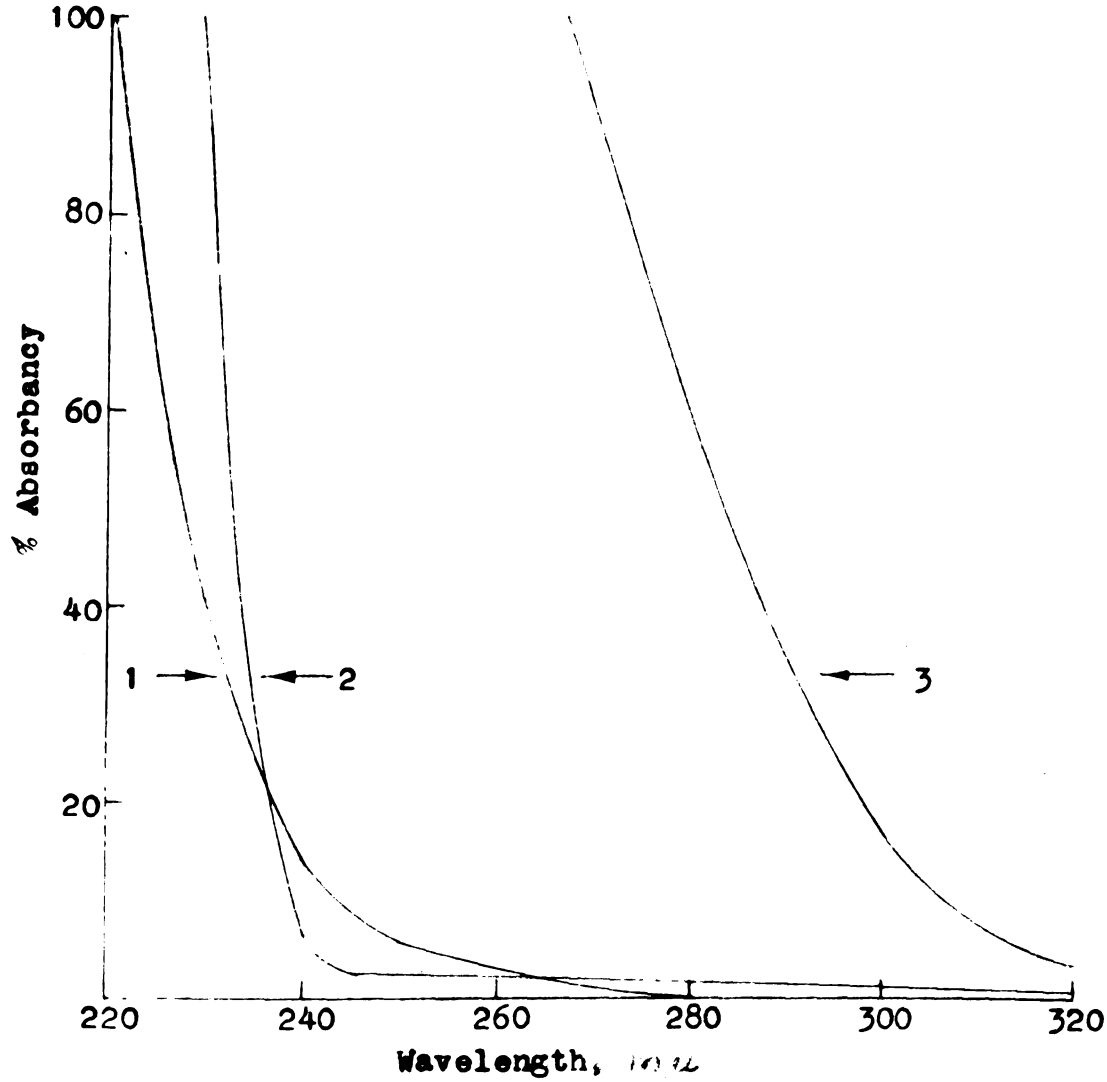
substrate had to be used. A linear reaction with time was obtained for only four minutes, probably because of an unfavorable equilibrium and product inhibition (104).

