METABOLIC STUDIES ON THE HONEY BEE, *APIS MELLIFERA*

by

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To Willie

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METABOLIC STUDIES ON THE HONEY BEE, APIS MELLIFERA

INTRODUCTION

The ultimate pathways of carbohydrate metabolism in most tissues investigated to date are included within the scope of three major metabolic schemes, namely, the tricarboxylic acid cycle, glycolysis, and the pentose cycle. A knowledge of the existence of any or all of these three schemes is essential to an understanding of the overall carbohydrate metabolism in any organism.

Evidence for the existence of the tricarboxylic acid cycle as an efficient mechanism by which pyruvic acid is oxidized to carbon dioxide and water and energy stored in the form of high energy phosphate compounds has accumulated for many types of tissue. Krebs, (13, pp.148-156) in 1937, established the cyclic nature of a series of reactions which had previously been associated with carbohydrate oxidations; thus, in 1911, Battelli and Stern (2, pp.478-505) had shown the rapid oxidation of citrate and several four carbon acids by a frog muscle preparation, and Martius in 1937 (17, pp.104-110) demonstrated the conversion of a six carbon acid, citrate, to a four carbon one, succinate. Numerous investigations (6, pp.140-146) have certified the existence of the tricarboxylic acid cycle in mammalian tissue, bacteria, molds, yeast, plant material

and certain invertebrates. Included in the last category are the insects, one of the most numerous of all forms of animal life. Despite the tremendous economic importance of these organisms, very few complete reports on their oxidative metabolism have been forthcoming. An understanding of the fundamental processes by which insects metabolize food stuffs could be of great value in the systematic development of antimetabolites for insect control. Of importance in this work would be any observed variation between insect and plant or animal metabolic patterns and also any variation among the various stages of insect growth.

Among the earliest reports of significance on tricarboxylic acid cycle activity in insects was that of Barron (2, pp.57-76) and Tahmisian using cockroach muscle. These investigators demonstrated a stimulation of respiration by several Krebs cycle intermediates, acetate and glutamate, and an inhibition of glucose oxidation by malonate. Data were also given on respiratory quotients relative to vertebrate and invertebrate tissue. In

addition, higher R. Q.[1] values were found for male than for female cockroaches.

In 1951, Watanabe and Williams (30, pp.675-689) succeeded in localizing Krebs cycle activity in the sarcosomes of insect flight muscle. The similarity between these particles and mammalian mitochondria was demonstrated. These particles possessed enzymatic activity for the oxidation of malic, succinic, and pyruvic acids.

Spirites (26, p.251) in the same year, using a less well-defined system of unwashed homogenates, water extracts, and acetone powders, was able, by Warburg monometry and spectrophotometric techniques, to demonstrate considerable Krebs cycle activity in the fly, Drosophila melanogaster. He observed activity for the enzymes aconitase, isocitric dehydrogenase, succinic dehydrogenase, fumarase, malic dehydrogenase, and the citrate condensing

1 Abbreviations used in this report are:
R. Q. - respiratory quotient
DPNH - reduced diphosphopyridine nucleotide
G-6-P - glucose-6-phosphate
G-1-P - glucose-1-phosphate
HDP - hexose diphosphate
EDTA - ethylenediamine tetraacetate or "Versene"
TRIS - trimethyolaminomethane
TPN - triphosphopyridine nucleotide
DPN - diphosphopyridine nucleotide
TTZ - triphenyl tetrazolium chloride
TPP - thiamine pyrophosphate
AMP - adenosine monophosphate
ATP - adenosine triphosphate
F-6-P - fructose-6-phosphate
R-5-P - ribose-5-phosphate

enzyme. Other ancillary enzymes were also investigated.

Intimately associated with Krebs cycle activity is the generation of high energy bonds in the nucleotide, adenosine triphosphate. This phenomenon has been demonstrated recently in insect sarcosomes by Lewis and Slater (15, p.xxvii). A relatively low P/O ratio of 1.80 was noted in the absence of any adenosine triphosphatase activity. They suggest that this may be a property of insect sarcosomes *in vivo*, differing from other living forms where P/O ratios of 3 or higher have been recorded.

