THE ENZYMATIC ACTIVITY IN A PARTICULATE FRACTION FROM SEEDLINGS OF BLACK VALENTINE BEANS (PHASEOLUS VULGARIS)

by

GEORGE STANLEY BEAUDREAU

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1954

APPROVED:

Redacted for Privacy

Associate Professor of Chemistry Department

In Charge of Major

Redacted for Privacy

Chairman of Department of Chemistry

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

Date thesis is presented October 21, 1953

Typed by Margaret Barber

ACKNOWLEDGMENT

I wish to express my appreciation to my major professor, Dr. L. F. Remmert, for his attentive instruction and generous aid during the course of this study.

TABLE OF CONTENTS

THE ENZYMATIC ACTIVITY IN A PARTICULATE FRACTION FROM SEEDLINGS OF BLACK VALENTINE BEANS (PHASEOLUS VULGARIS)

INTRODUCTION

Krebs and Johnson (31, pp.152-153) originally proposed the cycle known as the "citric acid cycle" or the "Krebs cycle." Using minces of pigeon breast muscle, these investigators obtained convincing evidence for the series of reactions composing the cycle. The subject has been examined by many investigators, and the Krebs cycle is now generally accepted as the principal mechanism by which carbohydrates are oxidized in animals. Evidence for this has been summarized by Krebs (28, pp.166-170) under three headings, namely:

1. All of the reactions which constitute the cycle have been demonstrated to occur in muscle tissue at rates which are sufficient to account for the maximum rate of respiration.

2. The di- and tricarboxylic acids of the cycle have been found to exert a catalytic effect upon muscle respiration, that is, they stimulate muscle respiration more than can be explained by stoichiometric reactions of the added material.

3. In the presence of 0.01 M. malonate the respiration of muscle tissue is inhibited and succinate accumulates.

This inhibition is partially or completely abolished by the addition of any of the intermediates of the cycle, but these no longer act catalytically but by more or less stoichiometric reactions.

That the Krebs cycle plays a major role in plant metabolism has not been generally accepted. Evidence for operation of the cycle in plants has been, until recently, largely circumstantial. Most of it has consisted of demonstrations of the occurrence in plants of individual enzymes and intermediates common to the cycle. In reviewing this information it is well to keep in mind the type of evidence, mentioned above, which has caused the acceptance of the cycle with respect to animal metabolism.

Most of the enzymes which catalyze the Krebs cycle have been demonstrated to occur in plants. Succinic dehydrogenase has been found in plants by many investigators (13, pp.348-359; 16, pp.275-276; 20, p.726; 40, pp.125-131; 39, pp.152-157; 41, pp.857-858; 47, pp.170-171; 53, p.737). Vennesland and coworkers (12, pp.196-197; 26, p.591; 60, p. 597; 61, p.314) and Speck (52, p.323) have reported the presence of malic dehydrogenase, the malic enzyme, and oxalacetic carboxylase in a number of plants. Perhaps the best evidence for a condensation reaction in plants, to form citrate from pyruvate and some other intermediate, is that provided by Millerd, et al (39, pp.153 and 159;

41, pp.857-858). They found that malate, and other intermediates of the Krebs cycle, catalyzed the oxidation of pyruvate by particles from mung beans (Phaseolus aureus). Pucher, et al (50, p.492; 49, pp.574-575; 48, p.533) used excised leaflets of tobacco and Bryophyllum calycinum plants and found evidence for the conversion of malate to citrate, which might further indicate that the condensation reaction occurs in plants. Berger and Avery (4, p.18) reported evidence for the presence of aconitase and isocitric dehydrogenase in Avena coleoptiles. The latter enzyme was found in a number of plants by Whatley (62, p.261). Respiratory responses upon adding α-ketoglutarate have been reported by Boswell (7, p.533) who used slices of the "roots" of Brassica napus L, and by Millerd, et al (39, pp. 152-157; 41, pp.857 and 861) who used particulate material from mung bean seedlings. It is true, then, that many of the reactions which the Krebs cycle includes have been demonstrated in plants.

Additional support for a contention that the Krebs cycle is operative in some species of the plant kingdom has been accumulated using tissue slices or organs from plants, or whole plants. Since it would require many pages to catalog completely the information of this type, only a few points will be cited. It has been demonstrated many times that addition of Krebs cycle intermediates to media in

which plant sections are incubated will, under certain circumstances, increase the respiratory rate of such sections (3, pp.385 and 389; 5, pp.318-326; 6, pp.506 and 510; 7, p.533; 15, p.288; 19, p.532; 42, p.314). Inclusion of malonate in the incubation medium has been shown to inhibit the respiration of certain plant materials (58, pp.296-297) and this inhibition may be partially or completely abolished by the simultaneous inclusion of members of the Krebs cycle (5, p.322; 6, pp.511-512; 34, p.601; 35, pp.293-295). As with materials of animal origin, plant sections poisoned with malonate have been shown to accumulate excessive amounts of succinate, and this increase is made greater by the presence in the medium of pyruvate, fumarate, or α-ketoglutarate (5, p.323; 34, p.601; 33, pp.10-15).

Unfortunately, many of the experiments with plant tissue sections have tended to be inconclusive. In the first place, there is the ever-present possibility that the added substrate may have exerted its effect upon respiration by some means other than its own oxidation. Secondly, respiratory responses from added intermediates are, with plant sections, the exception rather than the rule, probably because of the large amounts of substrate normally present in plant cells. Finally, even the inhibition by malonate cannot always be demonstrated (58, pp.296-297).

Perhaps the most convincing evidence for operation of the Krebs cycle in plants is that provided recently by Millerd, et al (39, pp.151-162; 41, pp.856-861). These investigators prepared particulate material from etiolated seedlings of the mung bean (Phaseolus aureus) and found that the particles catalyzed the oxidation of citrate, α-ketoglutarate, succinate, fumarate, malate and pyruvate. The oxidation of pyruvate was increased by the simultaneous oxidation of a small amount of any one of the other intermediates named, and these acted catalytically. The respiratory quotient for oxidation of pyruvate was found to be 1.3, a value close to the theoretical ratio of 1.2 for complete oxidation of pyruvate. These facts were given as evidence for the complete oxidation of pyruvate to carbon dioxide and water (39, p.150), and they indicate strongly that the Krebs cycle does function in plants. Further support for this contention was provided recently by Davies (14, pp.175-182) who presented evidence for operation of the Krebs cycle in pea seedlings.

The purpose of the investigation to be reported was to verify and increase existing knowledge of the oxidative reactions by which plants metabolize carbohydrates.

METHODS AND MATERIALS

The bean seedlings (Black Valentine, var. Phaseolus vulgaris) were grown in the dark in an inert soil (vermiculite). The etiolated seedlings were allowed to grow to a height of 8 to 12 inches before they were used for the experiments. This usually required a period of 10 to 14 days. During the winter months the temperature was regulated at 27° C. The seedlings were watered with tap water. The hypocotyls of the seedlings were the material used routinely for the preparations.

The measurement of the oxygen taken up during the oxidation of the substrate was made with a Warburg constant volume respirometer. The flasks were incubated in a circular constant temperature bath with a reciprocating shaking motion.

The diphosphopyridine nucleotide (DPN) was obtained from the Sigma Chemical Company and it was 90% pure. The triphosphopyridine nucleotide (TPN), obtained from the Sigma Chemical Company, was 10% pure and contained about 8% of DPN. The cocarboxylase was obtained from Nutritional Biochemicals Corporation. The terramycin·HCl was from the Charles Pfizer Company. The liver concentrate was from Armour and Company and contained >10 Lipmann units of coenzyme A/mg.; >4% TPN and >7% DPN. Cytochrome c was

obtained from Sigma Chemical Company. The adenosine triphosphate (ATP) and the reduced glutathione (GSH) were obtained from Schwarz Laboratories, Inc.

Succinate was determined manometrically using a succinoxidase preparation obtained from fresh pig heart by the method of Cohen (59, p.168). This preparation utilized only succinate as a substrate.

For the isolation of succinic acid from incubation mixtures, the methods of Krebs (30, p.457) and Cohen (10, p.554) were used with some modifications. The contents of each Warburg flask were acidified with 3 drops of 50% H_2SO_4 and transferred to a 15 ml. conical centrifuge tube. The volume of the sample was increased to about 10 ml., and 0.25 ml. of 10% sodium tungstate was added. The tube was swirled to insure mixing and then centrifuged. The supernatant liquid was transferred to a Kutscher-Steudel extractor of the type described by Krebs (29, p.1044). The precipitate was washed with 3-4 ml. of water, and the wash was added to the extractor. Two ml. of 50% H_2SO_4 and 0.3 ml. of 1 M. $KHSO_3$ were added to the extractor.

The water solutions were extracted 8 hours with ether free of peroxides. About 1 ml. of 0.1 M. phosphate buffer (pH7.4) was added to the ether extract, and the ether was distilled off. The residue was transferred from the extraction flask to a 15 ml. graduated centrifuge tube.

About 5 ml. of water were used in the transfer. The solution was neutralized with 2 N. NaOH using phenol red as an indicator, and the volume was evaporated to 1 ml. in a 110° C. oven. One ml. of 4 N. HCl was added to the centrifuge tube and the tube was sealed with a marble held tightly in place by metal springs. The tube was autoclaved for 4 hours at 258° F. to destroy the malonate. The solution was evaporated nearly to dryness in a vacuum dessicator containing solid NaOH. The material was neutralized and made to 2.2 ml. volume. One ml. aliquots were used for the succinate analyses which were carried out as described by Krebs (27, p.2097).

PART I

THE ENZYMATIC OXIDATION OF SUCCINIC ACID

Method of Preparing the Enzymes

The insoluble particulate fraction was obtained by the method of Schneider (51, p.260), but certain modifications were necessary to facilitate the use of large quantities of plant material.

Three hundred grams of etiolated bean seedlings (the aerial portion, unless otherwise stated) were cut in small sections. The material was placed in a 2° C. cold room for 30 minutes. The 300 grams of tissue was then placed in a large Waring blendor with 200 ml. of ice cold 0.25 M. sucrose. This mixture was homogenized in short intervals for a period totaling 1 minute. The homogenized material was strained through 2 layers of cheesecloth into an ice chilled beaker. The strained homogenate was placed in 8 50 ml. cellulose nitrate tubes and centrifuged at 3,000 x g. for 10 minutes. The supernate was removed and centrifuged at 14,000 x g. for 15 minutes. The pellets from the second centrifugation were resuspended in 20 ml. of 0.25 M. sucrose and centrifuged at 14,000 x g. for 15 minutes. The washed pellet was resuspended in 0.25 M. sucrose. This suspension, referred to as the particulate fraction, was added to the Warburg flask. All operations in this

procedure were carried out at less than 5° C.

In experiments designed to increase the enzymatic activity, other additions to the homogenizing medium were made; however, these experiments were generally unsuccessful. The basic procedure was as described above.

The volume of the liquid phase in each flask was 3.4 ml. including 0.2 ml. of KOH in the center well. The experiments in this section were carried out at 20° C. Air was the gas phase. The shaking rate was 90 cycles per minute.

Experimental

Price and Thimann (47, p.170) have pointed out that many investigators have had difficulty in obtaining the succinic dehydrogenase system in vitro from plants. The particulate fraction obtained by the above method contained the succinoxidase system but did not utilize oxalacetate, citrate or α-ketoglutarate. As shown in Table 1 and Figure 1, 24 μ atoms of oxygen were taken up in 2 hours in the presence of succinic acid. After this time the rate of oxygen uptake decreased sharply. Not more than 50% of the theoretical oxygen uptake for the conversion of succinate to fumarate was obtained.

Table 1

Oxidation of Succinate

Time	Net µl. oxygen uptake
30 min.	143
50	188
90	232
120	264
160	278

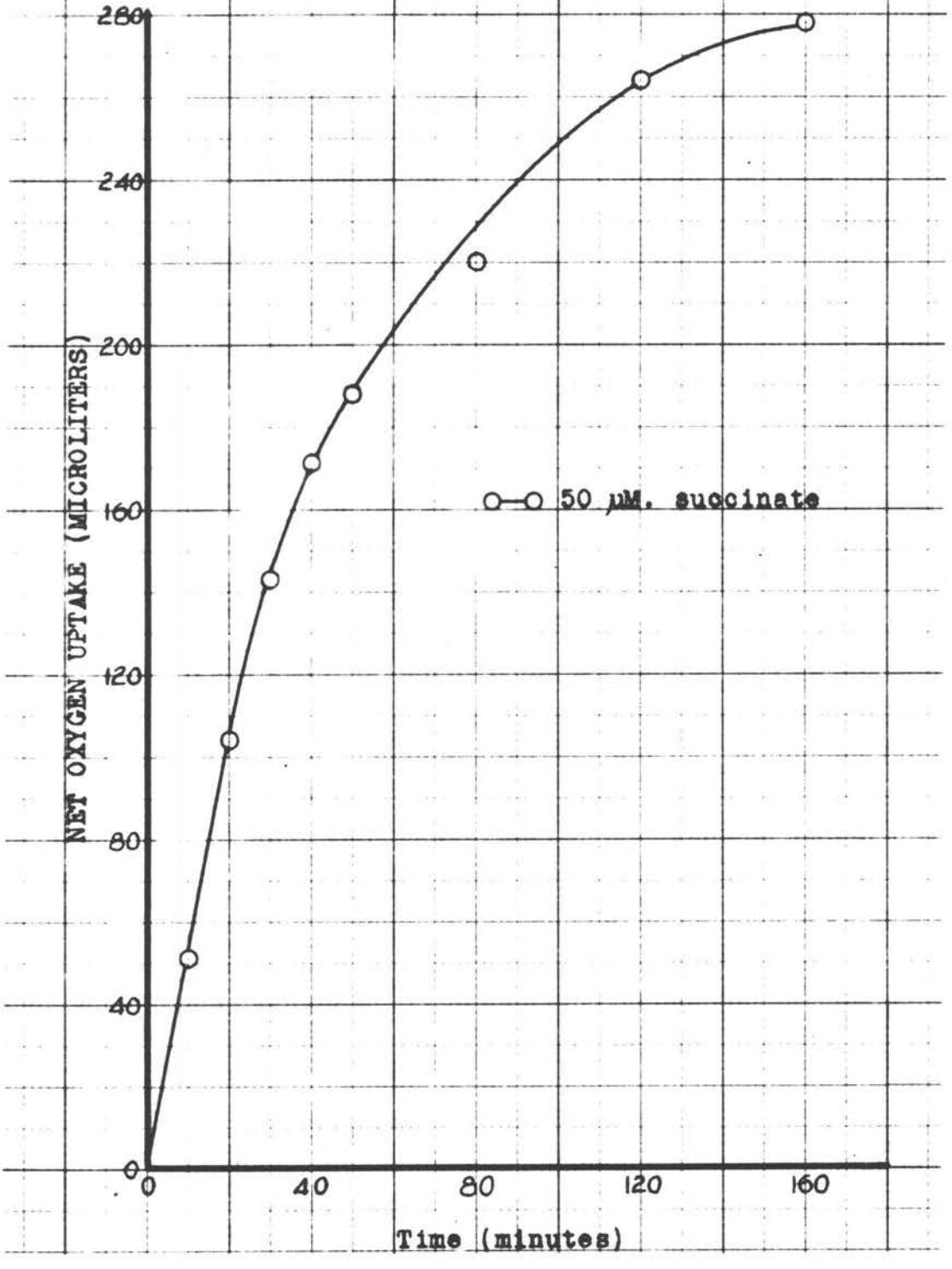
Additions were as follows: 0.1 ml. of 0.24 M. $MgSO_4$; 0.3 ml. of 0.04 M. K_4-ATP (pH 6.9); 0.3 ml. of 0.5 M. K-phosphate; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 0.5 M. K-succinate (pH 6.8); 0.2 ml. of 0.8% gelatin; 0.1 ml. of 5.44×10^{-3} M. DPN; 1.0 ml. of particulate fraction. The liquid volume in the main compartment was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 20° C.

Since succinic dehydrogenase had been reported as having an active sulfhydryl group (21, pp.613-619; 22, p. 1847), compounds known to protect this radical were added to the flask. No increase in enzymatic activity was obtained upon adding cysteine, glutathione, gelatin, or albumin. The latter 2 compounds were shown by Price and Thimann (47, p.170) to stabilize succinic dehydrogenase.

A cytochrome reductase preparation, obtained from pig heart according to Straub (55, p.789), failed to prevent the cessation of activity noted above.

In an attempt to supply missing co-factors a rat liver mitochondrial preparation was made. The enzymes

Fig. 1. The Oxidation of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings



were destroyed by heat and this preparation was added to the Warburg flasks. The rat liver mitochondrial preparation did not increase the enzymatic activity of the particulates from bean seedlings.

Inorganic ions (Fe^{++}, $HCO3^{-}$, Al^{+++}, Mn^{++}, Ca^{++}) did not increase the enzymatic activity when added singly or in combination.

To test the effect of Waring blendor speed during homogenization, each of 3 75-gram portions of bean tissue was homogenized in 50 ml. of 0.25 M. sucrose. Using a Waring blendor equipped with a Powerstat, the 3 portions were homogenized at different speeds. Subsequent fractionation of the 3 homogenates was done as described previously. The results, shown in Table 2, indicated that slow speed homogenization produced fractions less active than those obtained at higher speeds. This was probably due to the incomplete breakage of cells at the slower speeds.

Table 2

Oxygen Uptake by Particulates Prepared at Different Waring Blendor Speeds

Time	Net µl. O_2 uptake at Powerstat setting (50 V.)	(75 V.)	(110 V.)
20 min.	44	55	66
40	56	76	88
60	86	104	116
80	93	110	123
100	97	113	125

Additions were as follows: 1.0 ml. of particulate fraction; 0.1 ml. of 0.28 M. magnesium sulfate; 0.3 ml. of 0.5 M. phosphate buffer at pH 7.4; 0.3 ml. of 0.04 M. potassium adenosine triphosphate; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 0.5 M. potassium succinate + 0.05 M. sodium pyruvate; 0.2 ml. of 0.08% gelatin; 0.2 ml. of 0.0034 M. DPN. The liquid in the main compartment was made to 3.4 ml. with 0.25 M. sucrose.

Glutathione (Table 3) appeared to cause a slight increase in enzymatic activity in the particulate fraction; however, this did not prevent cessation of activity. DPN appeared to inhibit the enzyme system.

Table 3

Effect of Glutathione and Diphosphopyridine Nucleotide

Additions	Net oxygen uptake/2 hrs.
50 µM. K-succinate	21.9 µ atoms
50 µM. K-succinate + 16 µM. GSH	23.6
50 µM. K-succinate + 160 µM. GSH	25.0
50 µM. K-succinate + 16 µM. GSH + DPN	18.7
50 µM. K-succinate + 160 µM. GSH + DPN	20.4

Additions were as in Table 2 except that the liquid in the main compartment was 3.2 ml. DPN was added as indicated above. The homogenizing medium contained 1.0 gram of gelatin; 4 ml. of 1.0 M. $NaHCO_3$ and 2 ml. of 0.5 M. succinate in 200 ml. of 0.25 M. sucrose. The particulate fraction was suspended in 0.25 M. sucrose.

Table 4

Effect of Succinate Concentration and Cytochrome c

Additions	Conc. of cytochrome c	Net oxygen uptake/first hr.
5 µM. K-succinate	0.24 mg./flask	4.0 µ atoms
50 µM. K-succinate	0.24 mg./flask	16.8
100 µM. K-succinate	0.24 mg./flask	11.6
50 µM. K-succinate	0.00 mg./flask	3.1

Additions were as in Table 2. The particulate fraction was homogenized and washed in a solution of 0.25 M. sucrose and 0.5% gelatin, and suspended in 0.25 M. sucrose. The liquid volume in the main compartment was 3.2 ml.

The data in Table 4 showed that cytochrome c enchances the activity of the particulate fraction. The highest

percentage of succinic acid was oxidized when this acid was at a low concentration, and there appears to have been inhibition at the 100 μM. level.

The 3,000 x g. fraction and the supernate from the 14,000 x g. fraction were tested and found to contain no activity. When added to the 14,000 x g. preparation, these same fractions failed to cause any increase in activity.

Oxalacetate is known to inhibit succinic dehydrogenase (46, p.1094). The diminishing activity of the particulate fraction suggested the possibility of oxalacetic acid building up from succinate oxidation in sufficient quantities to inhibit the succinic dehydrogenase. Straub (56, p.148) suggested that in the presence of the proper transaminase the oxalacetate could be removed by addition of glutamic acid. The effect of glutamic acid on succinate oxidation is shown in Table 5 and Figure 2.

Table 5

Effect of Glutamic Acid and Fumaric Acid on Succinate Oxidation

Additions	Net µl. oxygen uptake 1 hr.	2 hrs.
50 µM. K-succinate	78	93
150 µM. K-succinate + 150 uM. K-glutamate	99	173
50 µM. K-succinate + 100 uM. K-fumarate	38	58

Additions were the same as in Table 2 except that 0.2 ml. of a cytochrome reductase preparation was added to each flask in this experiment. The liquid volume in the main compartment was 3.2 ml.

The presence of glutamic acid enabled the system to take up oxygen during the second hour at approximately the same rate as during the first hour. A rapid decline in activity was not observed. Fumaric acid appeared to inhibit the oxidation of succinic acid in this preparation.

Malic acid also appeared to inhibit succinic acid oxidation (Table 6 and Figure 3), and this inhibition was reversed by glutamic acid. Malic acid was oxidized slowly by the preparation. The malic dehydrogenase that was present appeared to be inhibited by oxalacetic acid since glutamic acid increased the malic acid oxidation. Only 4 µl. of oxygen uptake was observed when only glutamic acid was added to the flasks.