To explain the above results we have considered the fact that both glyoxylate and DPN are reported to be able to form complexes with other compounds. Glyoxylate condenses, for example, with tetrahydrofolate (105) and DPN with glycolaldehyde (107) or dihydroxyacetone (106). All of these condensations are non-enzymatic. In an attempt to reveal a condensation product of glyoxylate-DPN a different absorption spectrum from either oxidized or reduced DPN was sought by means of a Beckman DK-2 recorder. No change in the DPN spectrum in the region of 340 mμ was observed. Measurement at lower wave lengths was obscured by the discovery of a condensation of glyoxylate and the glycine buffer at pH 10.5 (Figure 18). The presence of such condensation products were confirmed chromatographically (Figure 19).

The explanation for glyoxylate inhibition of DPN linked dehydrogenases then becomes a question whether glyoxylate or a glyoxylate-glycine complex causes inhibition of substrate oxidation perhaps by removing the DPN from the enzymatic reaction. In order to answer this hypothesis other buffers than glycine were used. When carbonate-bicarbonate buffer was used, the inhibition of malate

FIGURE 18

Non-enzymatic condensation of glyoxylate and glycine, at pH 10.5, as reproduced from Beckman DK-2 chart.



1 - Glyoxylate, 0.01 M, in water or 0.1 M carbonate-bicarbonate buffer.

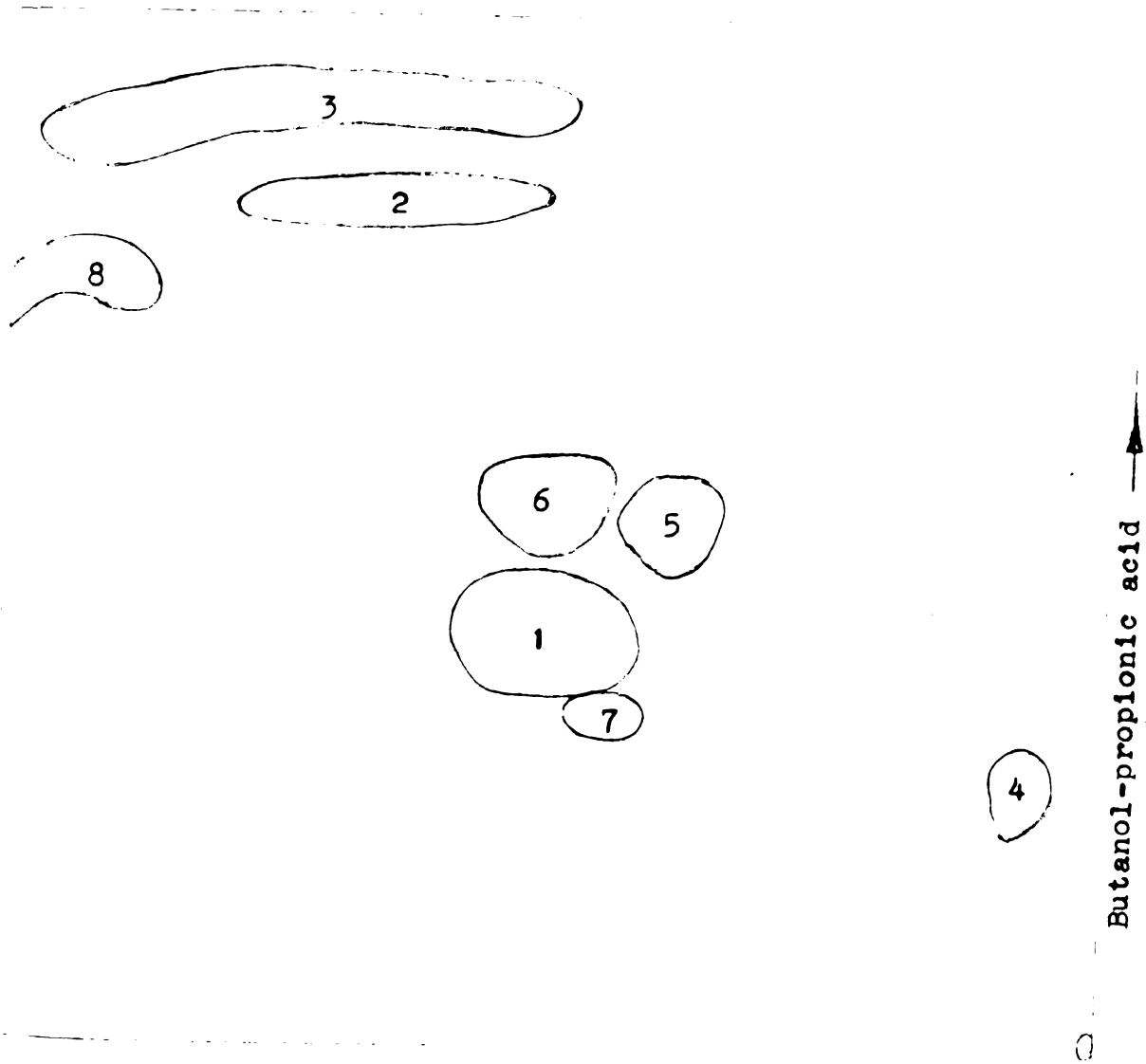
2 - Glycine, 0.1 M.