In 1953, Sacktor (22, pp.371-387) working with the housefly was able to show activity for several enzymes associated with the citric acid cycle. Among these were malic dehydrogenase, DPNH oxidase, cyctochrome c oxidase, and malic and succinic cytochrome c reductase. These enzymes were localized in a particulate fraction.

A recent study by McGinnis (18, pp.41-50) on the adult blowfly, *Phormia regina*, provides evidence for the complete oxidation of citric and α-ketoglutaric acids, and the inhibition of this oxidation by malonate. Citric acid oxidation and isocitric dehydrogenase activity was also demonstrated in mature larval preparations. Here, again, the Krebs cycle activity was shown to reside principally in a particulate fraction.

Glycolysis, or the anaerobic fragmentation of

sugars into lactate is also a widely distributed process. Thus, glycolytic activity has been demonstrated in mammalian tissue, protozoa, bacteria, yeasts, and numerous invertebrates (6, pp.100-108). An important contribution to knowledge of this process in insects was made by Barron and Tahmisian (2, pp.57-76) working with cockroach muscle. Using glucose as a substrate, these authors measured glycolytic activity both in terms of lactate formation and CO_2 production from a bicarbonate buffer. Considerably more CO_2 was evolved than lactic acid produced. The authors suggest that this could concievably be a function of considerable adenosine triphosphatase activity. This same phenomenon has been reported more recently by McGinnis (18, p.50).

That this increased CO_2 production, released by acid from a bicarbonate buffer, could be a function of the production of some acid besides lactic was shown by Humphrey (9, p.324) and Humphrey and Siggins (10, p.359). In the latter paper, working with the grasshopper, Migratoria locusta, these investigators were able to show the production of both lactic and pyruvic acids from glycogen, glucose, fructose, G-6-P^1, G-1-P^1, or HDP^1.

The occurrence of the third major carbohydrate pathway, the pentose cycle, has only recently been demonstrated in insect tissue. Previously this cycle, in which

hexoses may be oxidatively metabolized in the absence of a tricarboxylic acid cycle, had been demonstrated in higher animals (8, p.214), plants (1, pp.619-634), bacteria (7, pp.11-26), and the wheat smut fungus (20, p.27-35). The studies on insects have been limited to two organisms. The first demonstration of this cycle was with the pea aphid, Macrosiphum pisi, (19, pp.37-45) where oxidation of known intermediates in the cycle and interconversions among the cyclic constituents were shown. McGinnis (18, p.52) in his work on the blowfly was able to demonstrate the disappearance of ribose-5-phosphate and formation of sedoheptulose and hexose in all three life stages, egg, larva, and adult of this organism.

It can be seen that although information has been rapidly accumulating on the various phases of carbohydrate metabolism in insects, much work remains to be done. Such work would be important both from an economic viewpoint and in furthering the fundamental knowledge of comparative biochemistry. It was with this in mind that a study of metabolism in the honey bee was undertaken. This insect was chosen primarily for its year-round availability in quantities large enough for investigation and for its relative ease of handling under laboratory conditions.

METHODS AND MATERIALS

Preparation of Mitochondria

Mitochondria were prepared from the thoracic segment of the honey bee. Approximately three hundred bees were anesthetized with CO_2, transferred from the cage into a beaker and washed several times with distilled water. Heads and abdomens were removed and the thoraces ground in a cold mortar and pestle with a 0.9% KCl-0.01M EDTA[1] solution. The intestinal tract was removed essentially intact by the process of removing the head from the remainder of the body. The resultant brei was strained through eight layers of cheese cloth and centrifuged at 500 x g for five minutes to remove heavy cellular material. The supernatant liquid was then centrifuged at 10,000 x g for 10 minutes to separate the mitochondria. Before use, these were resuspended twice in the KCl-EDTA solution, recentrifuged at 10,000 x g and briefly homogenized in a loose-fitting Potter-Elvehjem glass homogenizer to obtain a uniform suspension. The mitochondria as viewed with a microscope at a 440-fold magnification, took up Janus green B stain and appeared to be homogeneous. All operations were carried out at 4°C.