Fig. 2. The Effect of Fumarate and Glutamate on the Oxidation of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

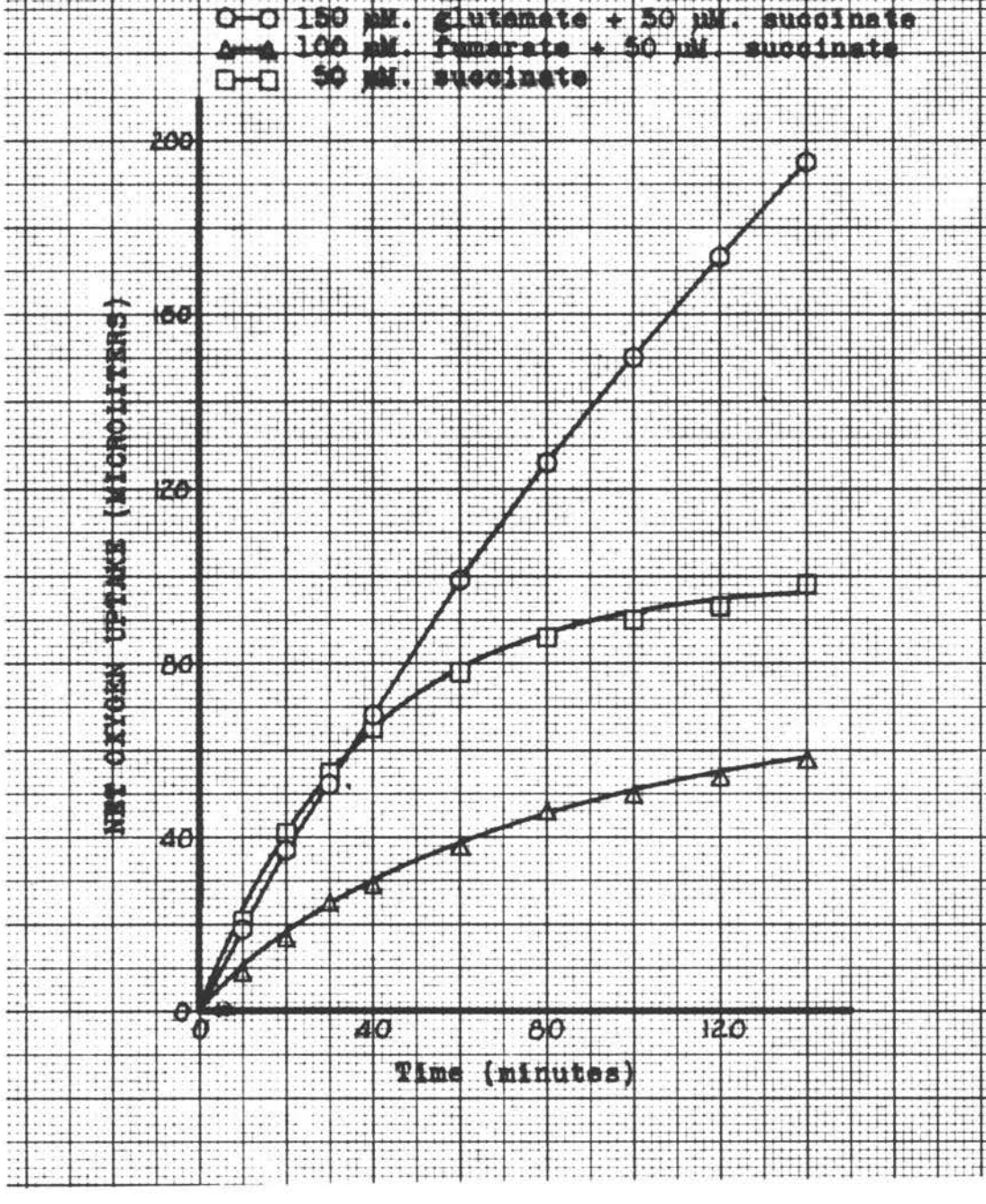


Table 6

Effect of Malate on Succinate Oxidation

Additions	Net µl. oxygen uptake 1 hr.	2 hrs.
50 µM. K-succinate (I)	92	112
50 µM. K-succinate + 50 µM. K-malate (II)	45	65
(II) + 150 µM. K-glutamate	81	136
50 µM. K-malate	8	10
150 µM. K-glutamate	4	0
50 µM. K-malate + 150 µM. K-glutamate	25	31

Additions were as in Table 5.

To further clarify the apparent inhibition noted, an experiment was designed in which the enzyme concentration was varied. Part of the enzyme preparation was diluted 1:3 at the end of the centrifugal fractionation. The concentrated enzymes were compared with the diluted enzymes, both with and without glutamic acid (Table 7 and Figure 4). The inhibition occurred at both enzyme concentrations and glutamic acid reversed the inhibition. In the presence of glutamic acid the concentrated enzyme catalyzed the uptake of 45 µ atoms of oxygen in 5 hours. Table 6 showed that malic acid is oxidized at a very slow rate; therefore, it is probable that approximately 90% of the 50 µM. of succinic acid was oxidized to fumaric acid.

Fig. 3. The Effect of Malate on the Oxidation of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

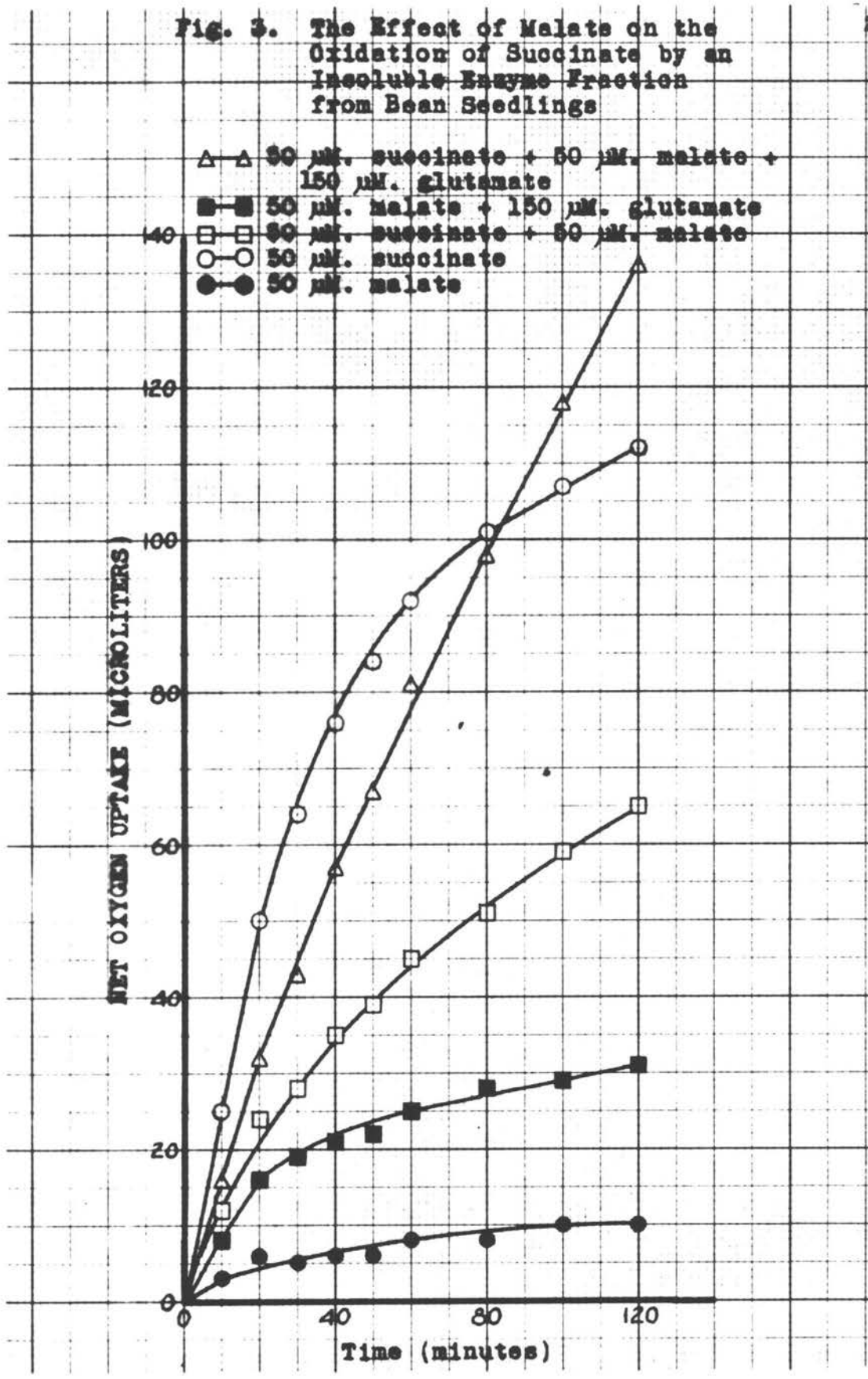


Table 7

Dilution of the Enzyme

Additions	Net µl. oxygen uptake 1	2*	3	4	5 hrs.
Conc. enzyme + 50 µM. K-succinate (I)	216	270	278	283	286
Dil. enzyme + 50 µM. K-succinate (II)	118	175	186	194	198
(I) + 150 µM. K-glutamate	240	398	474	495	505
(II) + 150 µM. K-glutamate	97	175	237	289	325

Additions were as in Table 2, except that DPN was omitted. The liquid volume in the main compartment was 3.2 ml.

*Value obtained from Figure 4. The experimental readings were made at 110 and 130 minutes.

For the flasks that contained glutamic acid, the times required at the 2 enzyme levels to take up fixed volumes of oxygen are compared in Table 8. The concentrated enzyme showed approximately 3 times the activity of that of the dilute enzyme. When glutamate was omitted, the time ratio of the dilute : concentrated enzyme did not remain constant for the first 200 µl. of oxygen uptake. The ratios were 1.9, 2.25, 2.54 and 6.0 for 50, 100, 150 and 200 µl., respectively. The early low ratios indicate an expected early inhibition of the concentrated enzyme. This may be due to a more rapid formation of oxalacetate by the concentrated enzyme.

Fig. 4. Comparison of Diluted and Concentrated Enzyme Preparations from Bean Seedlings

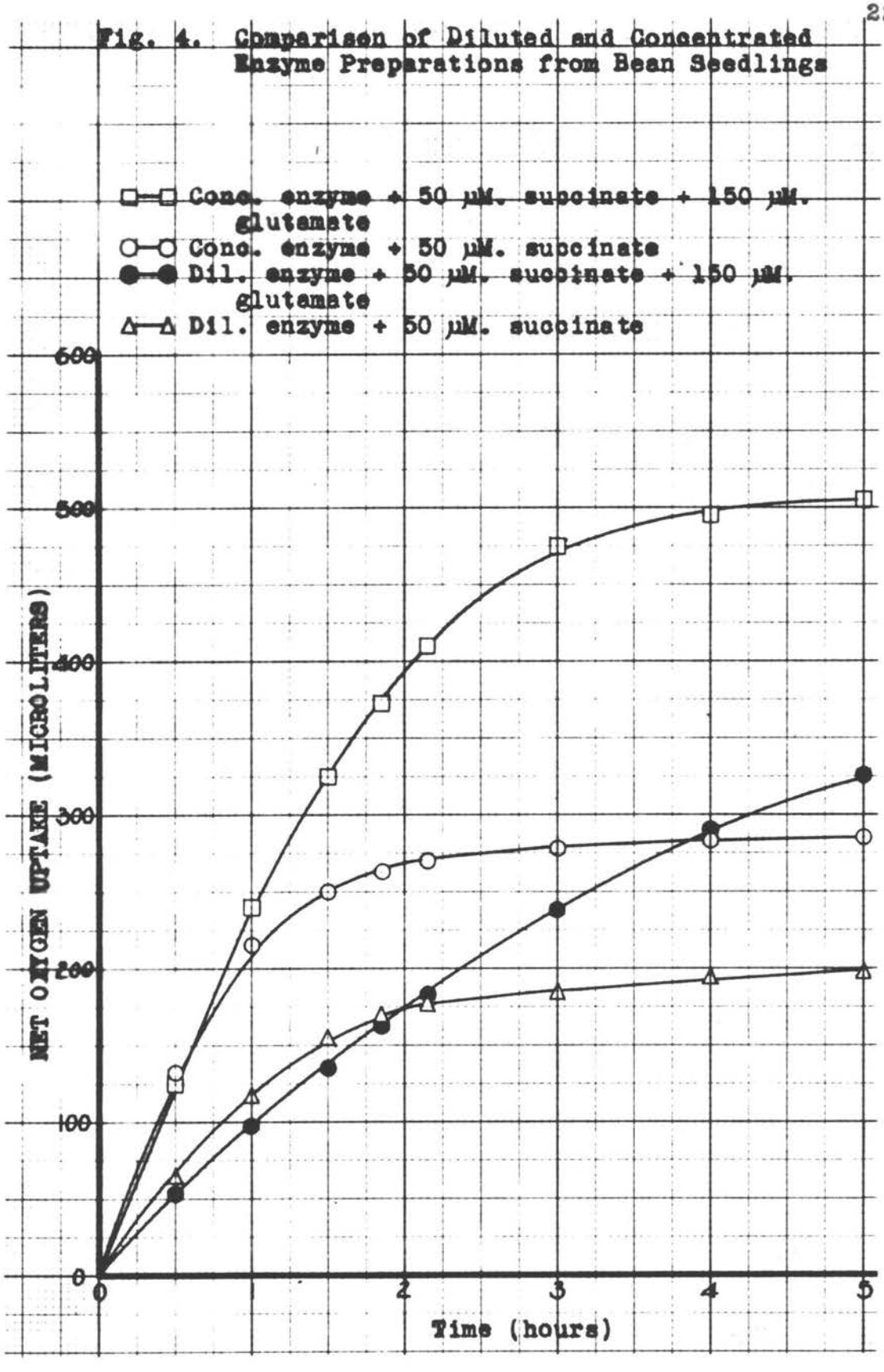


Table 8

Comparisons of Concentrated and Diluted Enzyme in the Presence of K-glutamate

Net µl. oxygen taken up	Dil. enzyme	Conc. enzyme	Dil. / Conc.
50 µl.	28 min.	10.5 min.	2.67
100	63	23.5	2.68
150	101	35	2.88
200	147	48.5	3.03
250	203	62.5	3.25
300	266	79	3.36

Arsenite at M./300 concentration is known to inhibit oxidation of fumaric acid (17, p.392). When the reaction was restricted by arsenite to the conversion of succinic acid to fumaric, the glutamic acid did not reverse the inhibition. Apparently fumarate inhibited succinic dehydrogenase in this case, and glutamate had no effect on this inhibition (Table 9).

Table 9

Fumarate Inhibition of Succinate Oxidation

Additions	Net μ atoms oxygen uptake 250 min.
50 μM. K-succinate	27.2
50 μM. K-succinate + M./30 arsenite (I)	23.1
(I) + 150 μM. K-glutamate	23.6
(I) + 50 μM. K-fumarate	16.9
(I) + 50 μM. K-fumarate + 150 μM. K-glutamate	17.2

Additions were as in Table 2. The liquid volume in the main compartment was 3.2 ml.

Oxalacetic acid had a very strong inhibitory effect which was reversed in part by glutamic acid (Table 10). The enzyme preparation appeared to slowly convert fumaric acid to oxalacetic acid which accumulated in sufficient quantities to inhibit the succinic dehydrogenase. The high inhibition (Table 11) by low concentrations of oxalacetic acid would explain the early inhibition of succinic dehydrogenase even though the preparation appeared to have a very low malic dehydrogenase activity (Table 6).

Table 10

Oxalacetate Inhibition of Succinate Oxidation

Additions	Net μ atoms oxygen uptake 260 min.
50 μM. K-succinate (I)	25.6
50 μM. K-succinate + 150 μM. K-glutamate (II)	43.5
(I) + 2 μM. oxalacetic acid	2.8
(II) + 2 μM. oxalacetic acid	34.8
(I) + 4 μM. oxalacetic acid	0.7
(II) + 4 μM. oxalacetic acid	18.3
(I) + 8 μM. oxalacetic acid	0.36
(II) + 8 μM. oxalacetic acid	5.9

Additions were as in Table 2. The liquid volume in the main compartment was 3.2 ml.

Table 11

Percentage Inhibition of Succinate Oxidation at Various Concentrations of Oxalacetate

Concentration of oxalacetate	% Inhibition
6.2×10^{-4}	93.6
2.5×10^{-4}	98.4
6.2×10^{-3}	99.1

Succinate concentration was 0.0156 M.

The figures for percentage inhibition (Table 11) were calculated from a 260-minute period; however, they correspond closely to the value, obtained by Swingle, Axelrod and Elvehjem (57, p.583), of 98% inhibition at 5×10^{-4} M.

oxalacetic acid. These workers used muscle tissue. The experiments were for one-hour periods. The calcium ion was used to relieve the inhibition.

Pardee and Potter (46, p.1085) noted an inhibition of succinic dehydrogenase by DPN similar to that reported in Table 3. Other research groups (57, p.590; 23, p.306; 37, p.512) have considered that this inhibition is probably caused by DPN stimulating the formation of oxalacetic acid.

Because of the economic importance of the phytotoxic agent, 2,4-dichlorophenoxyacetic acid (2,4-D), it was considered worthwhile to determine whether or not this chemical would affect the enzyme system developed to this point. Since the preparation apparently contained a transaminase for the removal of oxalacetic acid, it was considered that an inhibitor of either the succinoxidase system or the transaminase would cause a decrease in oxygen uptake. 2,4-D was added to the enzyme system at 2 concentrations. The results are shown in Table 12.

Table 12

Effect of 2,4-D on the Particulate Preparation

Additions	Net μ atoms oxygen uptake 60 min.	310 min.
50 μM. K-succinate (I)	18.7	39.5
(I) + 0.07 μM. 2,4-D	18.2	39.1
(I) + 0.7 μM. 2,4-D	16.7	35.2

The effect of 2,4-D on the oxygen uptake was not sufficient to be considered significant. This does not exclude the possibility of the conversion of 2,4-D by the intact plant to a compound that would affect this system.

The homogenizing medium was altered in an attempt to obtain a particulate fraction that would oxidize citric acid. An extraction of rat liver with 0.25 M. sucrose was made. The material was heated at 100° C. for 2 minutes and then centrifuged. The bean seedlings were homogenized in the supernate. Similar extracts were made with cabbage and bean plants. Citrate was not oxidized by particles prepared using these homogenizing media.

Citrate was oxidized when the following changes were made: (1) The homogenizing time was reduced to 25 seconds. (2) The bean sprouts were homogenized in 0.5 M. sucrose. (3) The particulate fraction was washed and suspended in 0.5 M. sucrose and 0.001 M. phosphate.

The experiments beyond this point were on a particulate fraction capable of catalyzing the oxidation of all members of the Krebs cycle. These experiments will be reported in Part II.

Discussion

In the preparation described apparently the only enzymes present and active were those required for conversion of succinate to oxalacetate. The enzymes of the succinoxidase system were present, and a small amount of malic dehydrogenase activity could be detected. There was evidence for conversion of succinate to oxalacetate, implying the presence of fumarase. Apparently the enzymes involved in the oxidation of citrate and α-ketoglutarate were absent. The procedure used in the preparation of the insoluble fraction must have either denatured the enzymes or made them soluble so that they were no longer in the particulate fraction. The long homogenizing time may have been responsible for either of these effects.

Succinate was oxidized at a constant rate for 1 to 2 hours; afterwhich, the rate of oxygen uptake decreased sharply (Table 1 and Figure 1). It was found that oxalacetate was a powerful inhibitor of the succinoxidase system (Tables 10 and 11). Malate inhibited succinoxidase, but at a much higher concentration than was necessary with oxalacetate (Table 6 and Figure 3). These facts would indicate that a sufficient quantity of oxalacetate to inhibit the succinoxidase was formed from succinate. The lag period of 1 to 2 hours before the inhibition occurred is

in accord with the hypothesis that oxalacetate causes the inhibition. In Table 3 it may be seen that DPN gave a slight inhibition. Since DPN is necessary for the conversion of malate to oxalacetate the concentration of the latter acid would be increased by DPN addition and a greater inhibition of succinate oxidation would be expected. Pardee and Potter (46, p.1085) have also observed this effect with animal tissue.

Glutamate reversed the inhibition which occurred when succinate was oxidized, and approximately 90% of the oxygen uptake required for the conversion of succinate to fumarate was obtained. Glutamate also reversed inhibitions induced by the addition of oxalacetate and malate, but glutamate had no influence on succinate oxidation. The latter fact was established by using arsenite at M./30 concentration to block the malate to oxalacetate step.

Glutamate must have removed oxalacetate by means of a transaminase reaction utilizing the glutamic-aspartic transaminase. This transaminase was found in oats by Albaum and Cohen (2, pp.26-27); later Leonard and Burris (36, p.708) surveyed an extensive group of plants and reported the transaminase present in most of the plants tested. No direct proof of the transaminase was demonstrated in this study; however, the reversal of oxalacetate inhibition by glutamate can best be explained in this

manner. Inhibition of the succinoxidase system in animal tissue, described by Swingle, et al (57, pp.588-590), was reversed by glutamic acid, and this was ascribed to a transaminase reaction.

The system may be summarized by the following reactions:

Oxalacetate could also be removed by either the condensation reaction to form citrate or by β-decarboxylation to form pyruvate. The enzymes for these reactions were not present in the insoluble fraction or some cofactors were missing.

Fumarate at high concentrations was found to inhibit succinate oxidation. The fumarate inhibition was not reversed by glutamate in the presence of M./30 arsenite. Apparently fumarate itself may inhibit the succinoxidase system; and it may also be converted to oxalacetate which is a more potent inhibitor.

Summary

1. Succinoxidase was present in the insoluble fraction prepared from bean seedlings.

2. Slight malic dehydrogenase activity was found.

3. Fumarate, malate and oxalacetate inhibited the succinoxidase. Inhibition by malate depended upon its conversion to oxalacetate.

4. When the concentration of succinate was 0.0156 M., 6.2×10^{-3} M. oxalacetate inhibited the succinoxidase 99.1%.

5. Glutamate reversed malate and oxalacetate inhibition; the inhibition that occurred during succinate oxidation was reversed by glutamate.

6. Cytochrome c was a necessary component of this system.

7. An outline of the enzymatic reactions that could be carried out by the insoluble fraction was presented.

PART II

THE DEMONSTRATION OF A KREBS CYCLE IN A PARTICULATE FRACTION OBTAINED FROM BEAN SEEDLINGS

Method

The particulate fraction was prepared in the following manner: 75 grams of bean seedling hypocotyls were homogenized in 50 ml. of a medium containing 1 M. sucrose and 0.1 M. phosphate at pH 7.0. The temperature was held below 5° C. The total period of homogenizing amounted to 6 seconds. Four homogenates, involving 300 grams of material, were combined for each experiment, strained through cheesecloth, and spun at 3,000 x g. for 10 minutes. The supernate was removed and centrifuged at 14,000 x g. for 15 minutes. The pellets from the second centrifugation were resuspended in 20 ml. of homogenizing medium and centrifuged for 15 minutes at 14,000 x g. The centrifugation was carried out at a temperature of less than 5° C. The washed particulate fraction was suspended in the homogenizing medium using a very loose Potter-Elvehjem type homogenizer. The amount of particulate suspension added to each flask contained from 0.8 to 1 mg. of nitrogen as determined by the semimicro Kjeldahl procedure.

The volume of the liquid phase was 3.4 ml., including 0.2 ml. of KOH in the center well. The experiments were

run at 30° C. The flasks were shaken at 100 oscillations per minute. Any variation from this general description will be noted in the text.

Experimental

1. Oxidation of Krebs Cycle Intermediates

Succinate, citrate and α-ketoglutarate were found to be oxidized by this modified preparation (Table 13). The substrates were not oxidized at a high rate and this did not appear to continue much beyond one hour. However, this was one of the first indications that the enzymes for the oxidation of substrates other than succinate were present. Pyruvate was probably not oxidized in this experiment.