3 - Glyoxylate, 0.01 M + glycine, 0.1 M, combined together for 5 minutes.

All reagents are listed as final concentrations.

FIGURE 19

Non-enzymatic reaction of 0.1 M glycine with 0.1 M glyoxylate at room temperature and pH 10.5.



1, 2, 3 - Spots when glycine was chromatographed.

4, 5, 6 - Glycine + glyoxylate reacted for 15 minutes.

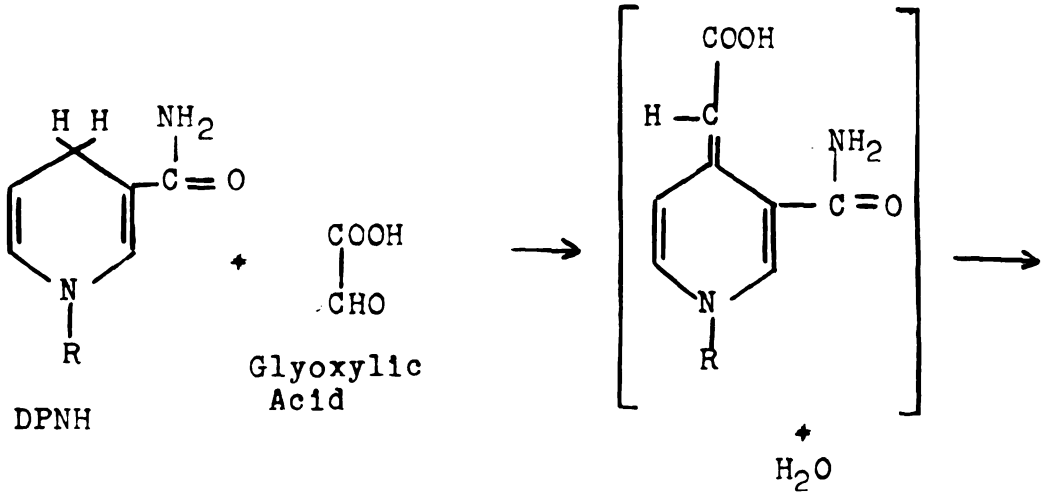
7 - Methyl red dye.

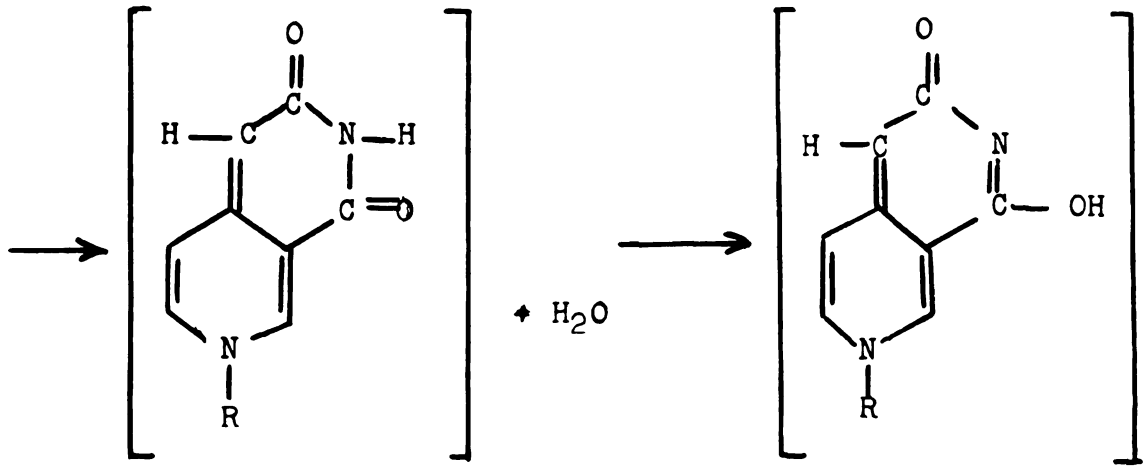
8 - Methyl orange dye.