Preparation of Acetone Powders

Acetone powders were used to a limited extent to determine activity and pyridine nucleotide specificity of pentose cycle enzymes. These were prepared by placing either whole bees, heads, abdomens or thoraces, separately, in a cold Waring blendor with 20 volumes of cold acetone blending for 30 seconds and filtering the resultant material. The powders were resuspended twice in 10 volumes of acetone and the entire operation repeated. The acetone powders were dried *in vacuo* and stored at -20°C. The preparations were stable for at least three months.

Preparation of Soluble Enzyme Systems

Soluble enzymes were prepared by one of three methods, namely, extraction of acetone powders of thoracic homogenates, differential centrifugation of thoracic homogenates, or alternate freezing and thawing of mitochondria as described previously.

For a few determinations of pentose cycle activity, acetone powders were used. Approximately one gram of material was suspended in 15 ml. of 0.2M TRIS[1] buffer, pH 8.0, homogenized in a Potter-Elvehjem homogenizer, and extracted for 30 minutes. This slurry was then centrifuged for 30 minutes at 3000 x g and the supernatant fluid

dialyzed two to three hours against 0.02 M TRIS[1] buffer, pH 8.0, to lower the content of pentose and hexose which would interfere with the colorimeter determinations. Considerable polyphenol oxidase activity was noted with a whole bee acetone powder extract as evidenced by a darkening of the solution during dialysis.

A soluble enzyme system from a thoracic homogenate was used for glycolytic measurements and to obtain evidence for the various enzymes of the pentose cycle. This system was prepared by the same general method as described for mitochondria with the exception that centrifugation was carried out at 10,000 x g for 45 minutes to remove all larger particles.

For the determination of certain chemical reactions associated with the particulate or mitochondrial fraction of a thoracic homogenate, especially those to be followed spectrophotometrically, a solubilization procedure had to be followed. The one used was alternate freezing and thawing of the particulate fraction. Both a slow freezing at -20°C. and a more rapid freezing with a chloroform-dry ice mixture were used. The slower process proved to be generally superior. A large amount of gelatinization occurred during the rapid procedure but was not evident during the slow procedure. The extract obtained in this manner was used to detect isocitric dehydrogenase,

α-ketoglutaric dehydrogenase, the acetate activating enzyme, the citrate condensing enzyme, and pyruvic oxidase. When pyridine nucleotide specificity was to be established, the extract was dialyzed for three to four hours against 0.02 M TRIS[1], pH 7 to 8. The acetate activating and citrate condensing enzyme systems could not be demonstrated without prior solubilization by this technique.

Warburg Manometry and Chemical Assays

A conventional Warburg apparatus was used to follow the oxidation of tricarboxylic acid cycle intermediates, utilization of HDP by the glycolytic pathway, arsenite and malonate inhibitions and formation of α-ketoglutaric acid from citric and isocitric acids. In all aerobic experiments, the flasks were oxygenated for five minutes and equilibrated for seven minutes prior to tipping of the substrate from the sidearm of the Warburg vessel. In all experiments, the bath temperature was 37°C.

Anaerobic measurements were made in an atmosphere of 95% nitrogen - 5% carbon dioxide. The production of CO_2 from a bicarbonate buffer was used as a measure of acid production from HDP during glycolysis. Concomitant production of lactic acid was measured by the method of Umbreit *et al.* (29, p.192). HDP was determined by the resorcinol method (29, p.191).