Table 13

Oxidation of Citrate, Succinate, and α-Ketoglutarate

Addition	Net μ atoms oxygen uptake 60 min.	180 min.
10 μM. K-succinate (I)	10.5	12.2
50 μM. Na_3-citrate (II)	2.5	5.0
50 μM. α-ketoglutarate (III)	4.6	5.3
I + II + III + 20 uM. Na-pyruvate	12.6	14.7

Additions were as follows: 0.1 ml. of 0.28 M. $MgSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN; 0.3 ml. of 0.5 M. K-glutamate; 2.0 ml. of particulate fraction. The liquid volume was made to the 3.2 ml. by addition of 0.25 M. sucrose. The temperature was 20° C. The entire bean plant was used in this experiment. 200 μM. of K-phosphate were added with the suspension of particulate fraction.

In an attempt to obtain a preparation more active on Krebs intermediates, the effect of adding other possible co-factors to the incubation medium was tested. In Experiment I, Table 14, TPN was added for the first time, while in Experiment II, Table 14, TPN, cocarboxylase and liver concentrate were added to the medium. While the oxidation of citrate was much improved (over that reported in Table 13), pyruvate apparently was not oxidized. The addition of cocarboxylase and liver concentrate appeared to have no effect on the oxidation.

Table 14

Oxidation of Citrate and Malate

Additions	Net μ atoms oxygen uptake 3 hrs. Exp. I	Exp. II
50 μM. Na_3-citrate	17.7	16.3
5 μM. Na_3-citrate	5.8	3.3
3 μM. K-malate + 50 μM. Na-pyruvate	2.2	3.8

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.2 ml. of 0.5 M. K-phosphate (pH 7.3); 0.3 ml. of 0.033 M. Na_4-ATP; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN and 5.38×10^{-4} M. TPN; 1.0 ml. of particulate fraction. 100 μM. of K-phosphate were added with the particulate fraction. The liquid volume was made to 3.2 ml. with 0.25 M. sucrose. In Experiment II each flask contained 1 mg. of liver concentrate and 0.1 ml. of 4.18×10^{-3} M. cocarboxylase per flask. The homogenizing medium contained 0.1 M. F^-.

In subsequent experiments the oxidation of citrate was found to occur at much higher rates. This was particularly true when glutathione was incorporated in the incubation medium (Table 15; Figure 5). The oxidation of citrate usually continued for about 4 hours before the rate began to decrease appreciably. An initial lag period usually occurred when citrate was the substrate.

Table 15

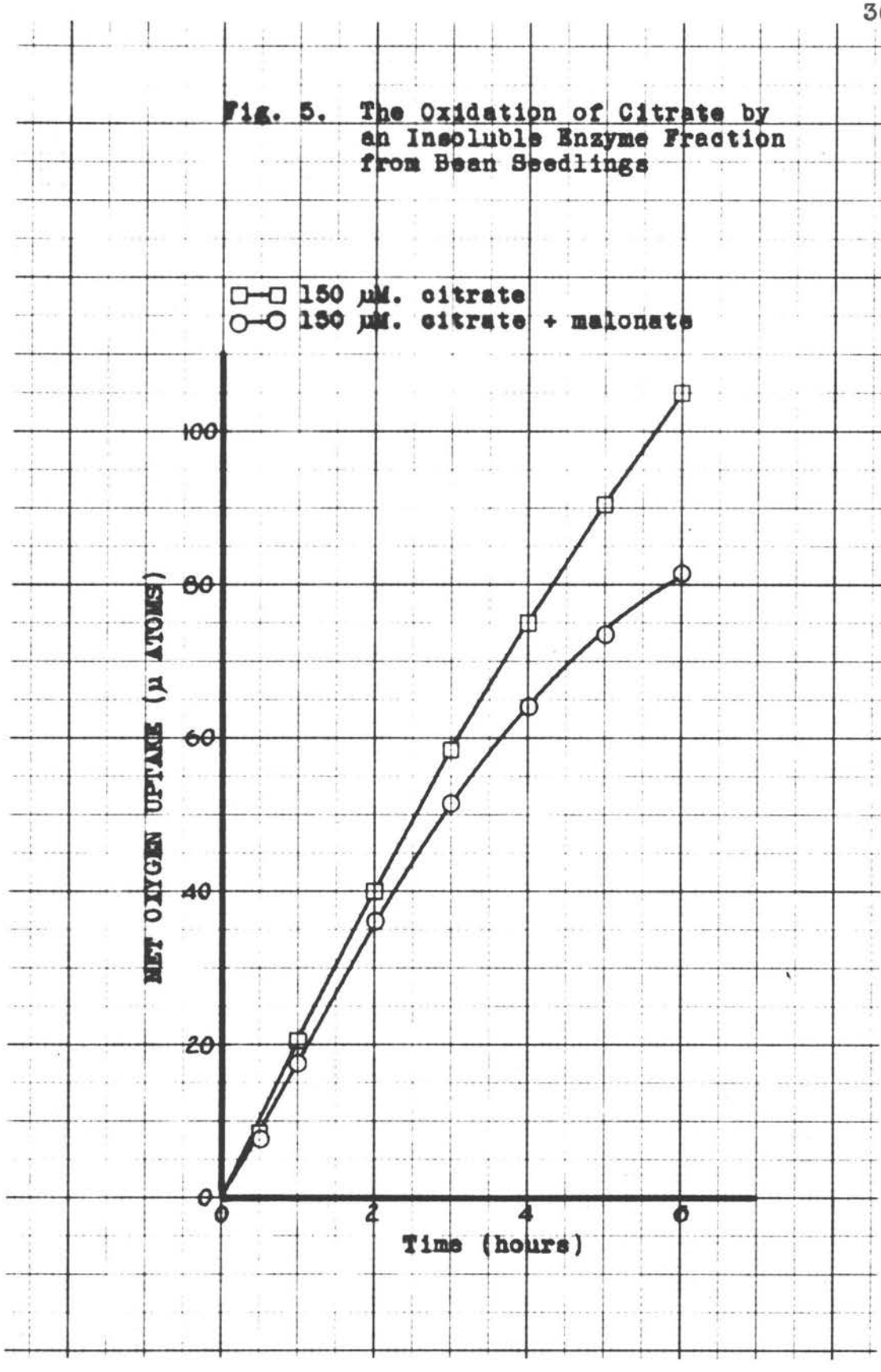
Citrate Oxidation

Additions	Net μ atoms oxygen uptake 1 hr.	6 hrs.
150 μM. Na_3-citrate	20.4	105.0
150 μM. Na_3-citrate + K-malonate	18.7	81.3

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.05 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.04 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1). K-malonate was added where indicated to give 0.033 M. concentration in the flask. The liquid volume was made to 3.2 ml. with distilled water. The experiment was carried out at 30° C.

When 5 μM. of succinate were used as substrate for this particulate fraction, oxalacetate apparently did not accumulate in sufficient quantities to inhibit the succinoxidase (Table 16; Figure 6). Fifty-seven percent of the succinic acid was oxidized in 3 hours when 5 μM. of

Fig. 5. The Oxidation of Citrate by an Insoluble Enzyme Fraction from Bean Seedlings



succinate was added.

Table 16

Succinic Acid Oxidation

Addition	Net μ atoms oxygen uptake 1 hr.	3 hrs.
5 μM. succinate	12.8	19.9

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.2 ml. of liver concentrate (5 mg./ml.); 0.1 ml. of 0.08 M. K-glutathione; 2.0 ml. of particulate fraction. 200 μM. of K-phosphate (pH 7.0) were added with the particulate fraction. The liquid volume was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

To determine whether succinate would be oxidized more efficiently at higher levels, succinic acid was added to the flasks at 10, 20 and 40 μM. amounts (Table 17; Figure 7).

Fig. 6. Oxidation of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

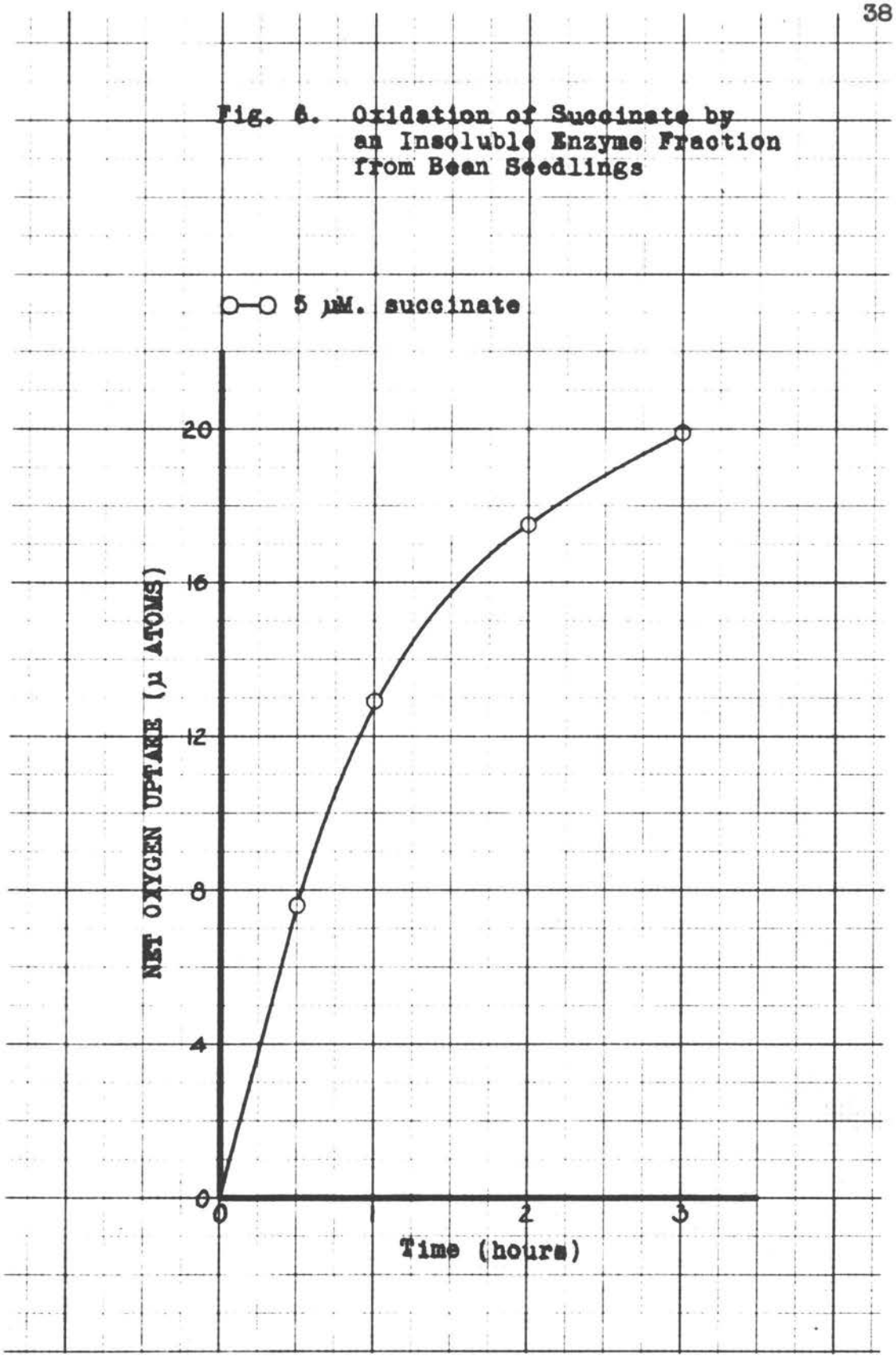


Table 17

Succinic Acid Oxidation at Various Concentrations

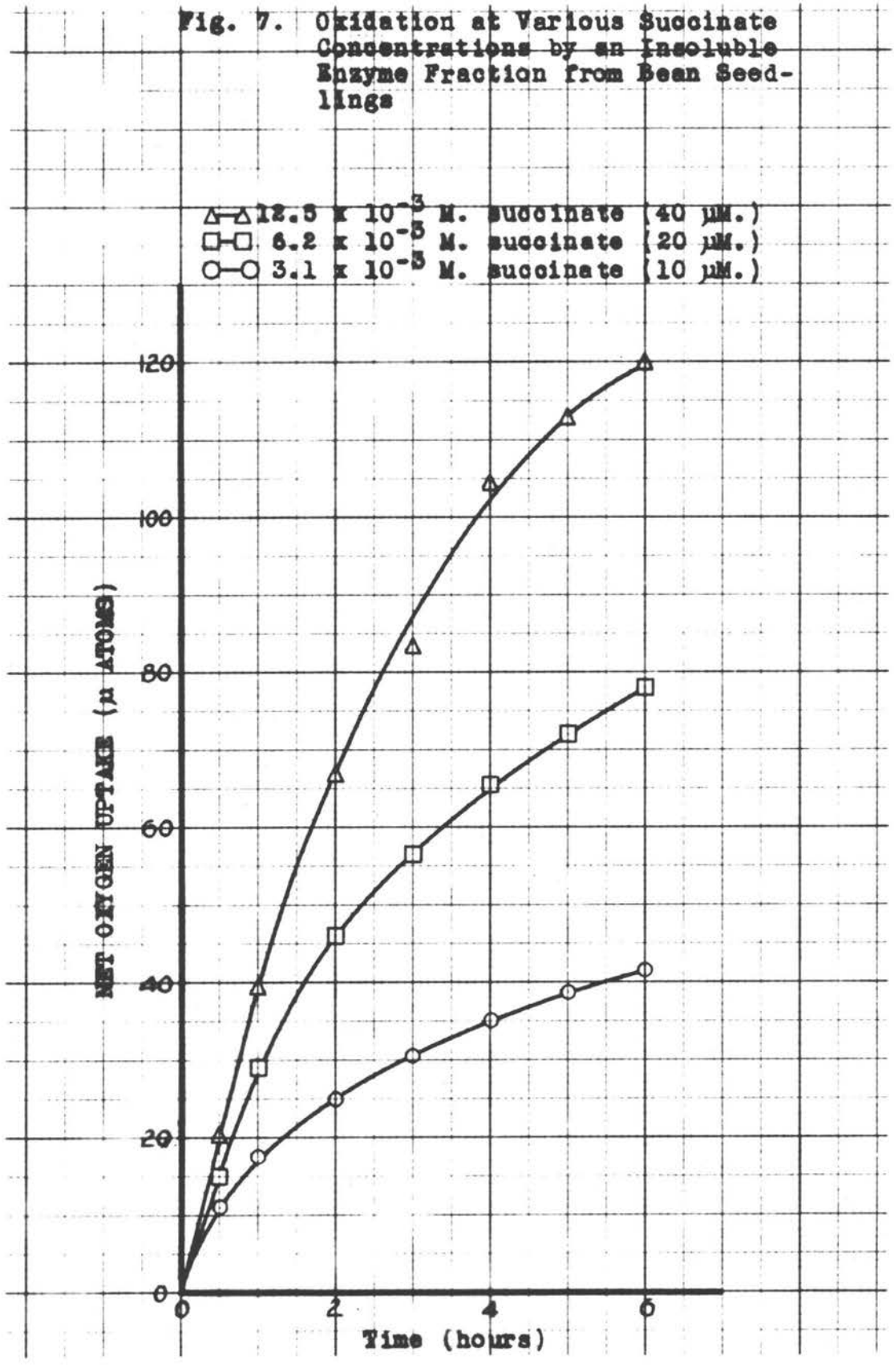
Succinate concentration	μ atoms oxygen uptake 1 hr.	6 hrs.
3.12×10^{-3} M. K-succinate (10 μM.)	17.5	41.7
6.24×10^{-3} M. K-succinate (20 μM.)	28.9	77.9
12.48×10^{-3} M. K-succinate (40 μM.)	39.3	120.0

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase + liver concentrate (10 mg./ml.); 2.0 ml. of particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The flasks were made to 3.2 ml. volume with 0.25 M. sucrose. The experiment was carried out at 30° C.

The enzyme system did not appear to be saturated even at the 20 μM. level of substrate. Figures 6 and 7 show typical succinate oxidation curves. The rate, which was high initially, decreased after 25-35% of the substrate was oxidized.

The break in the curve could be explained by the existence of two types of particulate matter in the insoluble fraction. If one type of particle contained the enzymes necessary to oxidize succinic acid to oxalacetate, while the other contained all of the Krebs cycle enzymes, a high initial rate would be expected. The evidence for the stability of succinic dehydrogenase supports this proposal.

Fig. 7. Oxidation at Various Succinate Concentrations by an Insoluble Enzyme Fraction from Bean Seedlings



Two conditions were found to be critical in pyruvate oxidation. The first condition was the presence of glutathione. The data for the effect of glutathione on pyruvate oxidation will be given in the next section. The second condition was the presence of another Krebs cycle intermediate. This means that the enzyme for the carboxylation of pyruvate did not exist in this preparation or that some cofactor had been lost. In Table 18 and in Figure 8, it may be seen that when 5 uM. of pyruvate were included with 5 uM. of succinate the oxidation was greater than could be accounted for by the succinate alone. This was demonstrated again, and perhaps more convincingly, in the experiment reported in Table 19 and Figure 9.

Table 18

Oxidation of Pyruvic Acid

Additions	Net μ atoms oxygen uptake 70 min.	280 min.
5 μM. K-succinate (I)	7.5	15.7
(I) + 5 μM. Na-pyruvate	10.9	28.4
(I) + F^-	8.0	16.1
(I) + 5 μM. Na-pyruvate + F^-	11.9	24.5

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na -ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of liver concentrate (20 mg./ml.); 0.1 ml. of 0.08 M. K-glutathione; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate (pH 7.0) were added with the particulate fraction. 0.1 ml. of 0.71% NaF was added where F^- is indicated. The liquid volume was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

Table 19

Oxidation of Pyruvic Acid

Additions	Net μ atoms oxygen uptake 1 hr.	6 hrs.
2 μM. K-succinate (I)	2.4	7.7
4 μM. K-succinate (II)	8.0	20.9
(I) + 4 μM. Na-pyruvate	7.5	22.3
(II) + 8 μM. Na-pyruvate	13.2	41.7

Additions were as described in Table 18.

Fig. 8. The Oxidation of Succinate and Pyruvate plus Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

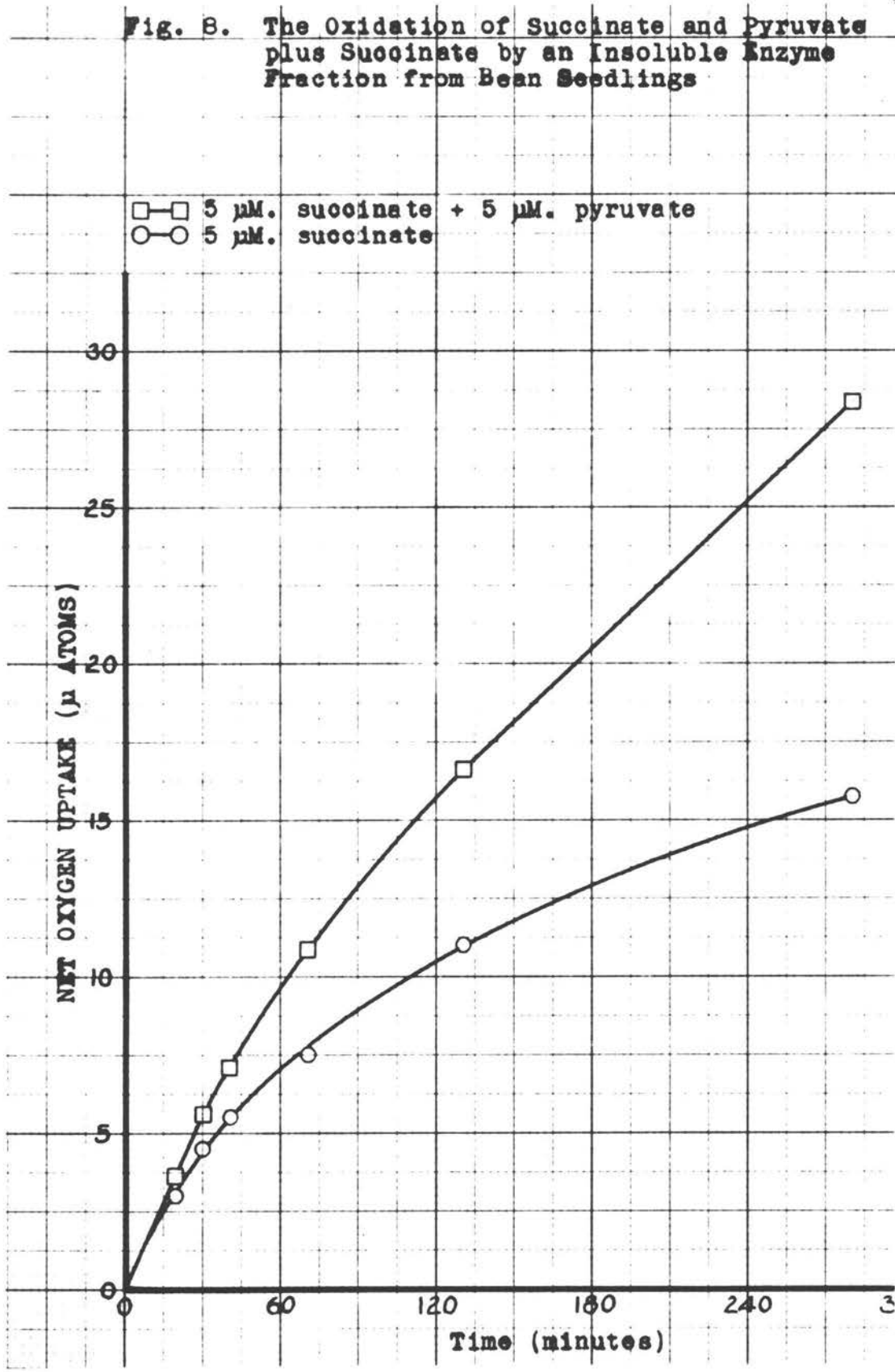
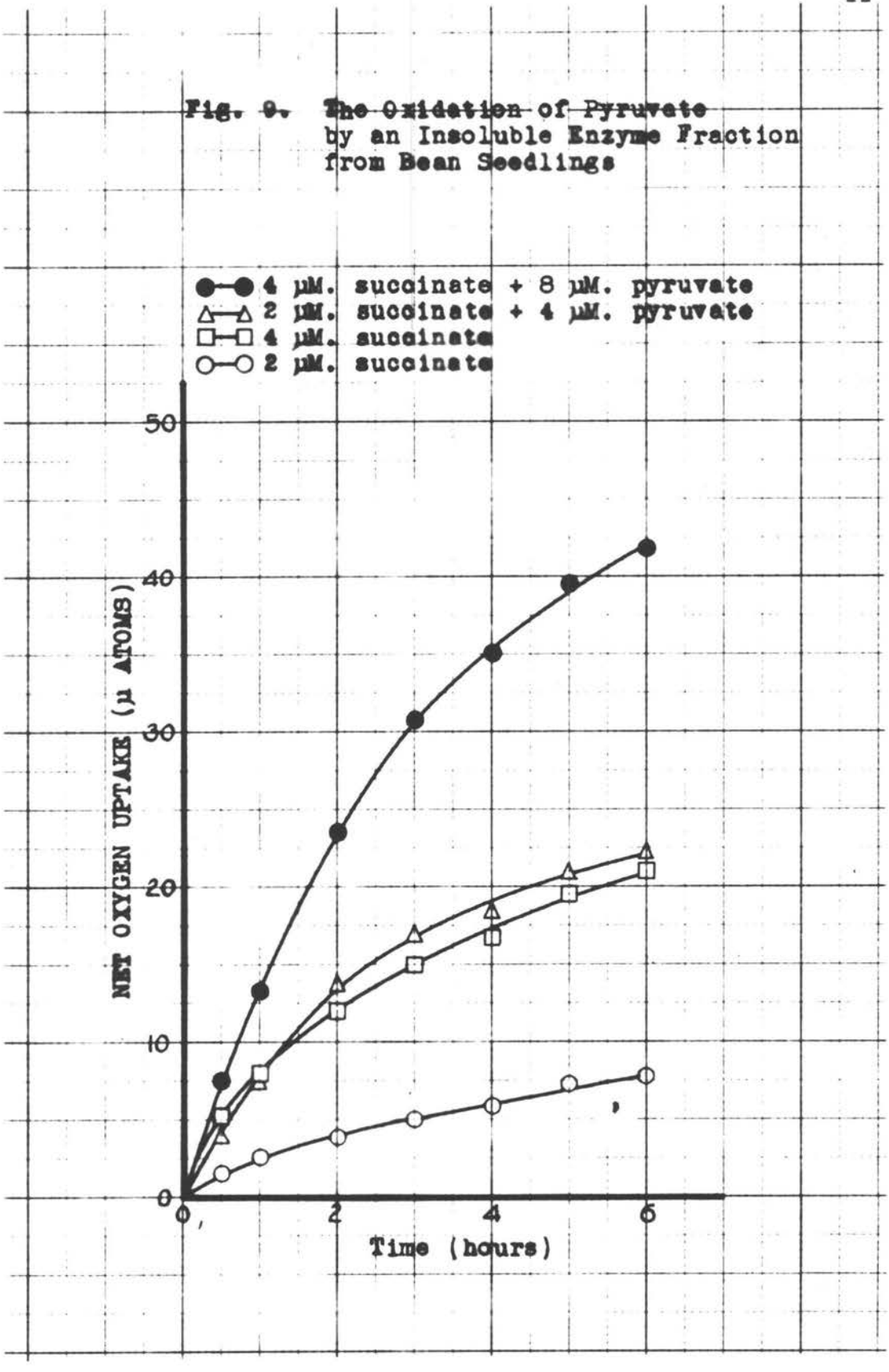


Fig. 9. The Oxidation of Pyruvate by an Insoluble Enzyme Fraction from Bean Seedlings



That oxidation of pyruvate is actually catalyzed by succinate was demonstrated clearly in an experiment reported in Table 20 and Figure 10. Pyruvate was not oxidized in the absence of succinate. The oxygen uptake, when both substrates were present, was much greater than could be accounted for by succinate alone. Catalysis of pyruvate oxidation by a member of the Krebs cycle is considered to be very good evidence that the cycle is operative in the system.