No spots detected with glyoxylate alone.

oxidation by glyoxylate and the reversibility of this inhibition by excess of added DPN was essentially the same as when glycine had been used as the buffer (Figure 16). However, no change in the absorption spectrum for glyoxylate was found when the reaction was run in water or in carbonate-bicarbonate buffer (Figure 18). These results show that the condensation product of glycine and glyoxylate was not responsible for the inhibition of the enzymatic reaction.

The ultraviolet absorption spectra of a solution of DPN or DPN + glyoxylate were essentially similar. Condensation of glyoxylate might have occurred with DPNH at the C_4 position of the nicotinamide ring without shifting the double bonds within the ring so that the final spectrum would be similar to that of reduced DPN. The following reactions would occur according to this hypothesis:





As a result glyoxylate would inhibit DPN catalyzed reactions either by removal of the DPNH or by formation of an inhibitory complex of DPNH.

This hypothesis has been supported by chromatographic assays of DPNH and glyoxylate mixtures and detection of the DPN component by 3660 A^{o} ultraviolet light. Ascending single dimensional chromatography was used with a solvent of the following composition: 600 grams of ammomium

sulfate were dissolved in one liter of 0.1 M sodium phosphate buffer, pH 6.8, and 20 ml of n-propanol were added. DPN and glyoxylate chromatographed as the DPN control which was a dark blue absorbing spot at R_f of 0.31. Apparently DPN and glyoxylate did not react. DPNH chromatographed with an R_f of 0.12 and showed a light blue fluorescent color. With DPNH plus glyoxylate a new spot appeared at the R_f of 0.19 which was also light blue fluorescent in color. Apparently DPNH and glyoxylate had formed non-enzymatically a complex with the same ultraviolet fluorescent characteristics as DPNH. That it was not DPNH but a new compound was known from its chromatographic properties. It is proposed that this new compound is the stable form of one of the structures suggested. Since a condensation product has the fluorescent color of DPNH it also should contain a quinone structure.

Having demonstrated a complete reversibility of the glyoxylate inhibition of malic and lactic dehydrogenase by excess DPN we then examined whether excess DPN could reverse glyoxylate inhibition of mitochondria preparations. In the experiment with malate as a substrate for mitochondrial preparation, only partial reversibility (47%) of the glyoxylate inhibitory effect was obtained by employing DPN in excess (Figure 20).

Experiments with pure malic dehydrogenase were run at pH 10.5 and with mitochondria preparations at pH 7.3 and

FIGURE 20

Effect of glyoxylate and DPN on malate oxidation by green pea mitochondria.