α-Ketoglutaric acid formation from citric and isocitric acids was measured under anaerobic conditions on the Warburg apparatus using substrate quantities of TPN[1] and DPN[1]. The α-ketoglutarate formed was assayed by the method of Brummond and Burris (4, pp.754-759). This method consisted essentially of precipitating the α-ketoglutarate as the 2,4-dinitrophenyl hydrazone, extracting with ether in a liquid-liquid extractor, evaporating to dryness and dissolving in 3-5 ml. of an 85% $CHCl_3$-15% butanol mixture. The latter is the organic phase of a previously prepared silica gel column. The α-ketoglutarate derivative formed was separated on the column from the unreacted dinitrophenyl hydrazine and measured on a Beckman spectrophotometer against a standard curve prepared with known quantities of the hydrazone derivative of α-ketoglutarate.

Acetyl coenzyme A was measured by the hydroxamic acid method as described by Lipmann and Tuttle (16, pp. 21-28) with the exception that the incubation time was extended to two hours to allow maximum development of color. The enzyme system was a solubilized preparation from frozen and thawed mitochondria. Dialysis time was twelve hours against 0.02 M TRIS and 0.001 M EDTA, pH 8.1.

Citric acid was measured by the method of Saffran and Dendstedt (23, pp.844-855). This method consists of

dissolving the deproteinized sample in acetic anhydride and developing the color with anhydrous pyridine at 60°C. A complete standard curve was carried out with each determination. All colorimetric readings were made on a Bausch and Lomb colorimeter.

The activity of several enzymes was measured as a function of the reduction of DPN or TPN at 340 mu. These included glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, isocitric dehydrogenase, and α-ketoglutaric dehydrogenase. All analyses were made with a Beckman Model B spectrophotometer.

TTZ[1] was used as the electon acceptor in measuring pentose cycle activity. A complete system for this experiment contained, in addition to substrate, phosphate buffer, pH 7.2, $MgCl_2$, Armour coenzyme concentrate, thiamine pyrophosphate, TTZ, and methylene blue. This mixture was incubated for one hour at 37°C. in vacuo to effect the oxidation, 5 ml. of acetone added, the resultant precipitate centrifuged and the optical density of the reduced dye (formazan) determined at 550 mu.

A standard curve was prepared by reduction of TTZ with excess $Na_2S_2O_4$. Known amounts of TTZ were dissolved in 1% NaOH. The $Na_2S_2O_4$ solution and 5 ml. of acetone were added and the solution aerated for five minutes with oxygen in order to remove excess hydrosulfite.

The disappearance of ribose-5-phosphate and formation of sedoheptulose and hexose were measured as a function of time, the reaction being stopped with trichloracetic acid. Ribose-5-phosphate was incubated at 37°C with the enzyme preparation, $MgCl_2$, TPP[1], and TRIS buffer, pH 8.0. Aliquots were removed for analyses at appropriate times. Pentose was determined by the Meijbaum orcinol method as described by Umbreit et al. (29, p.191). Sedoheptulose and hexose were assayed by the method of Axelrod et al (1, pp.619-634). Optical density measurements were taken on a Bausch and Lomb Spectronic 20.

In all experiments in which hexose or pentose was measured it was found necessary to starve the bees for several days prior to use. This procedure, coupled with a dialysis of the enzyme preparation against five or six changes of one liter of TRIS buffer, pH 8.0, over a period of 12 to 16 hours, served to reduce the endogenous oxidation by the tissue to a point where colorimetric measurements could be made.

All protein determinations were made by the method of Weichselbaum (31, pp.40-79).

RESULTS AND DISCUSSION

The Tricarboxylic Acid Cycle

Although several reports have been presented on tricarboxylic acid cycle activity in insects, much of the information available concerns itself with ill-defined systems or fragmentary data. It was felt that a more thorough investigation into the nature of oxidative metabolism in insects might be in order. Thus, any small deviation from known pathways in other organisms or plant tissue might be of importance in the development of insect antimetabolites.