Table 20

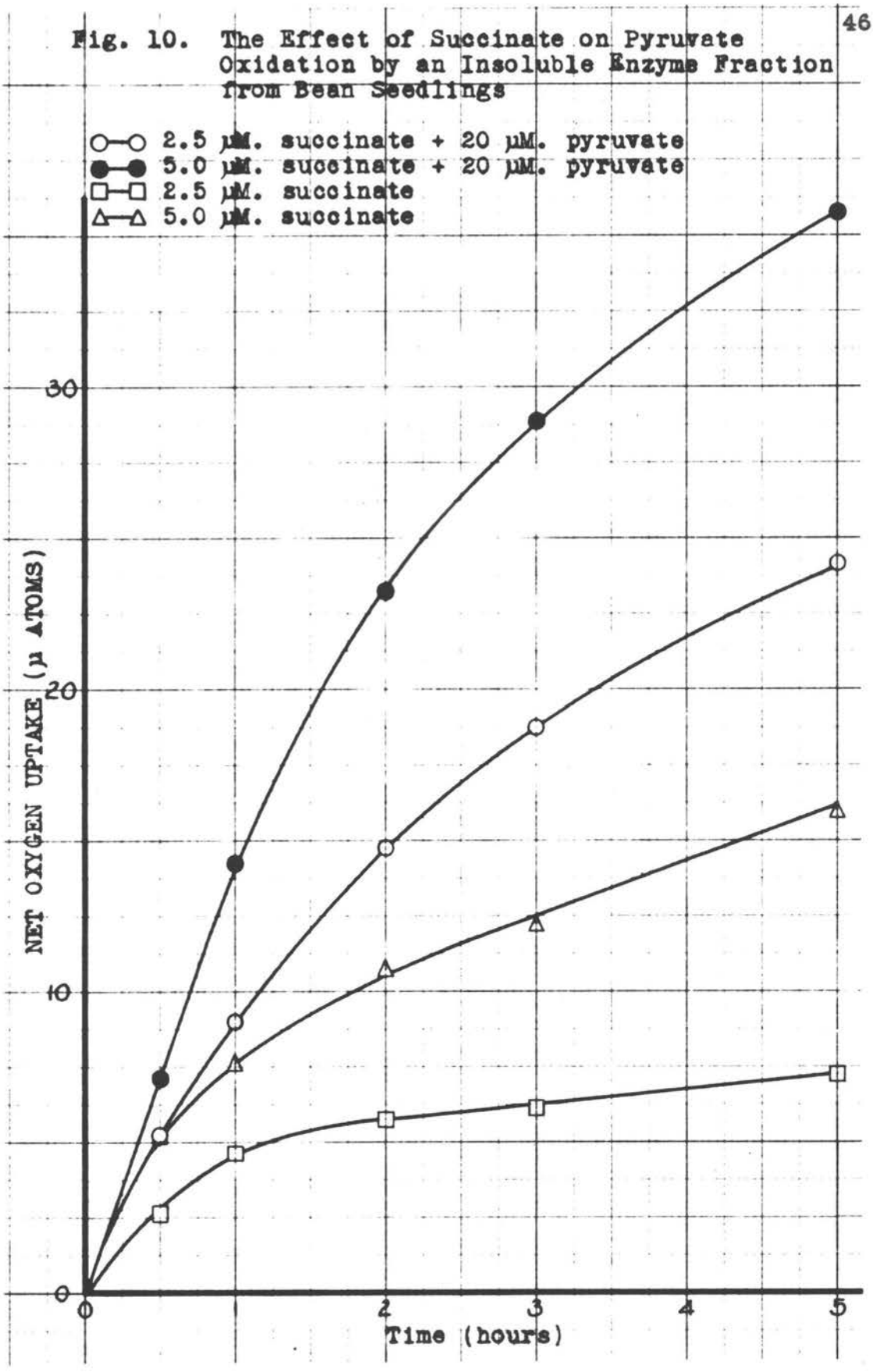
Catalytic Effect of Succinate on Pyruvate Oxidation

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
2.5 μM. K-succinate (I)	4.6	7.2
5.0 μM. K-succinate (II)	7.6	14.9
(I) + 20 μM. pyruvate	9.0	24.1
(II) + 20 μM. pyruvate	14.3	35.8
20 μM. pyruvate	0.0	0.0

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.1 ml. cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

That α-ketoglutarate was oxidized by the preparation is shown in Tables 13, 29 and 30.

Fig. 10. The Effect of Succinate on Pyruvate Oxidation by an Insoluble Enzyme Fraction from Bean Seedlings



Fumarate was oxidized by the particulate preparation (Table 46).

Malate was utilized as a substrate by this preparation (Tables 14 and 30).

Succinate, pyruvate, citrate, α-ketoglutarate, malate, lactate and acetate were used as substrates in a single experiment (Table 21; Figure 11).

Table 21

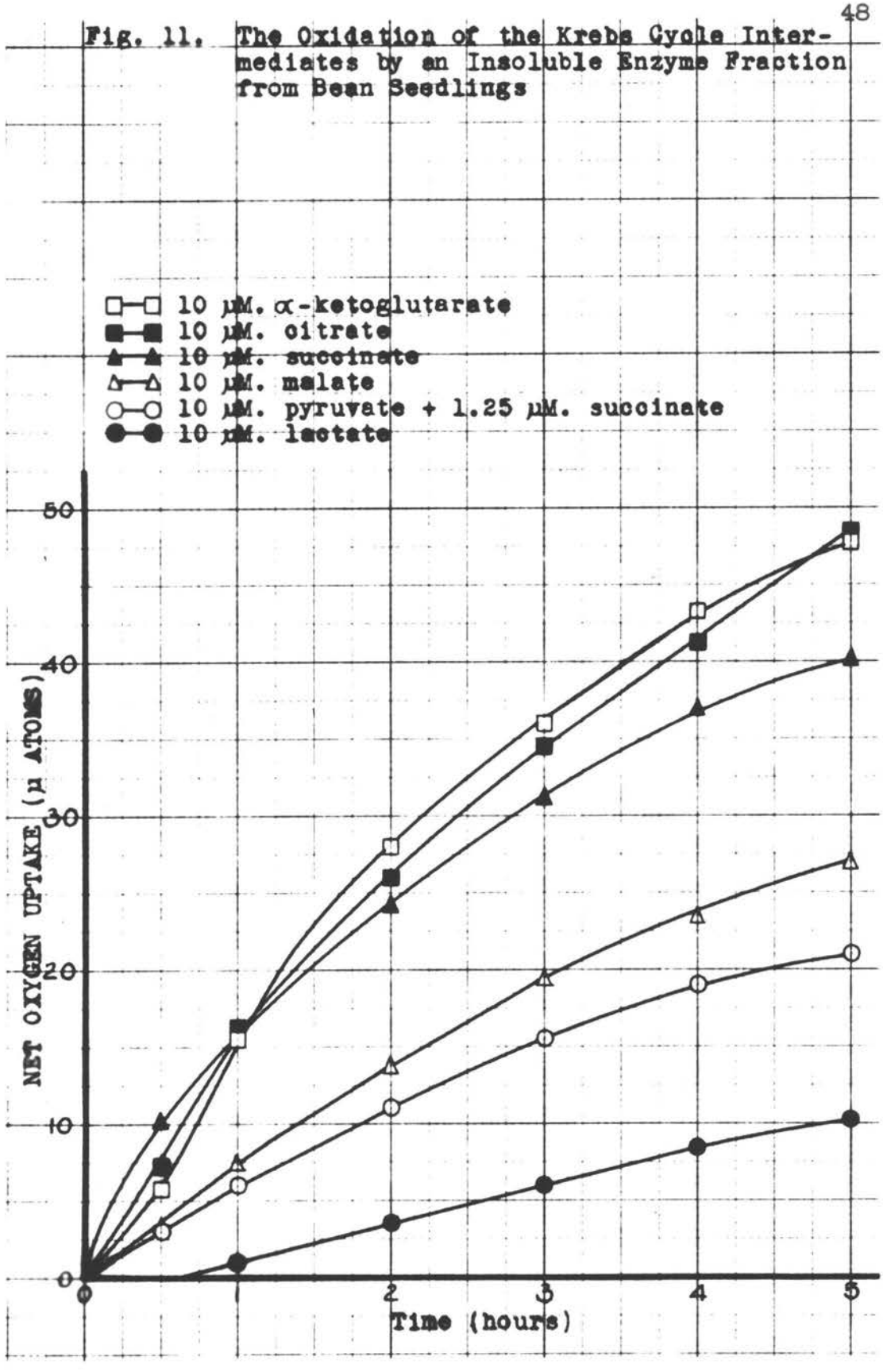
Oxidation of Krebs Cycle Substrates

Additions	Oxygen uptake (net μ atoms) 1 hr.	5 hrs.	% Theoretical 5 hrs.
10 μM. K-succinate	15.7	40.2	57.0
10 μM. Na_3-citrate	16.3	48.4	54.0
10 μM. α-ketoglutarate	15.5	47.8	60.0
10 μM. K-malate	7.5	27.0	45.0
10 μM. Na-pyruvate + 1.25 μM. K-succinate	6.0	20.9	42.0
10 μM. K-lactate + 1.25 μM. K-succinate	1.1	10.3	--
10 μM. K-acetate + 1.25 μM. K-succinate	0.0	0.0	--

Additions were as in Table 17.

It may be seen that the preparation was capable of catalyzing the oxidation of all of the Krebs cycle members tested. Succinate, citrate, and α-ketoglutarate were found to be oxidized at a high rate. Malate and pyruvate were oxidized at a much lower rate. The low concentration

Fig. 11. The Oxidation of the Krebs Cycle Intermediates by an Insoluble Enzyme Fraction from Bean Seedlings



of succinate added to the pyruvate oxidation. All of the substrates except lactate and acetate were oxidized up to about 50% of the theoretical value. Lactate oxidation occurred only after the first hour and acetate was not oxidized. The oxidation of lactate would indicate that a small amount of lactic dehydrogenase was present in the particulate fraction.

In this section the evidence for the following points have been presented: (1) The enzymes necessary for the oxidation of the Krebs cycle intermediates, citrate, α-ketoglutarate, succinate, fumarate, malate and pyruvate, were present in the preparation described. (2) Another Krebs cycle intermediate was necessary for pyruvate oxidation. (3) Acetate was not oxidized under the conditions described. (4) Lactic acid was oxidized at a slow rate. These points are regarded as good evidence that a Krebs cycle is operative in the particulate fraction from bean seedlings.

2. Glutathione Requirements

Glutathione appeared to give an increase in oxygen uptake in an experiment in which succinate and pyruvate were used as the substrates. This effect was tested and the results were as shown in Table 22 and Figure 12.

Further evidence of the requirement for glutathione can be found in Table 34.

Table 22

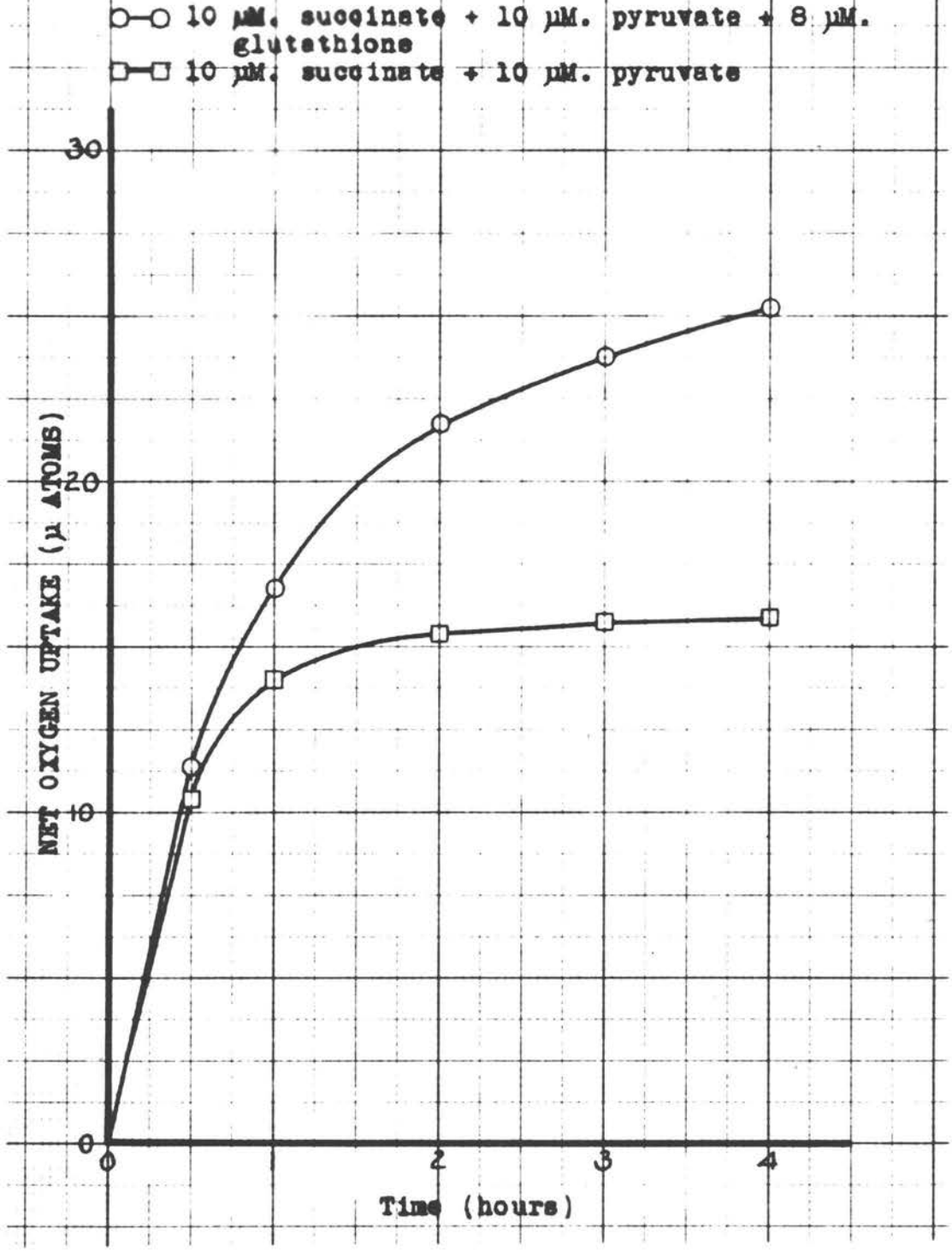
Effect of Glutathione on Oxygen Uptake

Additions	Net μ atoms oxygen uptake 1 hr.	4 hrs.
Experiment 1		
10 μM. Na-pyruvate + 10 μM. K-succinate (I)	14.0	15.8
(I) + 8 μM. K-glutathione	16.8	25.2
Experiment 2		
10 μM. Na-pyruvate + 10 μM. K-succinate (I)	9.8	11.3
(I) + 8 μM. K-glutathione	14.5	23.2

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×11^{-3} M. cocarboxylase + liver concentrate (10 mg./ml.); 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate (pH 7.0) were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. In Experiment 2 the homogenizing medium contained cysteine at 0.05 M. concentration. The glutathione was in the reduced state, and it was dissolved just before addition to the flasks. An equimolar quantity of $KHCO_3$ was added to the reduced glutathione.

Glutathione was added in 0, 8, 16, 32 and 64 μ mole amounts in an experiment in which succinate alone was used as substrate (Table 23; Figure 13). Although the 32 and 64 μM. amounts showed the largest oxygen uptake, the

Fig. 12. The Effect of Glutathione on the Activity of the Particulate Fraction from Bean Seedlings



increase over the 8 μM. level was not large enough to warrant use of a higher glutathione concentration. The increase in "endogenous respiration" was not appreciable when 8 μM. of glutathione was added to the flask. The mechanism of the glutathione effect has not been determined but the large stimulation at such low molar concentrations would suggest a catalytic action. During the first hour glutathione had very little effect on succinate oxidation, but at the end of the experiment the oxygen uptake was nearly doubled by the presence of glutathione.

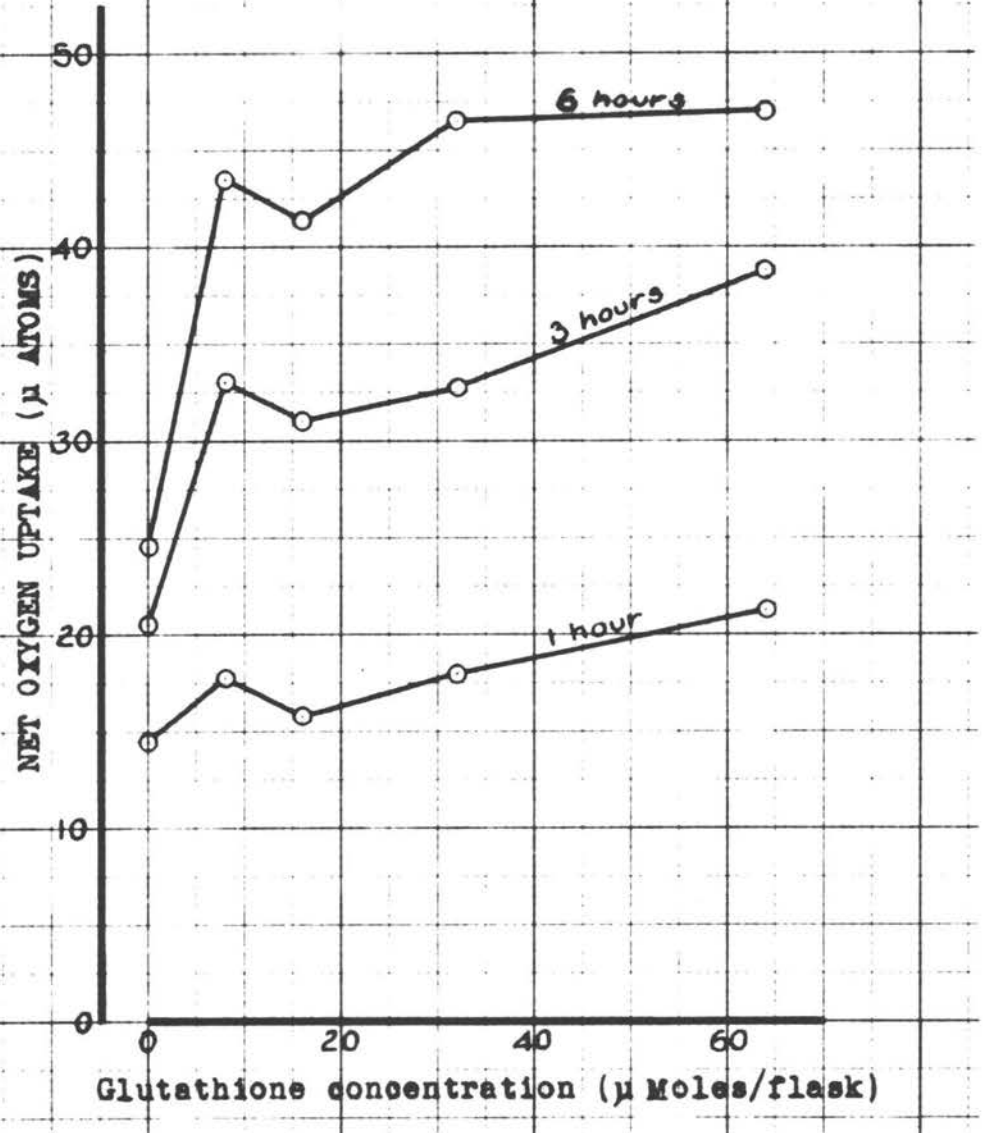
Table 23

Effect of Various Glutathione Concentrations on Succinate Oxidation

Additions	Net μ atoms oxygen uptake 1 hr.	6 hrs.
No glutathione	14.6	24.4
8 μM. glutathione	17.8	43.6
16 μM. glutathione	16.2	41.2
32 μM. glutathione	17.9	46.6
64 μM. glutathione	21.2	47.1

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP; 0.1 ml. of 0.1 M. K-succinate (pH 7.0); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of terramycin (0.16 mg./ml.); 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate (pH 7.0) were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

Fig. 13. The Effect of Glutathione at Various Concentrations on the Oxidation of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings



3. Effect of Fluoride on the Stability of Particles in the Homogenate

Kornberg and Pricer (25, pp.776-777) purified a nucleotide pyrophosphatase from potatoes. The enzyme hydrolyzed the pyrophosphate linkage from the coenzymes, DPN, TPN, FAD, ADP, ATP and TPP. Fluoride at 0.1 M. concentration was found to inhibit the enzyme, and phosphate was found to reduce the hydrolysis of DPN and FAD. Millerd and Bonner (39, p.155) have reported that fluoride will partially replace phosphate in the homogenizing medium for preparation of plant mitochondria. Consideration of these facts led to an attempt to increase the activity of the particulate preparation by adding fluoride to the homogenizing medium.