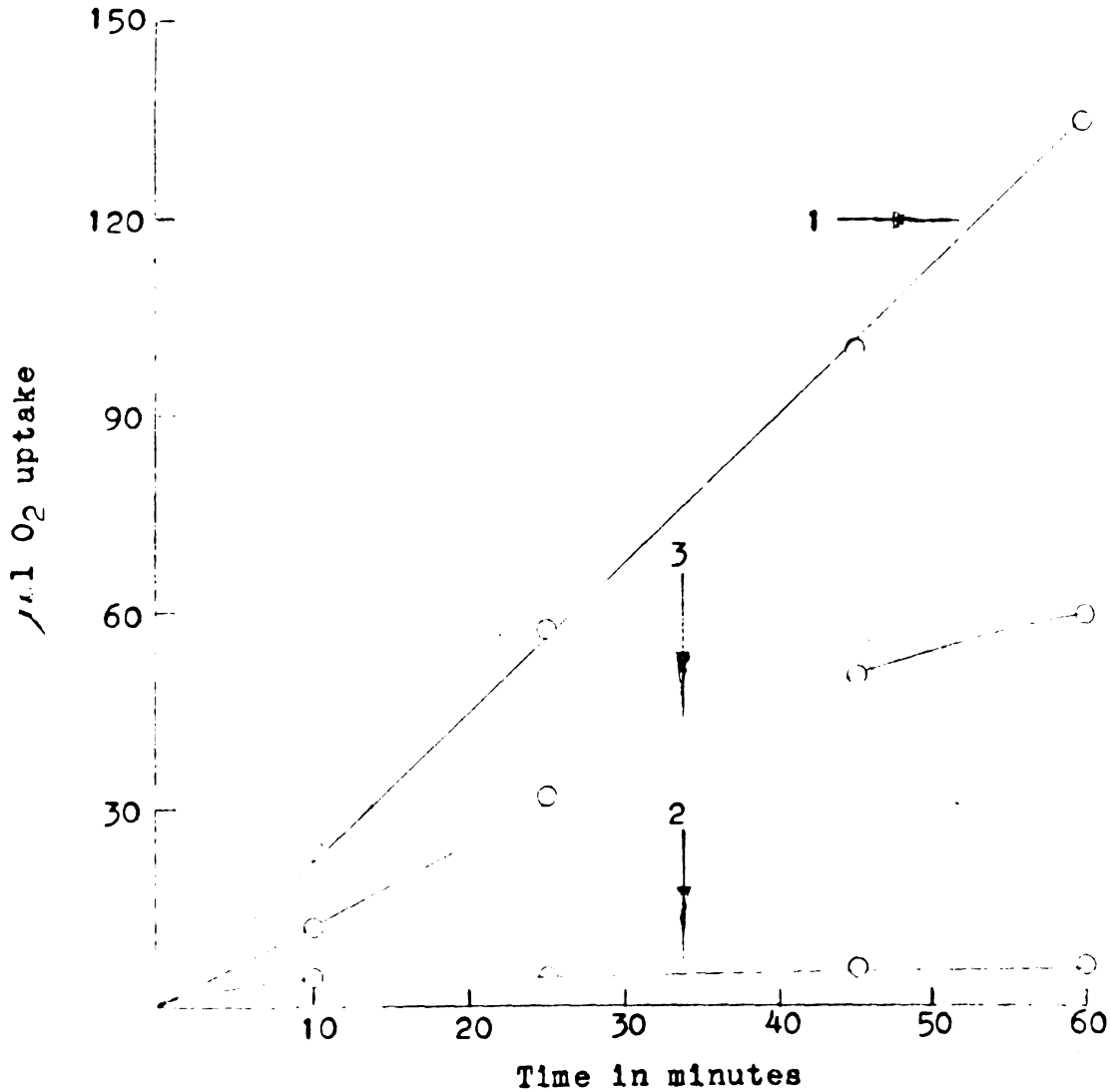
1 - 0.001 M or 0.002 M DPN

2 - 0.001 M DPN and 40 μ moles per flask of glyoxylate

3 - 0.002 M DPN and 40 μ moles per flask of glyoxylate

Each flask contained 10 μ moles of malate. Final pH was 7.3 and total volume of reactants 3.9 ml. Assay conditions and mitochondria preparation was as described in the Materials and Methods section.

FIGURE 20


150
120
90
60
30
µl O_2 uptake
1
2
3
10
20
30
40
50
60
Time in minutes

the higher pH would favor the condensation of glyoxylate with DPNH (106, 107). Thus we could not expect as complete reversibility by DPN of an inhibition with the complex mitochondrial system. The failure of excess of DPN to reverse the glyoxylate inhibition of succinate oxidation by mitochondria is consistent with the fact that succinic dehydrogenase requires FMN instead of DPN. Perhaps glyoxylate also complexes with FMN which hypothesis could be checked in a similar manner as done for glyoxylate condensation with DPN.

SUMMARY

Glycolic acid oxidase is active in the cell-free extracts from green leaves but not in sheaths of leaves or in etiolated leaves. The enzyme is predominantly located in the cytoplasm although some enzymatic activity is associated with mitochondria and chloroplast fractions.

An apoenzyme is present in sheaths or etiolated leaves, and upon addition of FMN in excess to the homogenates of these tissues, an active enzyme is obtained.

Exposure of etiolated plants to the light results in a several fold increase of active enzyme and of more apoenzyme. The increase in this enzyme is associated with greening of the plant. This change may be in part explained by the fact that green plants contain more FMN than etiolated plants. However, the content of FMN in types of plants is 10^{-8} M which is below the 10^{-4} M concentration of flavin necessary to activate the enzyme in vitro.