An important initial objective in this work was the development of an active particulate preparation for the demonstration of oxidation by citric acid cycle intermediates. Several attempts with varying grinding media failed to produce an active preparation until an isotonic KCl solution and high concentrations of EDTA were employed. Figure 1 shows the effect of varying the EDTA concentration on the oxidation of citrate by a thoracic homogenate. This oxidation was followed manometrically at 37°C. It can be seen that a ten fold increase in EDTA concentration, from 10^{-3}M to 10^{-2}M, results in approximately a 300% increase in total oxidation. When this information was applied to a mitochondrial system, it became possible to obtain consistently active preparations. The high endogenous shown

Figure 1. The effect of EDTA (Versene) upon the oxidation of citric acid by an Honey Bee homogenate. A complete system for these oxidations contained 14 μ moles phosphate buffer, pH 7.2, 20 μ moles Mg^{++}, 10 μ moles AMP, 1 mg. cytochrome c, 1 mg. of Armour coenzyme concentrate, and 3 μ moles citrate. Protein concentration was 50 mg./flask.

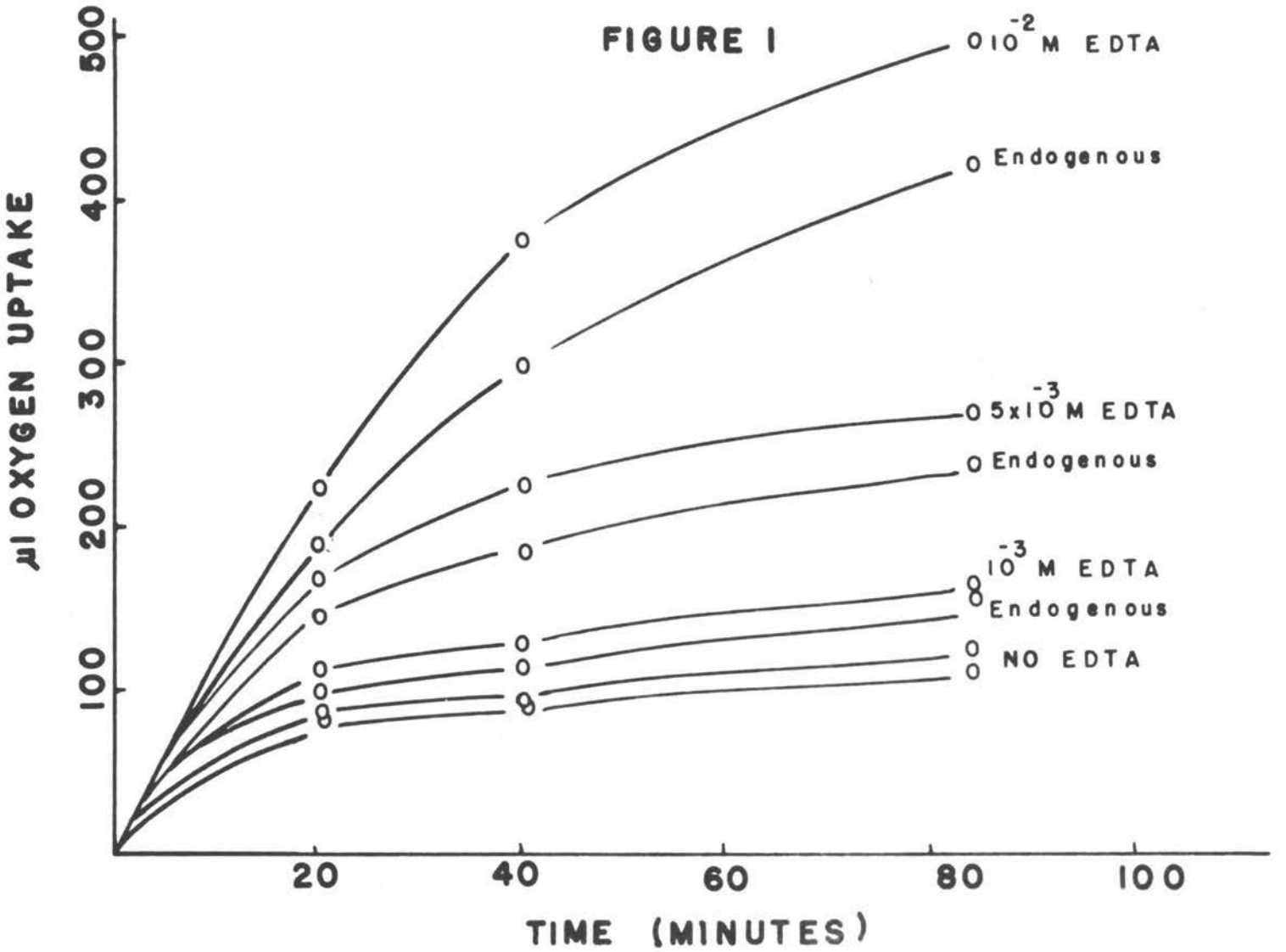


FIGURE 1
μl OXYGEN UPTAKE
100
200
300
400
500
20
40
60
80
100
TIME (MINUTES)
10^-2 M EDTA
Endogenous
5x10^-3 M EDTA
Endogenous
10^-3 M EDTA
Endogenous
NO EDTA