The experiment described in Table 24 was carried out as follows: The plant material was homogenized in 4 parts as described under Methods; however, each of the 4 media contained a different concentration of fluoride. The concentrations were 0.0 M., 0.05 M., 0.1 M., 0.15 M. The homogenates were carried separately through the fractionation. As much fluoride as possible was removed in the washing step. The fractions were suspended in 1.0 M. sucrose and 0.1 M. phosphate. The homogenizing medium in each case contained 0.05 M. cysteine.

Table 24

Effect of F^- in the Homogenizing Medium

Additions	F^- Conc.	Net μ atoms oxygen uptake/4 hrs.
50 μM. Na_3-citrate	0.0 M.	20.6
10 μM. Na-pyruvate + 10 μM. K-succinate	0.0	20.6
50 μM. Na_3-citrate	0.05	27.2
10 μM. Na-pyruvate + 10 μM. K-succinate	0.05	30.9
50 μM. Na_3-citrate	0.1	19.7
10 μM. Na-pyruvate + 10 μM. K-succinate	0.1	19.1
50 μM. Na -citrate	0.15	24.0
10 μM. Na-pyruvate + 10 μM. K-succinate	0.15	23.1

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44 x 10^{-3} M. DPN + 5.38 x 10^{-4} TPN + 4.18 x 10^{-3} M. cocarboxylase + liver concentrate (10 mg./ml.); 0.1 ml. of 0.08 M. glutathione; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

Since the results of the first experiment were not very convincing, the effect of fluoride was tested again with duplication of homogenates. The procedure was otherwise the same as in the first experiment. Two of the homogenates contained no fluoride and the other two contained 0.05 M. fluoride. The results are shown in Table 25.

Table 25

Effect of 0.05 M. Fluoride in the Homogenizing Medium

Fluoride concentration	Net μ atoms oxygen uptake 6 hrs.
0.0	27.0
0.0	30.9
0.05	25.0
0.05	29.3

Additions and conditions were as given in Table 24. 10 μM. Na-pyruvate + 10 μM. K-succinate were used as the substrates.

The 2 experiments with fluoride in the homogenizing medium failed to give any conclusive indication that this inhibitor should be so used. As expected, the results for duplicate homogenates were not identical. Fluoride failed to cause any large or consistent effect. When used in the flask media (Table 18), fluoride caused slight inhibition. Therefore, fluoride was not used routinely in subsequent experiments.

The loss of activity of the preparation in the latter stages could very well be contingent upon a catabolism of important co-factors by enzymes which are not affected by fluoride. An alternative pathway for the breakdown of DPN was demonstrated by Handler and Klein (18, pp.55-57), who studied a DPN nucleosidase which cleaved the glycosidic bond between nicotinamide and ribose. The enzyme was

inhibited by nicotinamide. This inhibitor possibly could increase the activity of the particulate fraction. In any event, the breakdown of co-factors must still be regarded as a possible explanation of the fact that complete oxidation of Krebs cycle intermediates has not been realized with plant particulate matter, as it has been with rat liver mitochondria.

4. Sucrose and Phosphate Concentrations in the Flask and in the Homogenizing Medium

The phosphate concentration was varied in the homogenizing media (Table 26). Four 75-gram portions of bean sprouts were homogenized separately in 50 ml. of the following media: 1 M. sucrose and no phosphate; 1 M. sucrose and 0.05 M. phosphate; 1.0 M. sucrose and 0.1 M. phosphate; 1.0 M. sucrose and 0.15 phosphate. The homogenates were fractionated separately. The particulate fractions were washed in 20 ml. of the homogenizing medium and suspended in 1 M. sucrose and 0.1 M. phosphate.

Table 26

Effect of Phosphate in the Homogenizing Media

Additions	Phosphate conc. in homogenate	Net μ atoms oxygen uptake 260 min.
10 μM. K-succinate	0.00 M.	10.2
10 μM. K-succinate	0.05	11.8
10 μM. K-succinate	0.10	13.3
10 μM. K-succinate	0.15	14.9
10 μM. Na_3-citrate	0.00	1.9
10 μM. Na_3-citrate	0.05	6.8
10 μM. Na_3-citrate	0.10	13.4
10 μM. Na_3-citrate	0.15	13.9

Additions were as follows: 0.1 ml. of 0.28 M. $MgSO_4$; 0.3 ml. of 0.033 M. Na -ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN; 0.3 ml. of 0.5 M. K-glutamate; 2.0 ml. of particulate fraction. 200 μM. K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 20° C.

The concentration of phosphate in the homogenizing medium did not appear to be too critical in the oxidation of succinate, but citrate oxidation was greatly diminished by the omission of phosphate from the medium. 0.1 M. phosphate was chosen for the concentration to be used in subsequent homogenizing media.

The phosphate concentration was varied in the flask medium with 20 μ moles of pyruvate and 5 μ moles of succinate being used as the substrates (Table 27; Figure 14).

Table 27

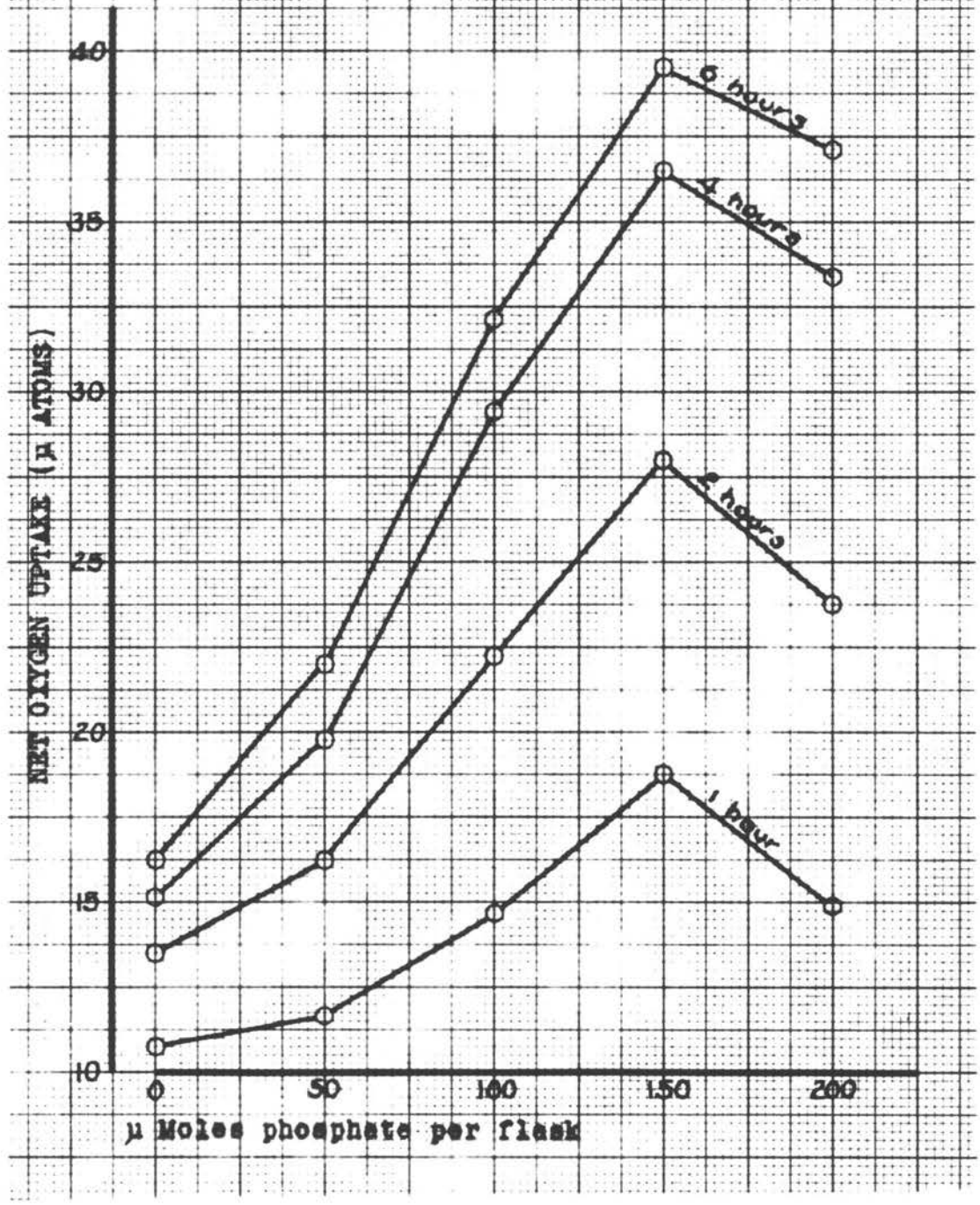
Effect of Phosphate on the Activity of the Particulate Fraction

Phosphate concentration	Net μ atoms oxygen uptake 1 hr.	6 hrs.
0 μM./flask	10.8	16.2
50	11.6	22.0
100	14.6	32.1
150	18.8	39.5
200	14.9	37.1

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of 0.2 M. Na-pyruvate; 0.2 ml. of 0.25 M. K-succinate; 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 1.0 ml. of the particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C. The buffer concentration was maintained at 200 μM./flask by the addition of 0.5 M. glycylglycine (pH 7.0). The washed particulate fraction was suspended in 1.0 M. sucrose.

Probably all of the phosphate concentrations were slightly higher than stated in Table 27, since some phosphate must have been carried over from the washing. The phosphate concentration which gave optimal activity was found to be 150 μM. per flask. As the experiment approached 6 hours the difference between 150 μM. and 200 μM. diminished. The decrease in the amount of phosphate below 150 μM. caused a sharp drop in the activity of the particulate fraction. A concentration of 200 μM. of phosphate

Fig. 14. The Effect of Phosphate on Oxidation of 6 μM. of Succinate plus 20 μM. of Pyruvate by an Insoluble Enzyme Fraction from Bean Seedlings



per flask was used in all subsequent experiments.

The sucrose concentration was varied in the homogenizing medium (Table 28). Citrate and succinate were used as the substrates. Four separate homogenates were made. Each of the homogenizing media contained a different concentration of sucrose (0.5, 0.75, 1.0 and 1.25 M.) and 0.1 M. phosphate. The homogenates were carried separately through the fractionating procedure. The fractions were washed in a medium containing 0.5 M. sucrose + 0.1 M. phosphate. The washed particulate fractions were suspended in a medium of 0.25 M. sucrose and 0.1 M. phosphate. The results in Table 28 do not establish conclusively that 1.0 molar is the best sucrose concentration to be used in the homogenizing medium. However, since the results with 1.0 M. sucrose were equal or better than those obtained at other concentrations, use of 1.0 M. sucrose in the homogenizing media was continued.

Table 28

Effect of Various Concentrations of Sucrose on the Activity of the Particulate Fraction

Additions	Sucrose conc. in homogenizing medium	Net μ atoms oxygen uptake 265 min.
50 μM. K-citrate	0.75	9.8
50 μM. K-citrate	1.00	16.1
50 μM. K-citrate	1.25	13.9
10 μM. K-succinate	0.50	12.8
10 μM. K-succinate	0.75	12.2
10 μM. K-succinate	1.00	13.5
10 μM. K-succinate	1.25	13.5

Additions were as follows: 0.1 ml. of 0.28 M. $MgSO_4$; 0.3 ml. of 0.033 M. ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN; 0.3 ml. of 0.5 M. K-glutamate; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate (pH 7.0) were added with the particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 20° C.

The sucrose concentration was varied in the flask. Citrate was used as the substrate in one experiment, and α-ketoglutarate was used as the substrate in the second experiment (Table 29).

Table 29

Effect of Sucrose on Citrate and α-Ketoglutarate Oxidation

Sucrose conc. in the flask	Citrate (μ atoms/200 min.)	α-Ketoglutarate (μ atoms/210 min.)
0.41 M.	7.7	4.7
0.51	7.9	4.7
0.61	7.9	5.3
0.71	7.7	5.4

Additions were as follows: 0.1 ml. of 0.28 M. $MgSO_4$; 0.3 ml. of 0.033 M. ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN; 0.3 ml. of 0.5 M. K-glutamate; 1.0 ml. of the particulate fraction; 0.2 ml. of 0.5 M. K-phosphate (pH 7.0). 100 μM. of K-phosphate were added with the particulate fraction. 50 μM. of the substrate were used. The liquid volume in each flask was made to 3.2 ml. with double distilled water. The experiment was carried out at 20° C. When α-ketoglutarate was used as the substrate, the K-glutamate was omitted and 0.1 ml. of 5.33×10^{-4} M. TPN was added.

Changes in the molar concentration of sucrose in this range had no effect on the activity of the preparation. The high initial concentration of sucrose was caused by the addition of one millimole of sucrose with the particulate fraction, which would give a 0.31 M. sucrose concentration in the flask.

5. Effect of Manganese Ions

Manganese has been reported to be necessary for citrate oxidation (1, p.1045; 43, pp.243-244). When half the magnesium ion concentration was replaced with

manganese ion an increase in oxygen uptake was noticed over that obtained when magnesium ion alone was used (Table 30; Figure 15).

Table 30

Influence of Manganese on Oxygen Uptake

Additions	Net μ atoms oxygen uptake 1 hr.	4 hrs.
10 μM. K-succinate + Mg^{++}	9.7	12.4
10 μM. K-succinate + Mg^{++} + Mn^{++}	11.0	15.6
50 μM. Na_3-citrate + Mg^{++}	8.9	19.1
50 μM. Na_3-citrate + Mg^{++} + Mn^{++}	10.6	25.8
50 μM. α-ketoglutarate + Mg^{++}	7.8	12.0
20 μM. K-malate + 20 μM. Na-pyruvate + Mg^{++}	5.2	12.1

Additions were as follows: 0.1 ml. of 0.28 M. $MgSO_4$ or 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 20° C.

A second experiment was made for the purpose of determining the concentration of manganese which would cause a maximum activity of the particulate preparation. Citrate, at a concentration of 30 μ moles per flask, was used as substrate (Table 31; Figure 16).

Fig. 15. The Effect of Manganese Ion on the Oxidation of Succinate and Citrate by an Insoluble Enzyme Fraction from Bean Seedlings

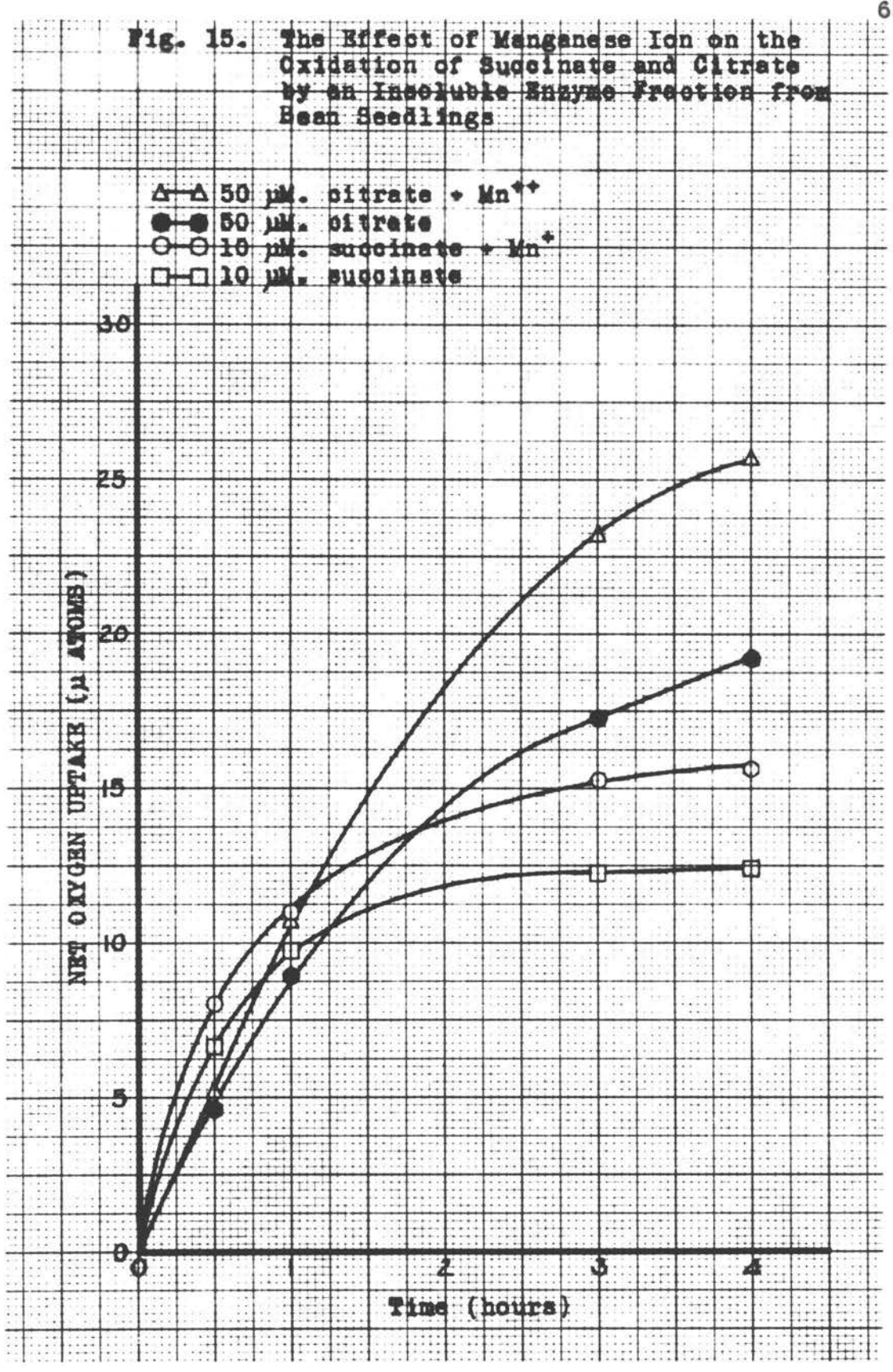


Table 31

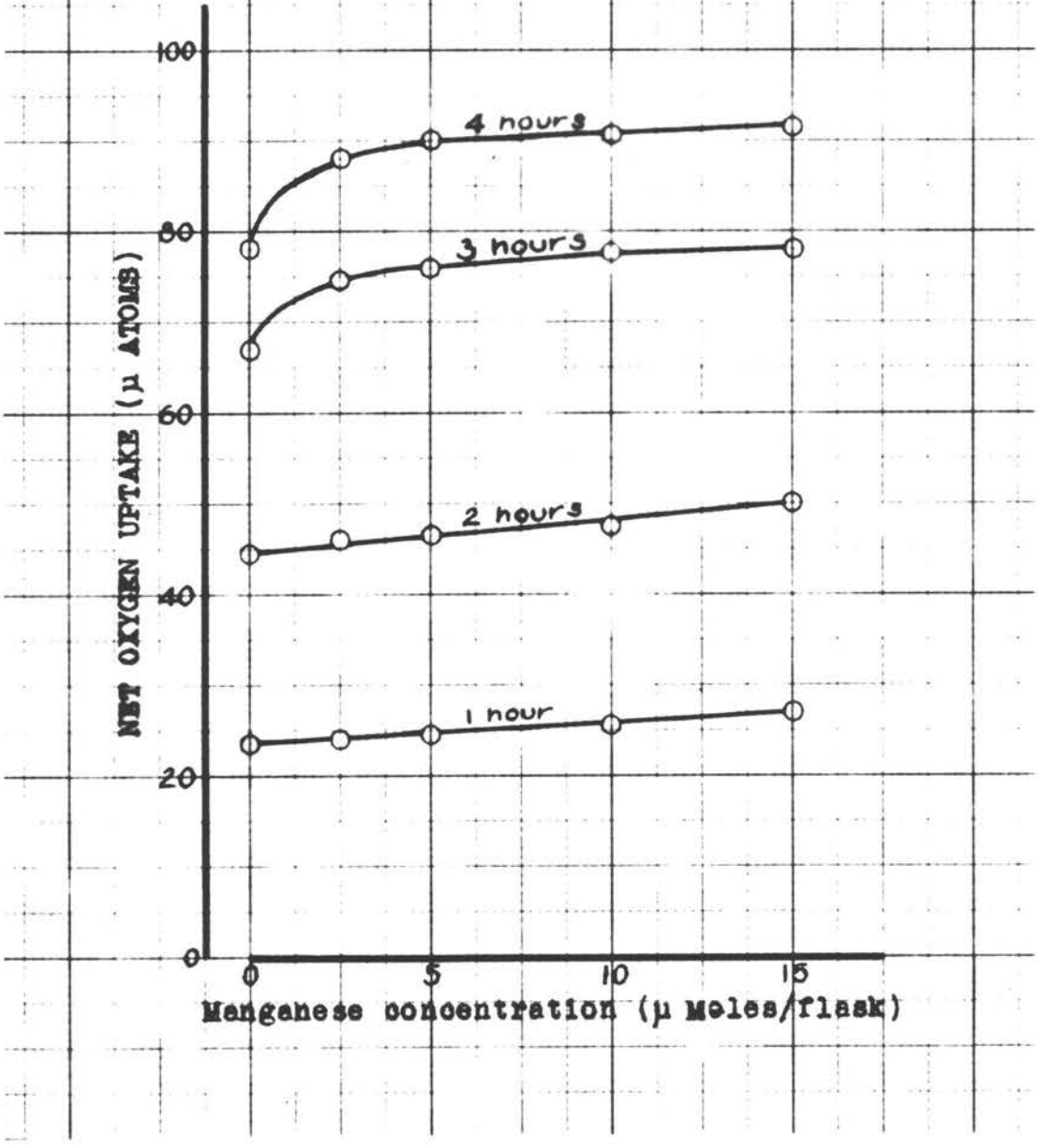
Effect of Manganese on the Activity of the Particulate Fraction

Mn^{++} concentrations	Net μ atoms oxygen uptake 1 hr.	6 hrs.
0.0 μM.	23.6	78.1
2.5	24.0	88.1
5.0	24.4	90.0
10.0	25.5	90.5
15.0	27.1	91.4

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4- ATP (pH 7.1); 0.3 ml. of 0.1 M. Na_3-citrate; 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

Manganese was shown to increase the oxygen uptake when either succinate or citrate was used as the substrate (Table 30). The increase in oxygen uptake was greater with citrate than with succinate. While a high oxygen uptake may be obtained without added manganese, addition of this element does increase the activity of the preparation. It is possible that manganese is tightly bound to one or more enzymes and that it is not entirely lost during the preparation. If this is the case, only a slight stimulation would be expected upon adding more manganese. The

Fig. 16. The Effect of Various Concentrations of Manganese on Oxidation of Citrate by an Insoluble Enzyme Fraction from Bean Seedlings



optimal amount of added manganese was 5 μM. per flask since larger additions caused no appreciable increase in oxygen uptake (Table 31).