Enzyme can be activated in vitro by preincubation of a cell-free sap in the cold with its substrates, glycolate, lactate or α-hydroxybutyrate. Ammonium sulfate precipitates of this apoenzyme were not activated by incubation with glycolate. The enzyme can also be activated in vivo without light by feeding etiolated plants glycolate, lactate,

α-hydroxybutyrate or even glyoxylate.

Both glycolate and glyoxylate were shown to be effective inhibitors of the oxidation of citric acid cycle intermediates by plant mitochondria. Glyoxylate was the more potent inhibitor. Because glycolate was oxidized by plant mitochondria by the associated glycolic acid oxidase it was assumed that inhibitory effects of glycolate could be ascribed to formation of glyoxylate. Glyoxylate also severely inhibited isolated malic and lactic dehydrogenase. Upon the addition of DPN in excess to these enzymes or to mitochondria with malate as a substrate, the inhibitory effect of glyoxylate was reversed. The inhibitory action of glyoxylate was caused by a non-enzymatic formation of complex with reduced DPNH. This complex was detected by paper chromatography.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Benson, A. A., and Calvin, M., J. Exp. Bot. 1, 63 (1950).

2. Bean, R. C., and Hassid, W. Z., J. Biol. Chem., 212, 411 (1955).

3. Calvin, M., Bassham, J. A., Benson, A. A., Lynch, V. H., Quellet, C., Schou, L., Stepka, W., and Tolbert, N. E., Symposia Soc. Exper. Biol., 5, 284 (1951).