in figure 1 disappeared in the washed mitochondria. Although the exact function of EDTA is not known, it can be assumed that a chelation of injurious metal ions is its principal role. Since insect thoraces have been shown to contain large amounts of calcium, the lowered activity in the absence of EDTA may be the result of this metal as suggested for similar animal systems (24, p.119).

An attempt was next made to demonstrate the complete oxidation of compounds directly and peripherally associated with the citric acid cycle. Table 1 shows the completeness of oxidation of these intermediates while figure 2 also shows rates of oxidation. It can be seen from Table 1 that with the exception of oxalacetate and α-ketoglutarate, all oxidations are complete. The oxidation of isocitrate, based on utilization of the L-isomer only, is considerably above theoretical. It is possible that some mechanism may be available for the conversion of the DL- to the L-isomer, or else that the enzyme in bee mitochondria is non-specific for the optical configuration.

A reaction characteristic of TCA cycle activity is the competition of malonic acid with succinic acid for the enzyme succinic dehydrogenase. This reaction displayed unusual features when quantities of succinate were used which did not saturate the enzyme surface of succinic dehydrogenase. Added malonate had the effect of increasing the

Figure 2. Oxidation of several Krebs cycle intermediates by a mitochondrial preparation from the Honey Bee. Additions to Warburg vessels are identical to those shown for figure 1. Protein concentration was 50 mg./flask.