6. Effect of ATP

ATP has been found to be a requirement or a desirable additive in nearly all of the preparations of tissue homogenates and particulate fractions in which Krebs cycle activity has been demonstrated. To determine the optimal concentration of ATP this co-factor was added to the flasks at various molar concentrations (Table 32; Figure 17). Twenty μ moles of pyruvate and 5 μ moles of succinate were used as the substrates. The concentration necessary for the maximum oxygen uptake was found to be 3.09×10^{-3} M. Na_4-ATP.

Table 32

Effect of Different Concentrations of ATP on the Activity of the Particulate Fraction

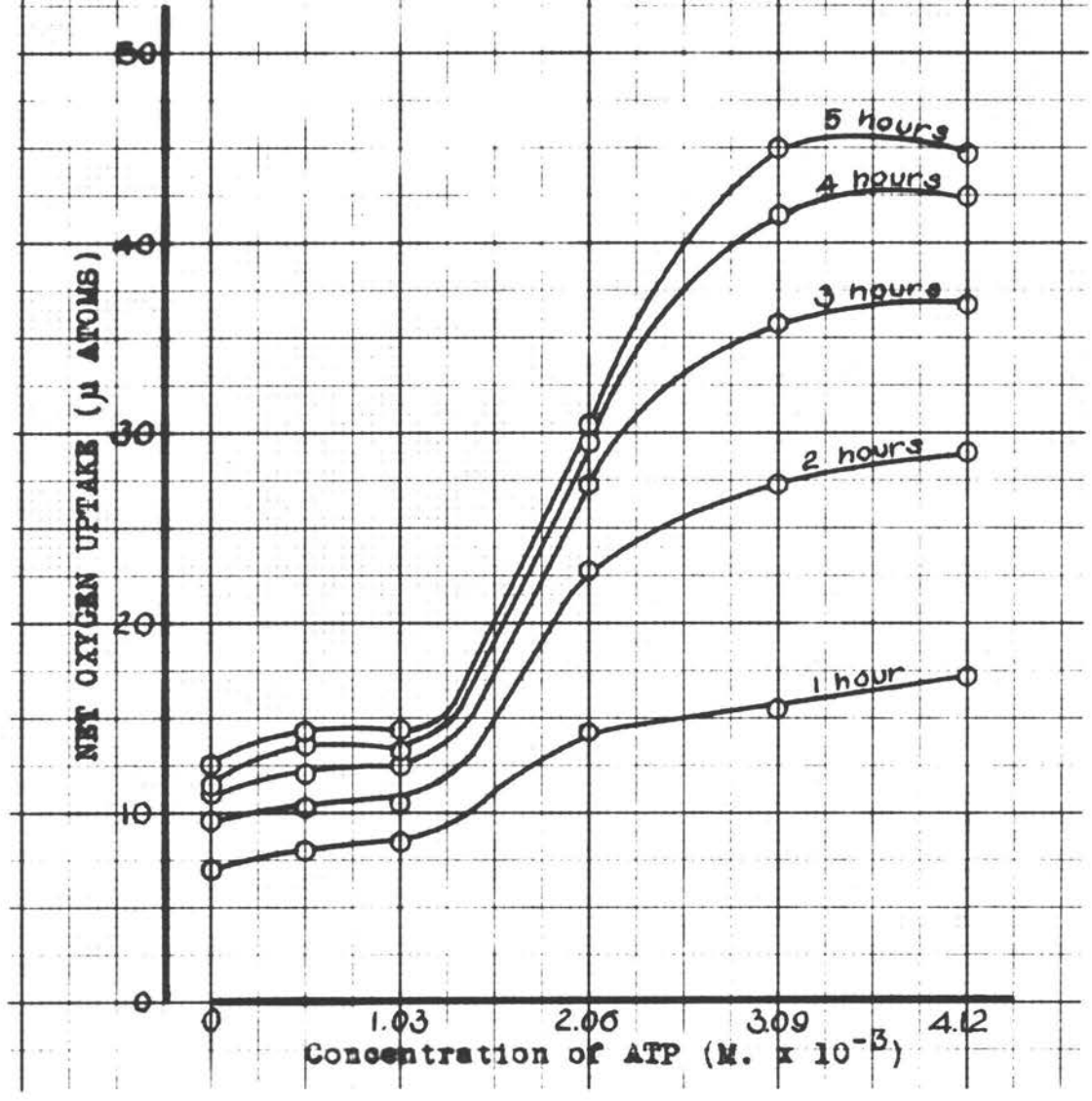
ATP concentration	Net μ atoms oxygen uptake 1 hr.	5 hrs.
0.0 M.	7.0	12.6
0.52×10^{-3}	7.9	14.3
1.03×10^{-3}	8.6	14.2
2.06×10^{-3}	14.2	30.5
3.09×10^{-3}	15.6	45.1
4.13×10^{-3}	17.2	44.8

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 1.56×10^{-3} M. MnSO ; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.2 ml. of 0.025 M. K-succinate; 0.1 ml. of 0.2 M. Na-pyruvate; 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

7. Addition of Various Coenzymes

A number of experiments were carried out to demonstrate the need for DPN, TPN, cocarboxylase and Co A. This was found to be difficult because coenzyme preparations completely free of other coenzymes were not available. The DPN was a 90% pure product and was probably free of other coenzymes. The cocarboxylase was highly purified. The source of Co A was a liver concentrate which contained >7% DPN, >4% TPN and >10 Lipmann

Fig. 17. The Effect of Adenosinetriphosphate on the Oxidation of 5 μM. of Succinate plus 20 μM. of Pyruvate by an Insoluble Enzyme Fraction from Bean Seedlings



units of Co A per milligram. The TPN preparation contained only 10% TPN, and 8% DPN was present as an impurity.

When DPN, TPN, cocarboxylase and liver concentrate were omitted, the oxygen uptake was greatly diminished (Table 33; Figure 18). This indicated that one or more of the co-factors was necessary.

Table 33

Omission of the Coenzymes

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
5 μM. K-succinate + coenzymes	9.0	22.8
5 μM. K-succinate - coenzymes	5.0	7.0

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38 M. $\times 10^{-4}$ TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of liver concentrate (10 mg./ml.); 0.1 ml. of 0.08 M. glutathione; 2.0 ml. of the particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. 200 μM. of K-phosphate were added with the particulate fraction. The experiment was carried out at 30° C.

In a second experiment the coenzymes were omitted individually and the results compared with those for the complete system (Table 34; Figure 19).

Fig. 18. The Effect of Coenzymes on the Oxidation of 5 μM. of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

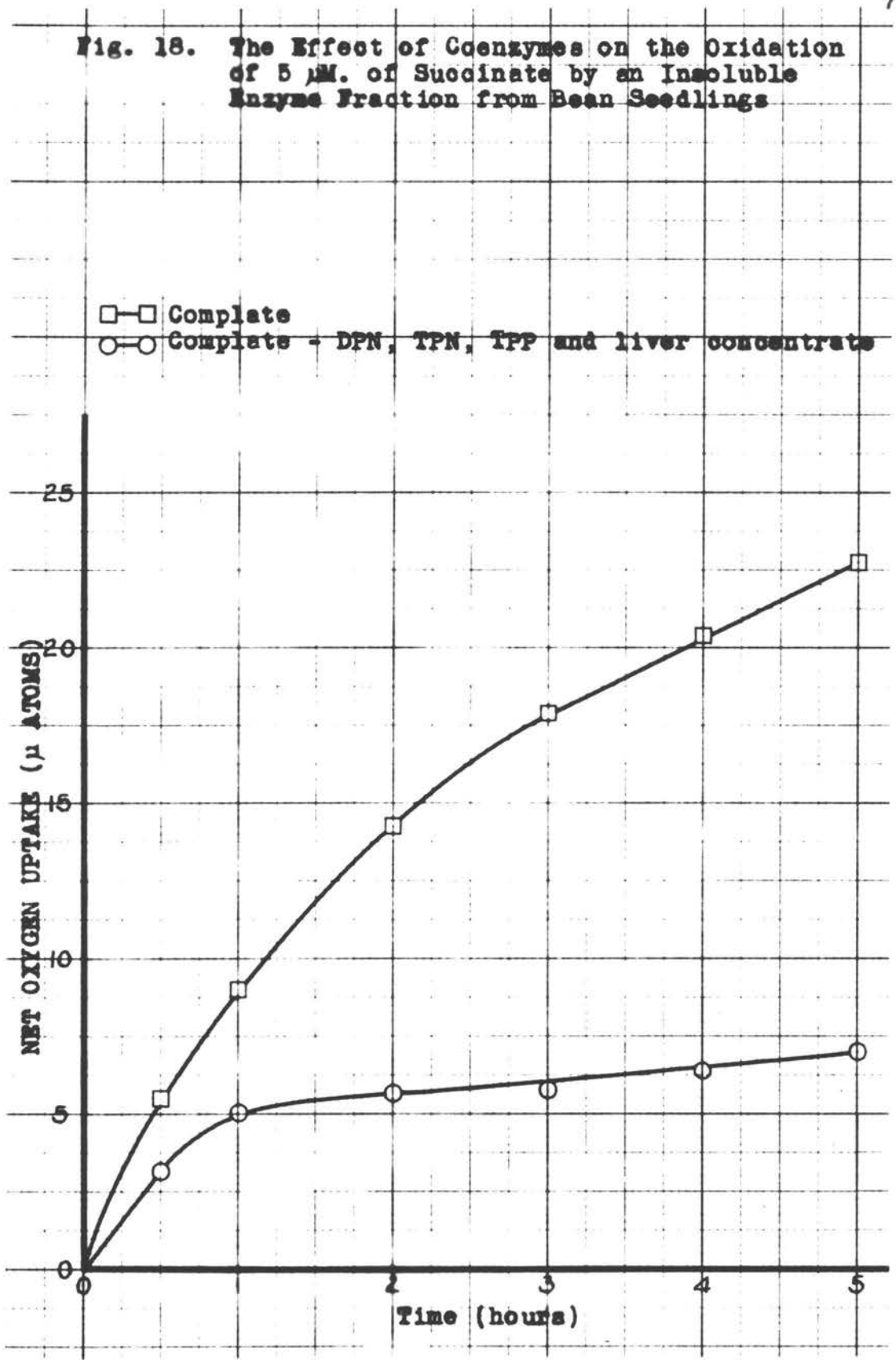


Table 34

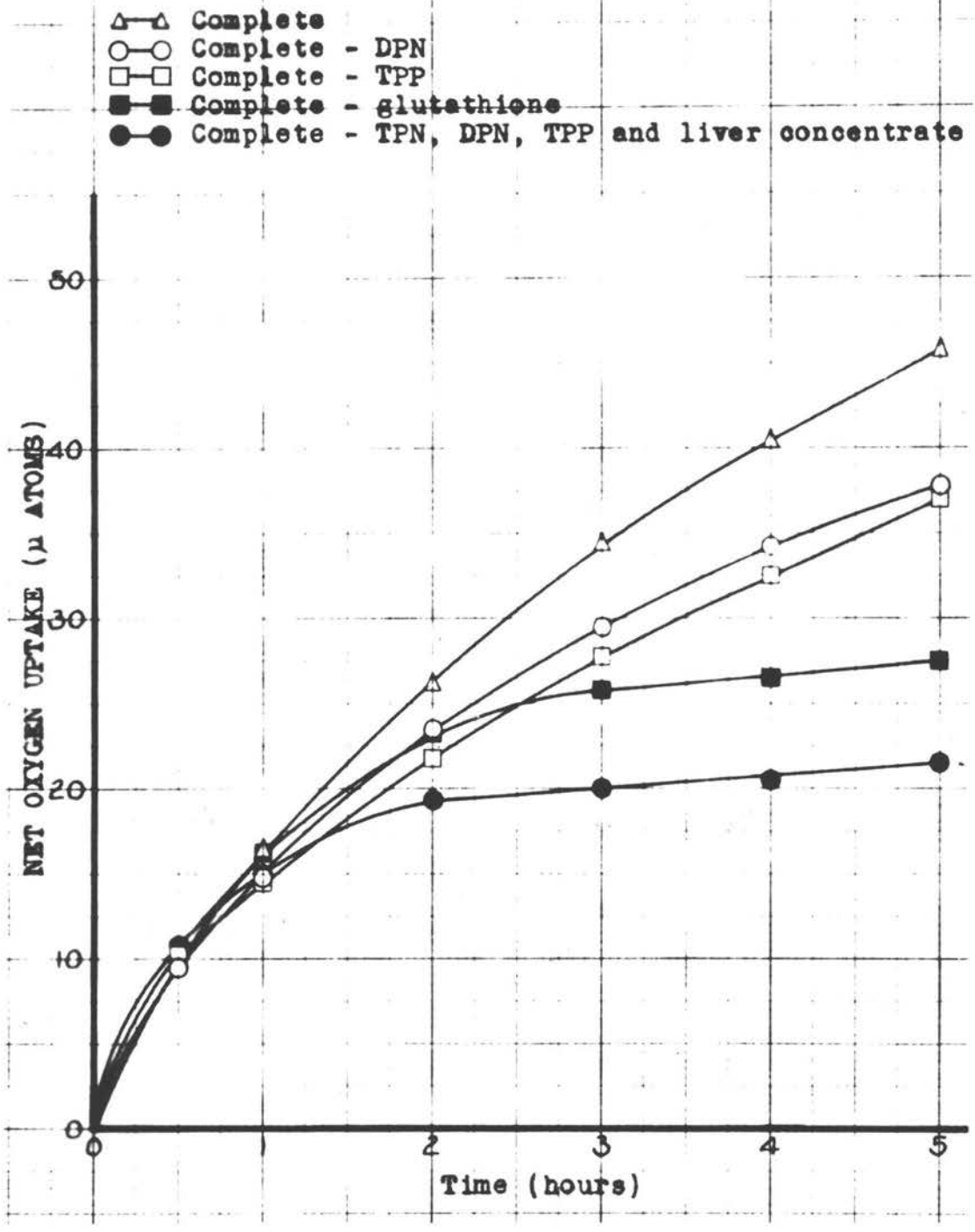
Coenzyme Activity

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
Complete	16.5	45.7
Complete- liver concentrate	16.6	43.5
Complete- DPN	14.7	37.7
Complete- TPN	16.5	44.6
Complete- TPP	14.5	37.0
Complete- TPN, DPN, TPP and liver concentrate	14.9	21.4
Complete- glutathione	16.2	27.4

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of liver concentrate (10 mg./ml.); 0.1 ml. of 0.08 glutathione; 0.1 ml. of 5.44×10^{-3} M. DPN; 0.1 ml. of 1.08×10^{-3} M. TPN; 0.1 ml. of 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.1 ml. of 0.1 M. K-succinate; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

The omission of either DPN or cocarboxylase caused the greatest decrease in activity, indicating that these co-factors are functional. When all of the coenzymes were omitted there was a decrease in oxygen uptake similar to that shown in Table 33. Glutathione was again shown to be necessary (Tables 22 and 23). The difference between the complete system and that in which the TPN preparation was omitted was very small, but in this case quite a large amount of TPN was placed in the flask with the addition of

Fig. 19. The Effect of Coenzymes on the Oxidation of 10 μM. of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings



1 mg. of liver concentrate. The removal of the liver concentrate did not affect the preparation significantly.

Using another preparation the results were found to differ from those given above (Table 35). However, the need for coenzymes might be different from one preparation to another because of variations in the preparative procedure.

Table 35

Coenzyme Activity

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
Complete	9.1	22.4
Complete- liver concentrate	9.7	25.2
Complete- DPN	5.9	14.5
Complete- TPN	11.2	28.2
Complete- TPP	9.2	23.3
Complete- liver concentrate, DPN, TPN and TPP	7.8	11.4

Additions and conditions were as in Table 34 except the concentration of TPN added was 5.33×10^{-4} M.

The coenzymes were studied in the absence of the liver concentrate (Tables 36 and 37; Figures 20 and 21). When either TPN or DPN was omitted the oxygen uptake was less than that obtained with the complete system. An even lower rate of oxygen uptake was obtained when both TPN and DPN were omitted. The TPN contained 8% DPN. When TPN was omitted there was also an omission of 32 μ grams of DPN.

It is doubtful that this reduction in the amount of DPN could cause the decrease in oxygen uptake observed. Added cocarboxylase appeared to be unnecessary in the experiment reported in Table 36.

Table 36

Coenzyme Activity in the Absence of Liver Concentrate

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
Complete	15.9	37.6
Complete- DPN	15.5	30.4
Complete- TPN	15.8	32.9
Complete- TPP	17.1	37.9

Additions and conditions were as in Table 35, except for the omission of the liver concentrate.

Table 37

Influence of TPN and DPN on Oxygen Uptake

Additions	Net μ atoms oxygen uptake 1 hr.	5 hrs.
Complete	17.9	43.5
Complete- DPN	15.5	32.1
Complete- TPN	15.1	35.0
Complete- TPN-DPN	14.4	22.0

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of 0.1 M. K-succinate; 0.1 ml. of 5.44×10^{-3} M. DPN; 0.1 ml. of 5.33×10^{-4} M. TPN; 0.1 ml. of 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 0.1 ml. of terramycin (0.16 mg./ml.); 2.0 ml. of particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C. 200 μM. of K-phosphate were added with the particulate fraction.

The liver concentrate was tested for activity at 2 concentrations (Table 38). Since Co A was present in such small amounts in the liver concentrate, the quantity added was increased ten-fold. No effect was observed with this increased concentration.

Fig. 20. The Effect of DPN and TPN on the Oxidation of 10 μM. of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

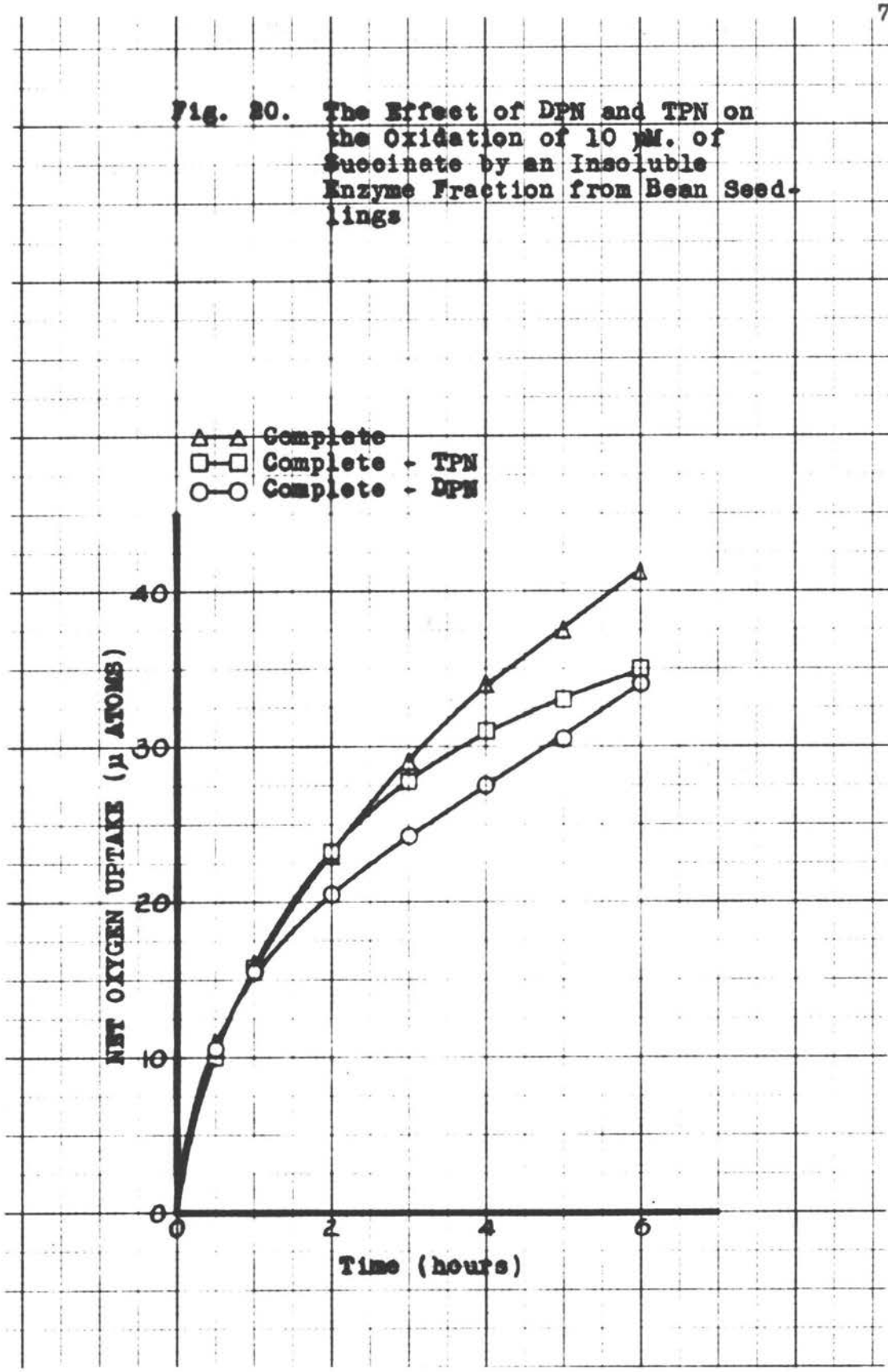


Fig. 21. The Effect of DPN and TPN on the Oxidation of 10 μM. of Succinate by an Insoluble Enzyme Fraction from Bean Seedlings