4. Noll, C. R., Jr., and Burris, R. H., Plant. Physiol., 29, 261 (1954).

5. Tolbert, N. E., and Cohan, Marjorie, S., J. Biol. Chem., 204, 639 (1953).

6. Sze-Chuh Cheng, Plant Physiol., 29, 458 (1954).

7. Zelitch, I., J. Biol. Chem., 224, 251 (1957).

8. Nagar, R. R., Ram, N., and Rhattacharia, A. K., Indian J. Agr. Sci., 23, 121 (1953).

9. Bernhauer, K., and Scheuer, Zofia, Biochem. Z. 253, 11 (1932).

10. Bolcato, V., and Scevola, M. E., Naturwiss., 44, 539 (1957).

11. Palleroni, N. J., and Doudoroff, M., J. Bacteriol. 74, 180 (1957).

12. Corpe, W. A., and Stone, R. W., J. Bacteriol. 80, 452 (1960).

13. Wright, Barbara F., Arch. Biochem. Biophys., 31, 332 (1951).

14. Campbell, L., Jr., J. Bacteriol. 71, 81 (1956).

15. Nakada, H. I., and Weinhouse, S., J. Biol. Chem. 204, 831 (1953).

16. Bachrach, V., Biochem. J. 66, 559 (1957).

17. Wong, T. O., and Ajl, S. J., J. Am. Chem. Soc., 78, 3230 (1956).

18. Kornberg, H. L., Biochem. Biophys. Acta, 24, 651 (1957).

19. Kornberg, H. L., and Krebs, H. A., Nature, 179, 988 (1957).

20. Saz, H. J., and Hillary, E. P., Biochem. J., 62, 563 (1956).

21. Krakow, Gladys, and Barkulis, S. S., Biochem. et Biophys. Acta, 21, 593 (1956).

22. Sieburg, E., and Vietense, K., Z. Physiol. Chem., 108, 207 (1919).

23. Nakada, H. I., and Sund, L. P., J. Biol. Chem., 233, 8 (1958).

24. Abbot, L. D., and Lewis, H. B., J. Biol. Chem., 137, 535 (1941).

25. Weinhouse, S., and Friedman, Bernice, J. Biol. Chem., 197, 733 (1952).

26. Nakada, H. I., Friedman, B., and Weinhouse, S., J. Biol. Chem., 216, 583 (1955).

27. Weinhouse, S., and Friedman, Bernice, J. Biol. Chem., 191, 707 (1951).

28. D'Abramo, F., and Tomasos, E., Boll. Soc. Ital. Biol. Sper. 31, 1609 (1955).

29. Mardashev, S. R., and Semina, L. A., Doklady Akad Nauk, U.S.S.R., 74, 537 (1950).

30. Flipse, R. J., Science, 124, 228 (1956).

31. Kolesnikov, P. A., Doklady Akad. Nauk, U.S.S.R., 60, 1205 (1948).

32. Delavan, L. A., and Benson, A. A., U. S. At. Energy Comm. BNL 512 (C-28), 259 (1958).

33. Kolesnikov, P. A., Biokhimia (Russ.), 13, 370 (1948).

34. Tolbert, N. E., and Burris, R. H., J. Biol. Chem., 186, 791 (1950).

35. Kolesnikov, P. A., Doklady Akad. Nauk, U.S.S.R., 60, 1353 (1948).

36. Kolesnikov, P. A., Biokhimia, 14, 124 (1949).

37. Kolesnikov, P. A., Doklady Akad. Nauk, U.S.S.R., 71, 1085 (1950).

38. Zbinovsky, V., and Burris, R. H., Plant Physiol., 27, 240 (1952).

39. Schou, L., Benson, A. A., Bassham, T. A., and Calvin, M., Physiol. Plant., 3, 487 (1950).

40. Rabson, R., and Tolbert, N. E., Plant Physiol., Suppl. 32, XXXV (1957).

41. Griffith, T., and Byerrum, R. U., J. Biol. Chem., 234, 762 (1959).

42. Tolbert, N. E., and Cohan, Marjorie S., Federation Proc., 11, 299 (1952).

43. Tolbert, N. E., and Cohan, Marjorie S., J. Biol. Chem., 204, 649 (1953).

44. Bradbeer, J. W., and Racker, E., Federation Proc., 20, 88 d (1961).

45. Tolbert, N. E., and Zill, L. P., J. Biol. Chem., 222, 895 (1956).

46. Tolbert, N. E., Brookhaven Symposia in Biology, 11, 271 (1958).

47. Warburg, O., and Krippahl, G., Z. f. Naturfor., 15b, 197 (1960).

48. Tanner, H. A., Brown, T. E., Eyester, C., and Treharne, R. N., Biochem. Biophys. Res. Com., 3, 205 (1960).

49. Tolbert, N. E., Clagett, C. O., and Burris, R. H., J. Biol. Chem., 181, 905 (1949).

50. Tolbert, N. E., J. Biol. Chem., 215, 27 (1955).

51. Tolbert, N. E., and Gailey, F. B., Plant Physiol., 30, 491 (1955).

52. Kolesnikov, P. A., Doklady Akad. Nauk, U.S.S.R., 96, 125 (1954).

53. Doyle, W. P., Huff, R., and Wang, C. H., Plant Physiol. 35, 745 (1960).

54. Wilson, D. G., King, K. W., and Burris, R. H., J. Biol. Chem., 208, 863 (1954).

55. Kornberg, H. L., and Beevers, H., Biochim. et Biophys. Acta, 26, 531 (1957).

56. Marcus, A., and Velasco, J., J. Biol. Chem., 235, 563 (1960).

57. Bradbeer, C., and Stumpf, P. K., J. Biol. Chem., 234, 498 (1959).

58. Yamamoto, Y., and Beevers, H., Plant Physiol., 35, 102 (1960).

59. Byerrum, R. U., Hamill, R. L., and Ball, C. D., J. Biol. Chem., 210, 645 (1954).

60. Byerrum, R. U., Dewey, L. J., Hamill, R. L., and Ball, C. D., J. Biol. Chem., 219, 345 (1956).

61. Wu, P. H. L., and Byerrum, R. U., Plant Physiol., 33, 230 (1958).

62. Zelitch, I., and Gotto, A. M., Federation Proc. 20, 375c (1961).

63. Donan, Janette Schoonover, J. Biol. Chem., 135, 793 (1940).

64. Clagett, C. O., Tolbert, N. E., and Burris, R. H., J. Biol. Chem., 178, 977 (1949).

65. Frigerio, N. A., and Harbury, H. A., J. Biol. Chem., 231, 135 (1958).

66. Chiba, H., Kawai, F., and Ueba, S., Bull. Research Inst. Food Sci., Kyoto Univ., 15, 89 (1954).

67. Zelitch, I., and Ochoa, S., J. Biol. Chem., 201, 707 (1953).

68. Izawa, S., and Tsukamoto, A., J. Biochem. (Japan), 41, 187 (1954).

69. Kolesnikov, P. A., Uspekhi Sovremennoi Biol., 38, 133 (1954).

70. Kun, E., J. Biol. Chem., 194, 603 (1952).

71. Kun, K., Dechary, J. M., and Pitot, H. C., J. Biol. Chem., 210, 269 (1954).

72. Kenten, R. H., and Mann, P. J. G., Biochem. J., 52, 130 (1952).

73. Richardson, K. E., Kuczmak, M., and Tolbert, N. E., Federation Proc., 18, 309 (1959).

74. Zelitch, I., and Ochoa, S., J. Biol. Chem., 201, 719 (1953).

75. Zelitch, I., J. Biol. Chem., 216, 553 (1955).

76. Mothes, K., and Wagner, A. N., Biokhimia (Russ.), 22, 171 (1957).

77. Graf, G. E., and Aronoff, S., Science, 121, 211 (1955).

78. Kolesnikov, P. A., and Emenova, S. V., Doklady Akad. Nauk, U.S.S.R., 109, 152 (1956).

79. Zelitch, I., J. Biol. Chem., 233, 1299 (1958).

80. Zelitch, I., and Barber, G. A., Plant Physiol., 35, 626 (1960).

81. Kleinzeller, A., Biochem. J., 37, 674 (1943).

82. Kursanov, A. L., and Kulaeva, O. N., Fiziol. Rastenii (Russ.), 4, 322 (1957).

83. D'Abramo, F., Romano, Marta, and Ruffo, A., Biochim. et Biophys. Acta, 24, 437 (1957).

84. D'Abramo, F., Romano, Marta, and Ruffo, A., Boll. Soc. Ital. Biol. Sper., 33, 1724 (1957).

85. Pierpoint, W. S., Biochem. J., 71, 518 (1959).

86. D'Abramo, F., Romano, Marta, and Ruffo, A., Biochem. J., 68, 270 (1958).

87. Gaull, G., and Villee, C. A., Biochim. et Biophys. Acta, 39, 560 (1960).

88. Ruffo, A., Romano, Marta, and Adinolfi, Anna, Biochem. J., 72, 613 (1959).

89. Hoagland, D. R., and Arnon, D. I., Univ. Cal. Agr. Exp. Sta. Circular, 347 (1938).

90. Arnon, D. I., Allen, M. B., and Whatley, F. R., Biochim. et Biophys. Acta, 20, 449 (1956).

91. Ohmura, T., Arch. Biochem. Biophys., 57, 187 (1955).

92. Official Methods of Analysis - A.O.A.C., 745 (1950).

93. Umbreit, W. W., Burris, R. H., and Stauffer, J. E., Manometric Techniques, 272 (1957).

94. Burch, H. R., Bessey, O. A., and Lowry, O. H., J. Biol. Chem., 175, 457 (1948).

95. Bessey, O. A., Lowry, O. H., and Love, R. H., J. Biol. Chem., 180, 755 (1949).

96. Methods of Biochem. Analysis, 5, 118 (1957).

97. Strominger, J., and Lowry, O. H., J. Biol. Chem., 213, 635 (1955).

98. Kolesnikov, P. A., Doklady Akad. Nauk, U.S.S.R., 112, 909 (1957).

99. Kolesnikov, P. A., Izvestia Akad. Nauk, Biol. Series, 14, 87 (1950).

100. Richardson, K. E., and Tolbert, N. E., J. Biol. Chem., 236, 1280 (1961).

101. Schrecker, A. W., and Kornberg, A., J. Biol. Chem., 182, 795 (1950).

102. Kornberg, A., and Pricer, W. E., Jr., J. Biol. Chem., 182, 763 (1950).

103. Comline, R. S., and Whatley, F. R., Nature, 161, 350 (1948).

104. Dixon N., and Webb, E. C., Enzymes, 81 (1958).

105. Ho, P. P. K., Scrimgeour, K. G., and Huennekens, F. M., J. Am. Chem. Soc., 82, 5957 (1960).

106. Burton, R. M., and Kaplan, N. O., J. Biol. Chem., 206, 283 (1954).

107. Davies, D. D., J. Exper. Bot., 11, 289 (1960).

108. Krakow, Gladys, Barkulis, S. S., and Hayashi, J. A., J. Bacteriol., 81, 509 (1961).

109. Neilands, J. B., J. Biol. Chem., 199, 373 (1952).

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