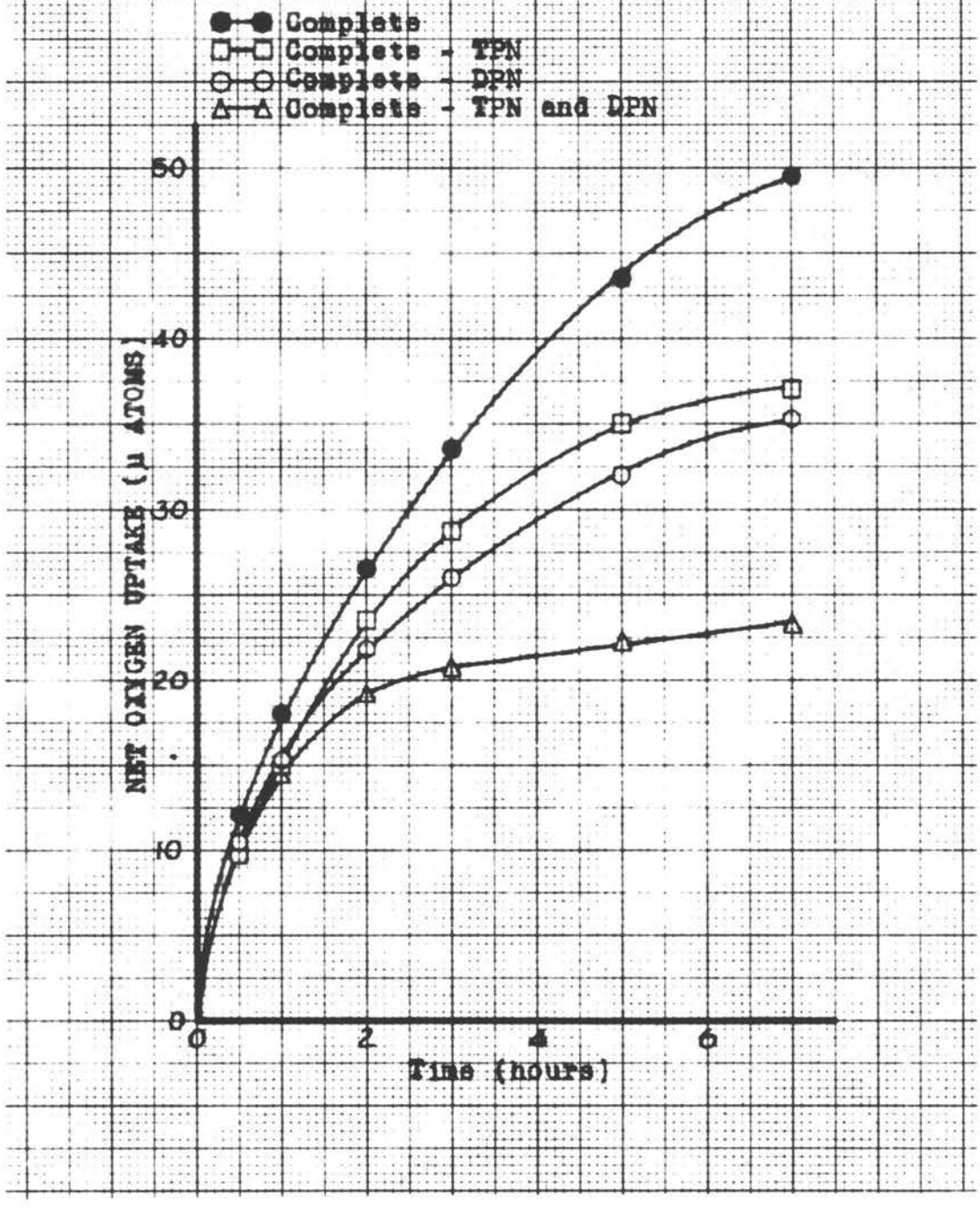


Table 38

Effect of Liver Concentrate on Oxygen Uptake

Additions	Net μ atoms oxygen uptake 1 hr.	4 hrs.
Liver concentrate 1 mg./flask	15.4	34.7
Liver concentrate 10 mg./flask	15.2	34.9

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.5 M. Na-pyruvate; 0.05 ml. of 0.1 M. K-succinate; 0.1 ml. of 0.08 M. glutathione; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

The coenzyme requirements of the particulate fraction were not completely resolved. DPN was shown to be a requirement for maximum activity of the preparation. TPN was found to increase oxygen uptake, but the possibility was not completely excluded that the effect was due to the addition of a small amount of DPN contaminating the TPN preparation. Liver concentrate, which was used as a source of Co A caused no significant response. It might well be that a response could have been obtained with a Co A preparation of higher purity. Cocarboxylase was shown to be active in some instances.

8. Bacterial Contamination in the Warburg Flasks

When the Warburg runs were continued beyond 5 hours a sharp rise in "endogenous oxidation" was observed. This suggested that a large bacterial contamination was obtained from the bean plants and was carried into the particulate fraction. Penicillin was tested, but failed to lower the bacterial count or the endogenous respiration.

Terramycin was next tested in the hope that this antibiotic might inhibit the increase in bacterial count and "endogenous metabolism," without interfering with substrate oxidation by the particulates from the seedlings (Tables 39 and 40; Figure 22). No apparent inhibition of substrate oxidation, by the terramycin, was observed, and even more important is the fact that the bacteria present apparently did not utilize succinate as a substrate. If the bacteria were oxidizing the succinate, the flasks which did not contain the terramycin (and, therefore, had a higher concentration of bacteria) would have had a higher rate of succinate oxidation than those with terramycin. The "endogenous oxidation" and the bacterial count were decreased by terramycin (Table 40; Figure 22).

Table 39

Effect of Terramycin on Oxidation of Succinate

Terramycin concentration	Net μ atoms oxygen uptake 1 hr.	3 hrs.	7 hrs.
0.0 ppm.	18.0	38.9	50.8
1.0	22.8	44.4	59.4
5.0	21.4	41.6	53.8
50.0	16.2	38.9	52.8

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$; 0.14 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of 0.1 M. K-succinate; 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

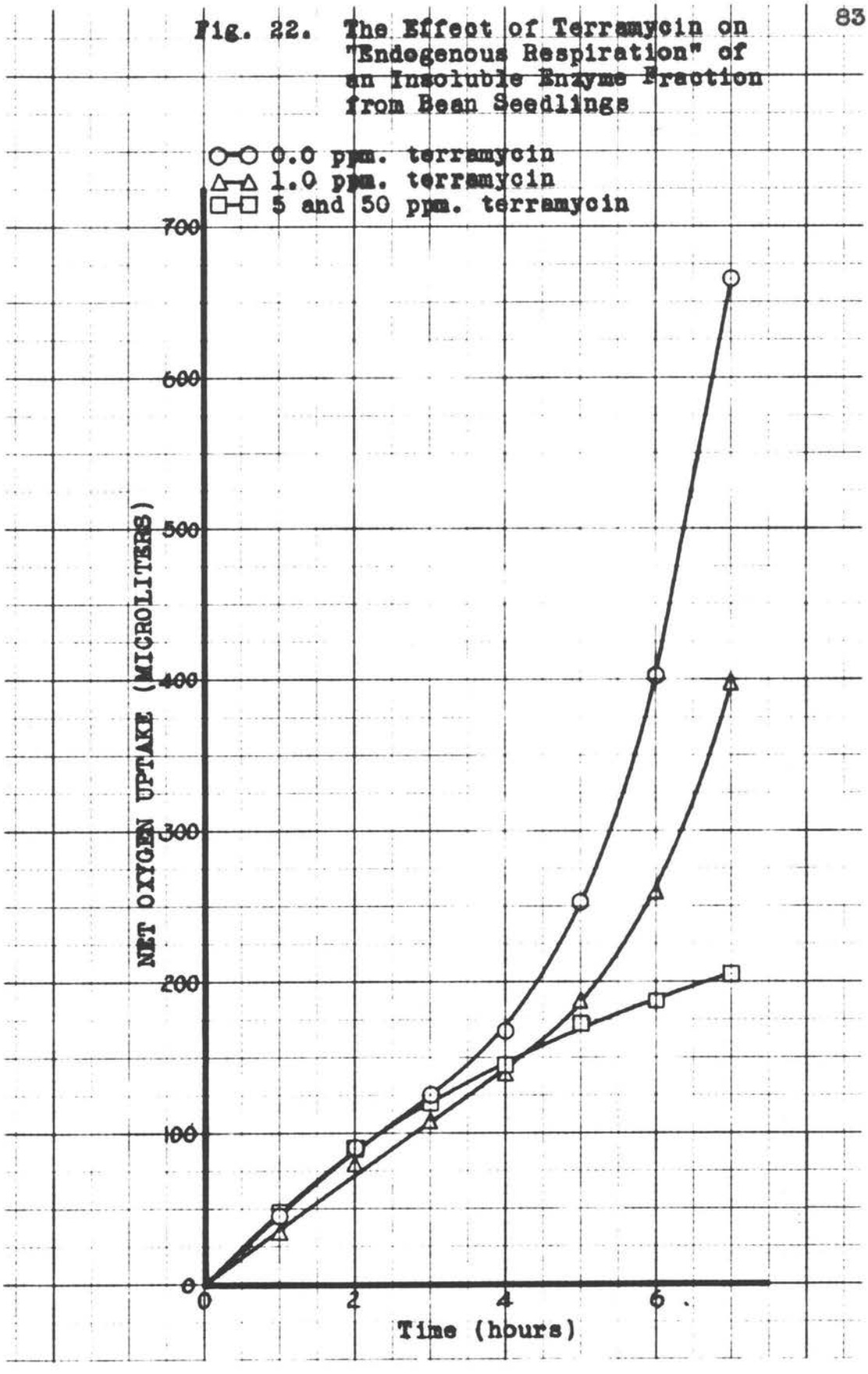
Table 40

Effect of Terramycin on "Endogenous Oxidation" and Bacterial Count

Terramycin conc.	Endogenous oxidation 1st hr.	7th hr.	Bacteria count per flask 7th hr.
0.0	44	264	960×10^6
1.0	49	139	461×10^6
5.0	46	19	36.8×10^6
50.0	35	15	16.3×10^6

23.8×10^6 bacteria were added with the 2.0 ml. of particulate fraction.

Fig. 22. The Effect of Terramycin on "Endogenous Respiration" of an Insoluble Enzyme Fraction from Bean Seedlings



9. Extent of Substrate Oxidation

A number of experiments were continued for 6 to 7 hours in an attempt to obtain the oxygen uptake theoretically required for complete oxidation of the substrate. The theoretical value was approached in many cases but was never attained. The results of these experiments are tabulated in Table 41.

Although the oxygen uptake was not found to equal the theoretical amount, 60 - 75% of theoretical oxidation may be considered sufficiently high to conclude that all of the enzymes for the Krebs cycle were active in this preparation. Evidence for pyruvate oxidation is also shown in Table 41. When succinate and pyruvate were added together the theoretical value for the succinate oxidation was greatly exceeded.

In one turn of the Krebs cycle, 50% of a given amount of succinate would be oxidized and 50% of the theoretical oxygen uptake could be expected. Two turns of the cycle would result in oxidation of a total of three-fourths of the succinate and 75% of the theoretical oxygen uptake would be obtained. Since some of the succinate is undoubtedly left unchanged or only partially oxidized, it may be assumed that when 75% of the theoretical oxygen uptake is obtained several turns of the cycle have occurred.

Table 41

A Comparison of Oxygen Uptake with the Theoretical Values

Substrate	Time	Net μ atoms oxygen uptake	Theoretical value	% Theoretical
2 μM. K-succinate	7 hrs.	9.4	14.0	67.1
4 μM. K-succinate	7	23.6	28.0	84.4
5 μM. K-succinate	6	26.0	35.0	74.3
5 μM. K-succinate	7	21.8	35.0	62.3
10 μM. K-succinate	7	54.6	70.0	78.1
10 μM. K-succinate	7	52.6	70.0	75.2
10 μM. K-succinate	7	51.4	70.0	73.4
10 μM. K-succinate	7	53.4	70.0	76.3
10 μM. K-succinate	7	49.4	70.0	70.6
2 μM. K-succinate + 4 μM. Na-pyruvate	7	24.7	34.0	72.7
4 μM. K-succinate + 8 μM. Na-pyruvate	7	46.6	68.0	68.7

10. First-Hour Rate of Oxygen Uptake

The $Q_O(N)$ was calculated from a number of experiments and the values are recorded in Table 42. The values of $Q_O(N)$ that were obtained with 40 and 50 μM. of substrate compare favorably with calculations by Brody and Bain (8, p.689) for brain mitochondria. The $Q_O(N)$ values of the bean seedling preparation are over three times those reported by Millerd (39, p.157) for mung beans, even when the comparison is made with Millerd's data for a higher substrate concentration.

11. Accumulation of Succinic Acid

Krebs and Eggleston (30, pp.448-449) have used malonate to demonstrate the conversion of fumarate to succinate by an oxidative pathway in muscle tissue. Malonate inhibits the forward and reverse reactions of succinic acid to fumaric acid (31, p.154). The aerobic conversion of fumarate to succinate in the presence of malonate was taken as evidence for a pathway of oxidation which is now known as the Krebs cycle (30, p.450).

Using the particulate fraction, and citrate and fumarate plus pyruvate as substrates, an attempt was made to demonstrate succinate accumulation in the presence of malonate. The details of the succinic acid analysis were given under Methods.

Table 42

First-Hour Rate of Oxygen Uptake by Particles from Bean Seedlings

Substrate	Flask conc.	µl./hour/mg.N
5 µM. succinate	1.56×10^{-3} M.	110
	1.56×10^{-3} M.	75
10 µM. succinate	3.12×10^{-3} M.	238
	3.12×10^{-3} M.	191
	3.12×10^{-3} M.	260
	3.12×10^{-3} M.	206
	3.12×10^{-3} M.	220*
20 µM. succinate	6.25×10^{-3} M.	364*
40 µM. succinate	1.25×10^{-2} M.	459*
50 µM. succinate	1.56×10^{-2} M.	565
30 µM. succinate	9.4×10^{-3} M.	308
30 µM. citrate	9.4×10^{-3} M.	338

*These were taken from a single experiment. The remainder are values from different preparations.

The effect of malonate on the oxygen uptake is shown in Table 43 and Figures 23 and 24. Citrate and succinate were used as the substrates. Citrate oxidation was affected by malonate which is said to be a specific inhibitor of succinic dehydrogenase (31, p.154). This indicates that citrate was oxidized by reactions involving succinate as an intermediate. The malonate concentration of 0.033 M. was used in the succinate accumulation studies since succinate oxidation was inhibited about 88.4%.

Table 43

Effect of Malonate on Citrate and Succinate Oxidation

Substrate	Malonate conc.	Net μ atoms oxygen uptake 6 hrs.	Malonate / No malonate
30 μM. Na_3-citrate	0.00 M.	69.7	----
30 μM. Na_3-citrate	0.01	42.9	0.615
30 μM. Na_3-citrate	0.033	25.4	0.364
30 μM. Na_3-citrate	0.1	7.9	0.113
30 μM. K-succinate	0.00	61.2	----
30 μM. K-succinate	0.01	23.8	0.389
30 μM. K-succinate	0.033	7.1	0.116
30 μM. K-succinate	0.1	0.4	0.007

Additions were as follows: 0.1 ml. of 0.14 M. $MgSO_4$ + 0.14 M. $MnSO_4$; 0.1 ml. of cytochrome c (2.4 mg./ml.); 0.3 ml. of 0.033 M. Na_4-ATP (pH 7.1); 0.1 ml. of 5.44×10^{-3} M. DPN + 5.38×10^{-4} M. TPN + 4.18×10^{-3} M. cocarboxylase; 0.1 ml. of 0.08 M. glutathione; 2.0 ml. of the particulate fraction. 200 μM. of K-phosphate were added with the particulate fraction. The liquid volume in each flask was made to a 3.2 ml. with 0.25 M. sucrose. The experiment was carried out at 30° C.

Succinate was found to be recovered almost completely by continuous ether extraction for 8 hours (Table 44).

Fig. 23. Malonate Inhibition of Succinate Oxidation by an Insoluble Enzyme Fraction from Bean Seedlings

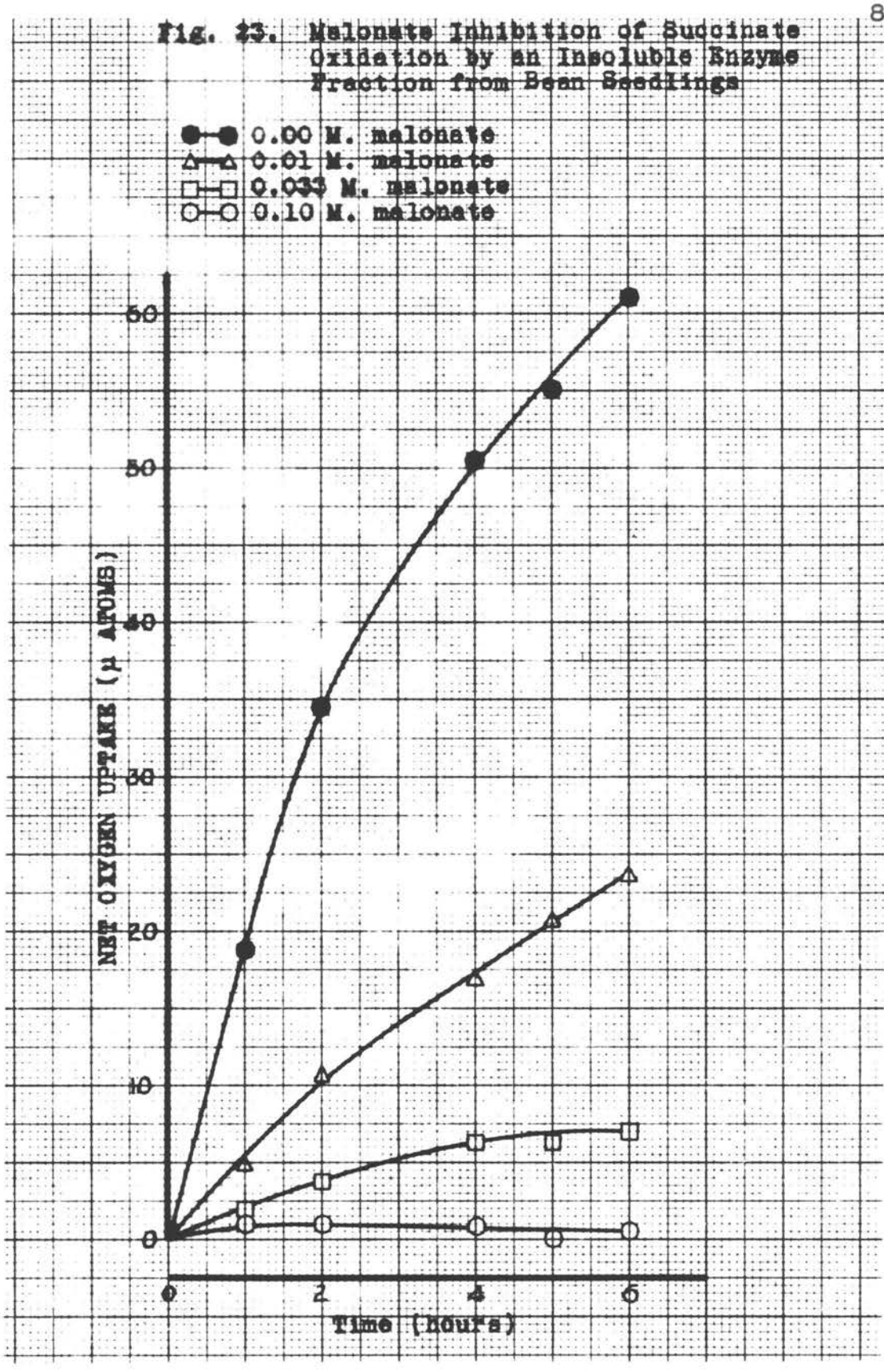


Fig. 24. Malonate Inhibition of Citrate Oxidation by an Insoluble Enzyme Fraction from Bean Seedlings

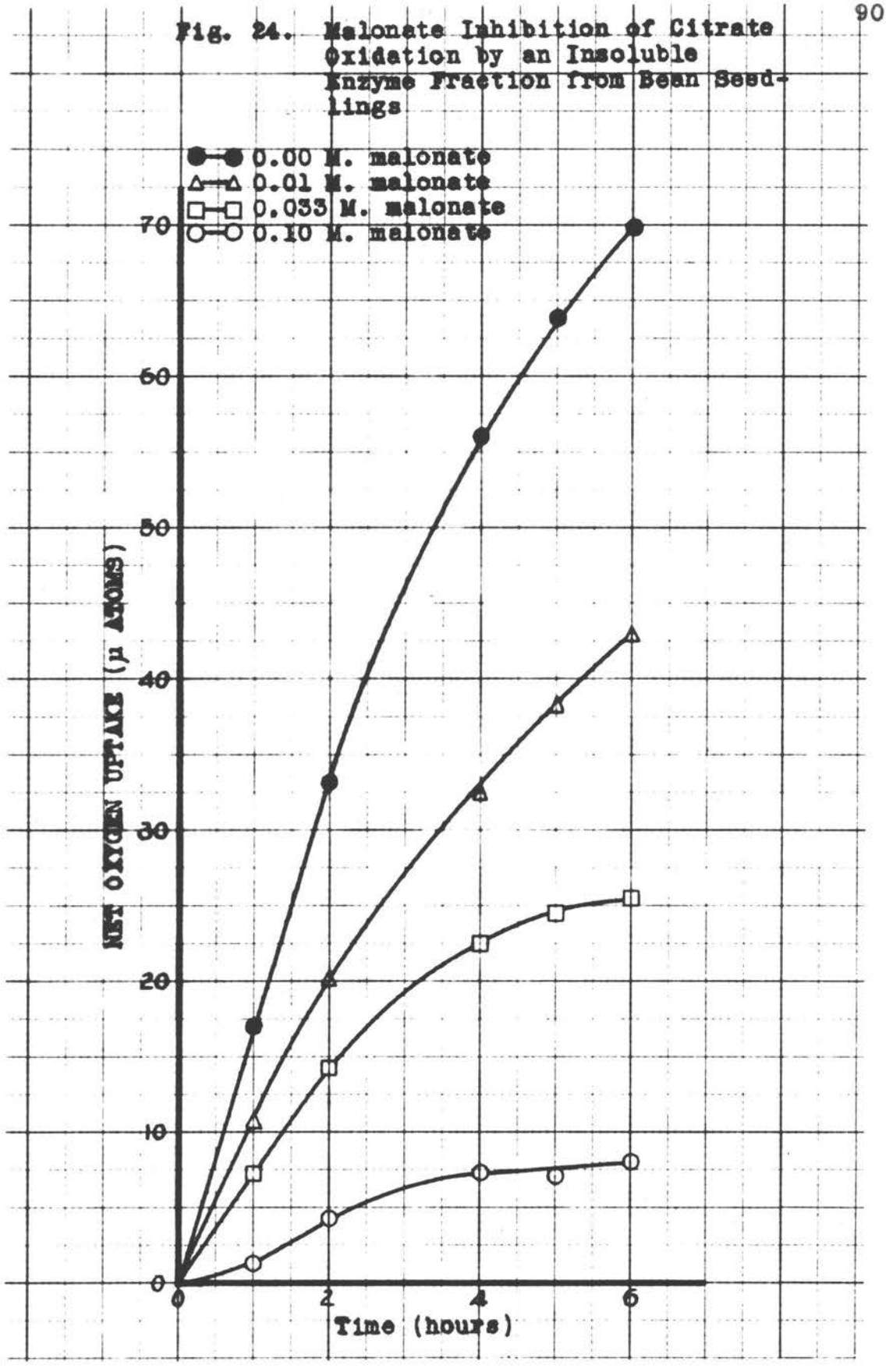


Table 44

Recovery of Succinate by Ether Extraction

Amount succinate added	Time of ether extraction	µM. succinate recovered	% Recovery (corrected)
20 µM.	8 hrs.	19.2	104.5
20	4	18.2	98.4
20	8	18.4	99.5
20	8	18.2	98.4
10	(not extracted)	9.25*	

*Average of 9 separate analyses.

When 10 µM. of succinic acid were analyzed by means of the pig heart succinic dehydrogenase preparation, an average of 92.5% of the theoretical oxygen uptake for conversion of succinate to fumarate was obtained (Table 44). No oxygen uptake was observed when malate and citrate were added to the succinic dehydrogenase preparation.

Table 45 gives the data for the accumulation of succinate from citrate. When only malonate was added to the particulate fraction, the amount of succinate that accumulated was too low for accurate determination. The succinate accumulated from the citrate represents the conversion of citrate to succinate. Though some succinate did accumulate when citrate was present and malonate was absent, the addition of the inhibitor did increase the amount of succinate accumulated. Correlation between

oxygen uptake data and succinate accumulation is difficult since the malonate did not completely inhibit succinate oxidation.

Table 45

Accumulation of Succinic Acid from Citrate

Additions	Net μ atoms oxygen uptake	Time	μM succinate
	(Experiment 1)		
150 μM. Na_3-citrate + malonate	99.2	6 hrs.	28.1
150 μM. Na_3-citrate	97.3	6	9.9
150 μM. Na_3-citrate	103.0	6	9.9
	(Experiment 2)		
150 μM. Na_3-citrate + malonate	114.0	6-1/4	33.4
150 μM. Na_3-citrate + malonate	108.0	6-1/4	33.1
150 μM. Na_3-citrate	159	6-1/4	17.4
150 μM. Na_3-citrate	159	6-1/4	13.9

When fumarate and pyruvate were used as the substrates, succinate accumulated; however, the presence of malonate did not seem to increase the amount of succinate (Table 46). The oxygen uptake was inhibited by malonate and this inhibition was appreciable even during the first hour. The flasks containing malonate showed only 63% as

much oxygen uptake during the first hour as did those without malonate. The data do not provide an explanation for the fact that the malonate inhibited oxygen uptake but had no effect on succinate accumulation. The important aspect of this experiment was that succinate was formed from fumarate under aerobic conditions and in the presence of malonate. Under these conditions the back reaction, fumarate⇌ succinate, may be assumed to be inoperative.

Table 46

Accumulation of Succinic Acid from Fumarate and Pyruvate

Additions	Net μ atoms oxygen uptake	Time	μM succinate
(Experiment 1)			
100 μM. K-fumarate + 100 μM. Na-pyruvate + K-malonate	34.1	7 hrs.	5.7
100 μM. K-fumarate + 100 μM. Na-pyruvate + K-malonate	40.6	7	5.8
100 μM. K-fumarate + 100 μM. Na-pyruvate	63.6	7	5.8
100 μM. K-fumarate + 100 μM. Na-pyruvate	68.8	7	5.1
(Experiment 2)			
100 μM. K-fumarate + 100 μM. Na-pyruvate + K-malonate	52.7	6-1/2	6.7
100 μM. K-fumarate + 100 μM. Na-pyruvate + K-malonate	50.8	6-1/2	6.2
100 μM. K-fumarate + 100 μM. Na-pyruvate	75.1	6-1/2	5.8

Discussion

The particulate fraction prepared as described in Part II was able to oxidize citrate, succinate, pyruvate, fumarate, α-ketoglutarate, malate and lactate. Acetate was not oxidized. The oxidation of pyruvate occurred only in the presence of some other Krebs cycle intermediate. Oxidation of all of these compounds by the enzyme fraction, and the fact that all of them are intermediates in the Krebs cycle, may be considered good evidence that the Krebs cycle is operative in Black Valentine bean seedlings. Although no attempt was made to identify the particles, the enzymatic activity in this fraction is similar to that ascribed to mitochondria in animal tissue.

Citrate was oxidized for 5 hours (Figure 5) with only a very slow decrease in rate. An early decrease in rate occurred with succinate. The decrease in rate when succinate is the substrate is discussed in Section 1.

When the different substrates were compared (Table 21), pyruvate had the lowest oxygen uptake. The condensation step is probably the limiting one in the series of reactions carried out by the insoluble enzyme fraction from bean seedlings. Attainment of a high percentage of the theoretical oxygen uptake with each of the substrates provides further evidence that the enzymes of the Krebs cycle were operative.

Glutathione stimulated oxygen uptake when succinate was used as the substrate. Before glutathione was used with the enzyme preparation, pyruvate oxidation could not be demonstrated. Figure 12 shows a sharp break in the rate of oxygen uptake when succinate was used as the substrate and glutathione was absent. This could indicate a break at the condensation step in the Krebs cycle. This break in the curve did not occur when glutathione was present. The low effective concentration of glutathione indicates a catalytic role. Glutathione may have an active role as a coenzyme in the condensation reaction.

The idea that glutathione may function as a coenzyme is not new. Glutathione has been demonstrated as a prosthetic group for glyceradehyde-3-phosphate dehydrogenase by Krimsky and Racker (32, p.729). Cavallini (9, pp.4-5) reported oxidative decarboxylation of pyruvic acid to acetate in the presence of glutathione and the cytochrome c-cytochrome oxidase system. Further, a glutathione reductase linked with TPN was found in wheat germ by Conn and Vennesland (11, p.27) and in peas by Mapson and Goddard (38, p.601).

Phosphate was found to be a necessary component of the homogenizing medium if citrate was to be oxidized by the particulate fraction isolated, but its presence during the preparation did not have a large effect on succinate

oxidation. The function of the phosphate in the homogenizing medium is not known.

When the phosphate concentration in the reaction medium is lowered (Figure 14), the activity of the enzyme fraction is greatly decreased. Still (54, p.281) and Koepsell and Johnson (24, p.383) showed a requirement for phosphate in the bacterial pyruvic acid oxidase. Ochoa (44, p.91) showed the need for phosphate in α-ketoglutaric acid dehydrogenase obtained from muscle tissue. Phosphate seems to be a necessary co-factor for Krebs cycle oxidation in bean seedlings.

Sucrose in the homogenizing medium appeared to be necessary for a preparation which would oxidize citrate. One molar was found to give the optimal activity with citrate as the substrate. Sucrose probably provides a proper molar concentration so that the particles remain intact and retain full enzymatic activity.

Manganese ions gave an increased oxygen uptake when either citrate or succinate was used as the substrate. Ochoa and Weisz-Tabori (45, pp.124-127) showed that the decarboxylation of oxalsuccinic and oxalacetic acids required manganese and that magnesium was ineffective at similar concentrations. Therefore, two points in the cycle are dependent on manganese. The activation of the oxalacetate carboxylase by manganese was shown by Speck

(52, p.323) in a preparation from parsley roots. Thus it is reasonable to assume that manganese ions are necessary for the activity of enzymes in the preparation from bean seedlings.

ATP was found to be necessary for full activity in the particulate enzyme fraction from bean seedlings. The addition of ATP has been necessary with most preparations of this type. This would indicate that ATP is easily dissociated from the particles in such preparations.

DPN was found to be required as a coenzyme for this enzyme fraction. DPN is known in animal tissue to be a required coenzyme for the enzymatic oxidation of malate to oxalacetate and pyruvate to acetate.

TPN was found to increase oxygen uptake by the fraction from bean seedlings. TPN has been shown to be required for the conversion of citrate to α-ketoglutarate (1, p.1044; 43, pp.243-244).

It is very fortunate that this enzyme preparation demonstrated the requirement for so many co-factors. The requirements for ATP, Mn, DPN, TPN and phosphate are all in accord with those known for animal tissue, but glutathione as a requirement for Krebs cycle enzyme activity has not been discussed in the literature. With the evidence of other workers cited above, and from the results presented in this report, it is reasonable to consider

that glutathione may act as a co-factor for Krebs cycle enzymes in the plant preparation.

The problem of bacterial contamination was considered to be a serious one. The difficulty arose from the fact that the contamination from the bean seedlings was concentrated in the enzyme fraction studied. Terramycin was found to inhibit growth of the bacteria during the Warburg determination, but fortunately it did not inhibit the enzymatic activity of the particles from bean seedlings. Apparently the bacteria did not oxidize the Krebs intermediates. The use of terramycin simplified interpretation of the data and allowed continuation of the experiments for long periods without interference from bacterial respiration.

Complete oxidation of succinate was not achieved. Eighty-four and four-tenths percent of the theoretical oxygen uptake was the highest value obtained, and in most determinations the figure was 70 to 80% of the theoretical amount. At the end of the determination the preparation was still active; however, the rate of oxidation was so low that a very long extension of the experimental time would have been required for complete oxidation of the substrate.

Green's cyclophorase system (17, p.400) was capable of complete oxidation of substrates in a short period.

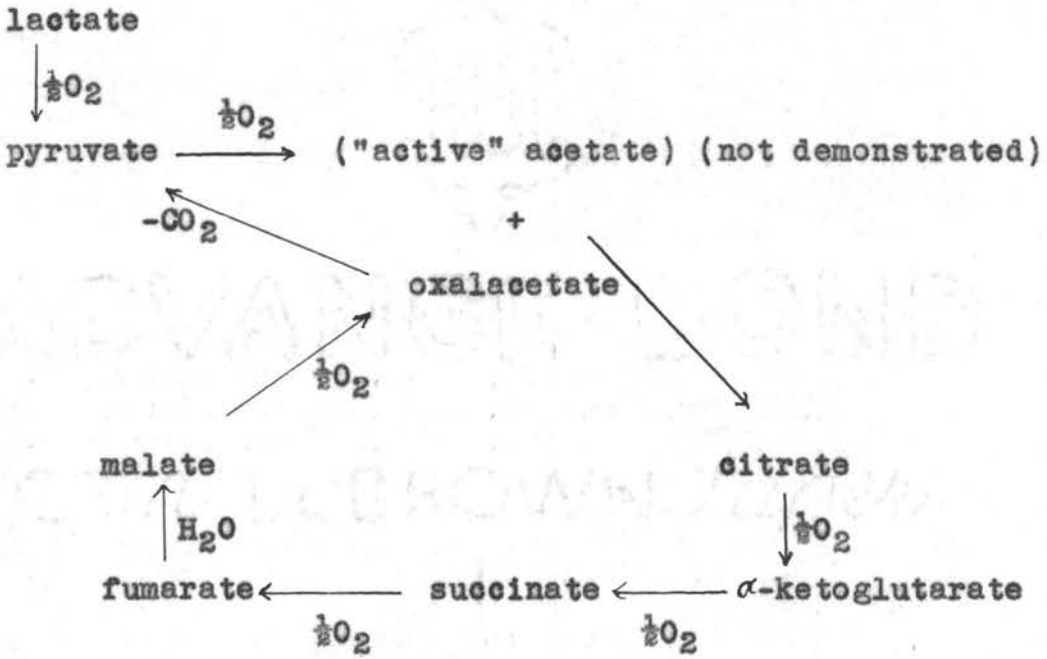
In the particles from bean seedlings a process of degradation of important co-factors or of the enzymes must occur to account for the low activity of the preparation during the latter hours of an experiment. Changing the succinate concentration from 2 to 10 μM. did not seem to affect the percentage oxidized (Table 41). This indicates that succinate can be oxidized at very low concentrations. Enough oxygen uptake was observed with all substrates to provide good evidence that the Krebs cycle is the oxidative pathway in the particles from bean seedlings.

The first-hour rate for the bean seedling preparation was found to be comparable to that for particulate preparations from other sources. It should be mentioned that most of the experiments were run with 3.12×10^{-3} M. substrate concentration and at this concentration a low $Q_o(N)$ value is to be expected. When the substrate concentration was raised to 1.56×10^{-2} M., the $Q_o(N)$ values rose and were found to be 2 to 3 times those reported by Millerd (39, p.157) who used a substrate concentration of 3×10^{-2}.

Citrate and a mixture of fumarate and pyruvate were oxidized to succinate in the presence of malonate. The accumulation of succinate indicates an oxidative path in which succinate is an intermediate. From the oxygen uptake data and the data for succinate accumulation, it can

be said that fumarate plus pyruvate required more oxygen for conversion to succinate than did citrate. An exact stoichiometric relationship was not established because malonate did not completely inhibit the succinic dehydrogenase; however, the data indicate that fumarate plus pyruvate requires about twice as much oxygen to form a micromole of succinate as does citrate. This is in accordance with the Krebs cycle oxidative pathway.

The data can be summarized in the following series of reactions which apparently occur in the preparation from bean seedlings:



The evidence for this scheme is as follows: (1) Enzymes were present which oxidized each of the intermediates when added. (2) Pyruvate oxidation required the presence of another intermediate. (3) The amount of oxygen uptake upon adding each of the intermediates was sufficient to indicate that at least 2 turns of the cycle had occurred. (4) Succinate was shown to be an intermediate of fumarate and citrate oxidation, and fumarate plus pyruvate required approximately twice the amount of oxygen per mole of succinate that citrate required.

Summary

1. An insoluble particulate fraction from bean seedlings was prepared. This fraction contained enzymes which catalyzed the oxidation of pyruvate, citrate, α-keto-glutarate, succinate, malate, fumarate, and lactate. An oxidative pathway for the breakdown of these intermediates was proposed.

2. When succinate was used as the substrate, 70-80% of the theoretical oxygen uptake for complete oxidation was obtained.

3. ATP, DPN, TPN, glutathione, phosphate and manganese were found to be necessary for maximum activity of the particulate fraction.

4. In the presence of malonate, succinate was accumulated from citrate and from a fumarate pyruvate mixture.

BIBLIOGRAPHY

1. Adler, Erich et al. Isocitric dehydrogenase and glutamic acid synthesis in animal tissue. Biochemical journal 33:1028-1045. 1939.

2. Albaum, Harry G. and Philip P. Cohen. Transamination and protein synthesis in germinating oat seedlings. Journal of biological chemistry 149:19-27. 1943.

3. Barron, E. S. Guzman et al. The metabolism of potato slices. Archives of biochemistry 28:377-398. 1950.

4. Berger, Julius and George S. Avery Jr. Glutamic and isocitric acid dehydrogenases in the Avena coleoptile and the effect of auxins on these enzymes. American journal of botany 31:11-19. 1944.

5. Bonner, James. Biochemical mechanisms in the respiration of the Avena coleoptile. Archives of biochemistry 17:311-326. 1948.

6. Bonner, James and S. G. Wildman. Enzymatic mechanisms in the respiration of spinach leaves. Archives of biochemistry 10:497-518. 1946.

7. Boswell, J. G. Metabolic systems in the 'root' of Brassica napus L. Annals of botany, New series 14:521-543. 1950.

8. Brody, T. M. and J. A. Bain. A mitochondrial preparation from mammalian brain. Journal of biological chemistry 195:685-695. 1952.

9. Cavallini, D. The coupled oxidation of pyruvate with glutathione and cysteine. Biochemical journal 49:1-5. 1951.

10. Cohen, Philip P. Microdetermination of glutamic acid. Biochemical journal 33:551-558. 1939.

11. Conn, Eric E. and Birgit Vennesland. Glutathione reductase of wheat germ. Journal of biological chemistry 192:17-28. 1951.

12. Conn, Eric E., Birgit Vennesland and L. M. Kraemer. Distribution of a triphosphopyridine nucleotide - specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. Archives of biochemistry 23:179-197. 1949.

13. Damodaran, M. and T. R. Venkatesan. Amide synthesis in plants, I. The succinoxidase system in plants. Proceedings of the Indian academy of sciences, Section B, 13:345-359. 1941.

14. Davies, D. D. The Krebs cycle enzyme system of pea seedlings. Journal of experimental botany 4:173-183. 1953.

15. Eny, Desire M. Respiration studies on chlorella. II. Influences of various organic acids on gas exchange. Plant physiology 26:268-289. 1951.

16. Glock, Eugene and Clifford O. Jensen. The colorimetric determination of plant succinic dehydrogenase. Journal of biological chemistry 201: 271-278. 1953.

17. Green, D. E., W. F. Loomis and V. H. Auerbach. Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. Journal of biological chemistry 172:389-403. 1948.

18. Handler, Philip and J. Raymond Klein. The inactivation of pyridine nucleotides by animal tissue in vitro. Journal of biological chemistry 143:49-57. 1942.

19. Henderson, James H. M. and John F. Stauffer. The influence of some respiratory inhibitors and intermediates on growth and respiration of excised tomato roots. American journal of botany 31:528-535. 1944.

20. Hill, R. and K. Bhagvat. Cytochrome oxidase in flowering plants. Nature 143:726. 1939.

21. Hopkins, Frederick Gowland and Edward James Morgan. The influence of thiol groups in the activity of dehydrogenases. Biochemical journal 32:611-620. 1938.

22. Hopkins, Frederick Gowland, Edward James Morgan and Cecilia Lutwak-Mann. The influence of thiol groups in the activity of dehydrogenases. Biochemical journal 32:1829-1848. 1938.

23. Keilin, D. and E. F. Hartree. Succinic dehydrogenase-cytochrome system of cells. Intercellular respiratory system catalyzing aerobic oxidation of succinic acid. Proceedings of the royal society of London, Series B, 129:277-306. 1940.

24. Koepsell, H. J. and Marvin J. Johnson. Dissimilation of pyruvic acid by cell-free preparations of Clostridium butylicum. Journal of biological chemistry 145:379-386. 1942.

25. Kornberg, Arthur and Pricer, W. E. Jr. Nucleotide pyrophosphatase. Journal of biological chemistry 182:763-778. 1950.

26. Kraemer, L. M., Eric E. Conn and Birgit Vennesland. The β-carboxylase of plants. III. Oxalacetic carboxylase of wheat germ. Journal of biological chemistry 188:583-591. 1951.

27. Krebs, Hans Adolph. The role of fumarate in the respiration of Bacterium coli commune. Biochemical journal 31:2095-2124. 1937.

28. Krebs, Hans Adolph. The tricarboxylic acid cycle. The Harvey lectures, Series 44:165-199. 1948-49.

29. Krebs, Hans Adolph, David Henry Smyth and Earl Alison Evans Jr. Determination of fumarate and malate in animal tissue. Biochemical journal 34:1041-1045. 1940.

30. Krebs, Hans Adolph and Leonard Victor Eggleston. The oxidation of pyruvate in pigeon breast muscle. Biochemical journal 34:442-459. 1940.

31. Krebs, Hans Adolph and W. A. Johnson. The role of citric acid in intermediate metabolism in animal tissues. Enzymologia 4:148-156. 1937.

32. Krimsky, I. and E. Racker. Glutathione, a prosthetic group of glyceraldehyde-3-phosphate dehydrogenase. Journal of biological chemistry 198:721-729. 1952.

33. Laties, George G. The oxidative formation of succinate in higher plants. Archives of biochemistry 22:8-15. 1949.

34. Laties, George G. The role of pyruvate in the aerobic respiration of barley roots. American journal of botany 34:601. 1947.

35. Laties, George G. The role of pyruvate in the aerobic respiration of barley roots. Archives of biochemistry 20:284-299. 1949.

36. Leonard, Mary Jane Kreko and R. H. Burris. A survey of transaminases in plants. Journal of biological chemistry 170:701-709. 1947.

37. Mann, P. J. G. and J. H. Quastel. Nicotinamide, cozymase and tissue metabolism. Biochemical journal 35:502-517. 1941.

38. Mapson, L. W. and D. R. Goddard. The reduction of glutathione by plant tissues. Biochemical journal 49:592-601. 1951.

39. Millerd, Adele. Respiratory oxidation of pyruvate by plant mitochondria. Archives of biochemistry and biophysics 42:149-163. 1953.

40. Millerd, Adele. Succinoxidase of potato tuber. Proceedings of the Linnean society of New South Wales 76:123-132. 1951.

41. Millerd, Adele et al. Oxidative and phosphorylative activity of plant mitochondria. Proceedings of the national academy of sciences 37:855-862. 1951.

42. Morrison, J. F. Enzymatic mechanisms in the respiration of rhubarb leaves. Part 2. Australian journal of experimental biology and medical science 28:311-320. 1950.

43. Ochoa, Severo. Isocitric dehydrogenase and carbon dioxide fixation. Journal of biological chemistry 159:243-244. 1945.

44. Ochoa, Severo. α-Ketoglutaric dehydrogenase of animal tissue. Journal of biological chemistry 155:87-100. 1944.

45. Ochoa, Severo and Erna Weisz-Tabori. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. II. Oxalsuccinic carboxylase. Journal of biological chemistry 174:123-132. 1948.

46. Pardee, Arthur B. and Van R. Potter. Inhibition of succinic dehydrogenase by oxalacetate. Journal of biological chemistry 176:1085-1094. 1948.

47. Price, Carl A. and Kenneth V. Thimann. The succinic dehydrogenase of seedlings. Archives of biochemistry and biophysics 33:170-171. 1951.

48. Pucher, George W., Alfred J. Wakeman and Hubert Bradford Vickery. The metabolism of organic acids of tobacco leaf during culture. Journal of biological chemistry 119:523-534. 1937.

49. Pucher, George W. and Hubert Bradford Vickery. The metabolism of organic acids of tobacco leaves. I. Effect of culture of excised leaves in solutions of organic acid salts. Journal of biological chemistry 178:557-575. 1949.

50. Pucher, George W. et al. Studies in the metabolism of crassulacean plants: The behavior of excised leaves of Bryophyllum calycinum during culture in water. Plant physiology 22:477-493. 1947.

51. Schneider, Walter C. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. Journal of biological chemistry 176:259-266. 1948.

52. Speck, John F. The effect of cations on the decarboxylation of oxalacetic acid. Journal of biological chemistry 178:315-324. 1949.

53. Stafford, Helen A. Intracellular localization of enzymes in pea seedlings. Physiologia plantarum 4:696-741. 1951.

54. Still, Jack Leslie. Pyruvic dehydrogenase of Bacterium coli. Biochemical journal 35:380-389. 1941.

55. Straub, Ferenc Bruno. Isolation and properties of flavoprotein from heart muscle tissue. Biochemical journal 33:787-792. 1939.

56. Straub, Ferenc Bruno. On the reoxidation of diaphorase-flavoprotein. Enzymologia 9:148-149. 1940.

57. Swingle, Karl F., A. E. Axelrod and C. A. Elvehjem. The mechanism of the effect of calcium salts on the succinoxidase system. Journal of biological chemistry 145:581-591. 1942.

58. Turner, J. S. and V. Hanly. Malonate and plant respiration. Nature 160:296-297. 1947.

59. Umbreit, W. W., R. H. Burris and J. F. Stauffer. Manometric techniques and tissue metabolism. Rev. ed. Minneapolis, Burgess, 1949. 227p.

60. Vennesland, Birgit. The β-carboxylases of plants. II. The distribution of oxalacetic carboxylase in plant tissues. Journal of biological chemistry 178:591-597. 1949.

61. Vennesland, Birgit, Miriam C. Gollab and John F. Speck. The β-carboxylases of plants. I. Some properties of oxalacetic carboxylase and its quantitative assay. Journal of biological chemistry 178:301-314. 1949.

62. Whatley, F. R. Isocitric dehydrogenase in green leaves. New phytologist 50:258-267. 1951.