FIGURE 2

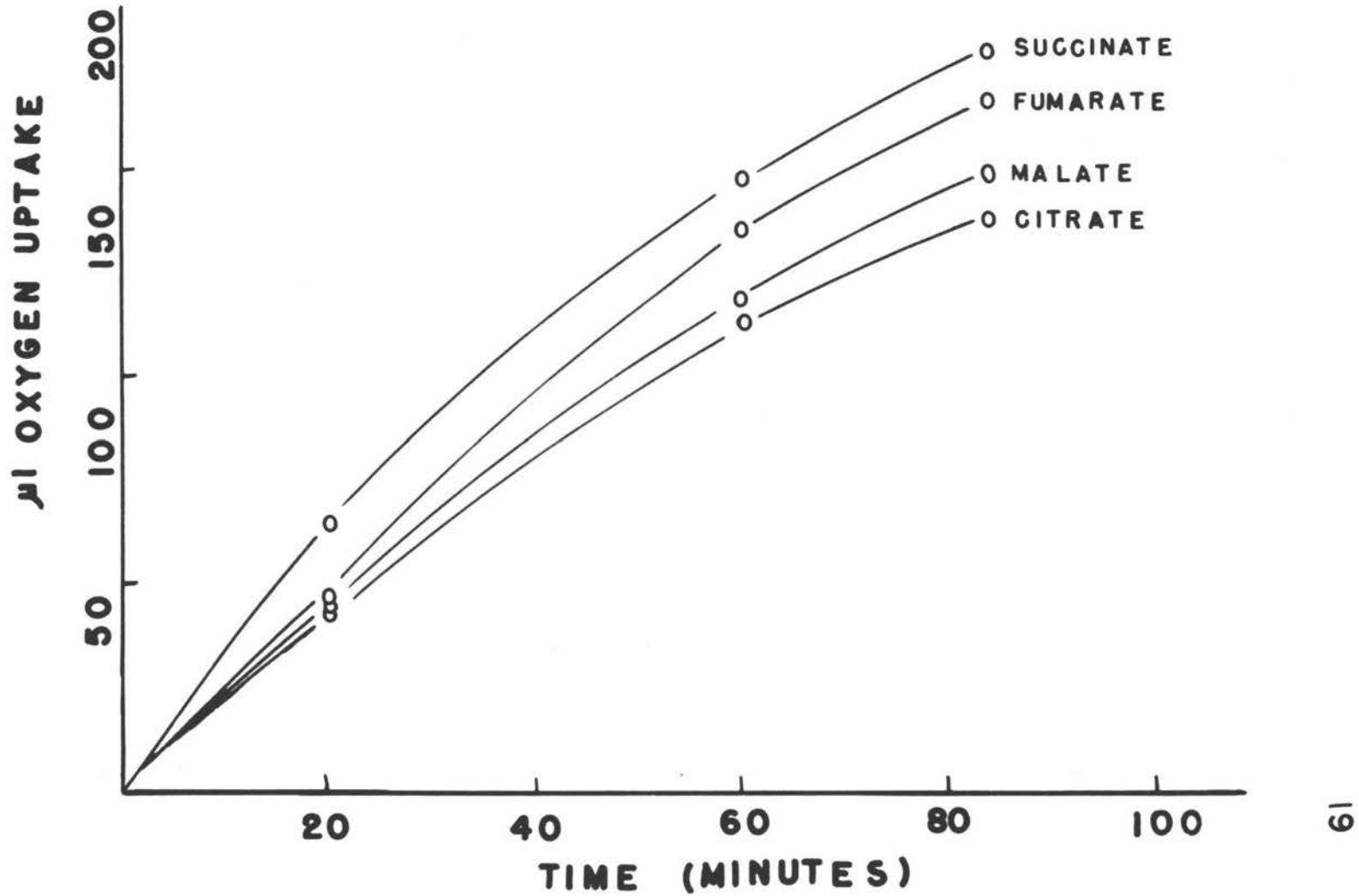

µl OXYGEN UPTAKE
50
100
150
200
20
40
60
80
100
TIME (MINUTES)
SUCCINATE
FUMARATE
MALATE
CITRATE

TABLE 1

Oxidation of Krebs Cycle Intermediates

by the Honey Bee

Substrate	μ moles added	μ atoms oxygen uptake	
		theory	observed
Citrate	3	27	26.3
Isocitrate (DL)	4	18*	25.6
α-Ketoglutarate	3	24	12.7
Succinate	3	21	21.4
Malate	3	18	18.4
Fumarate	3	18	20.3
Oxalacetate	2	10	5.8
Pyruvate	2	10	9.8
Acetate	20	80	29.6

* On basis of the utilization of L-isomer. For additions to Warburg vessels required for these oxidations, see figure 1. Protein concentrations averaged 40 to 60 mg./flask.

rate of oxidation of added substrate. These data are illustrated in figure 3. It was originally thought that malonate itself might be oxidized, possibly by a preliminary decarboxylation; however, experiments designed to test this theory showed neither oxidation of malonate itself nor CO_2 evolution as measured by the direct method for CO_2. A possible explanation of this phenomenon lies in the function of malonate as an uncoupling agent for oxidative phosphorylation. This has been reported to increase the rate of oxidation of tricarboxylic acid cycle intermediates (14, p.379).

Further work on this problem with a fully saturated enzyme established malonate as a potent inhibitor of succinate oxidation. This is shown in Table 2. The quantity 'K' shown in this table is a value which represents the ratio of succinate to malonate which will give a 50% inhibition of the oxidation of succinate alone. This value was derived by Krebs (12, p.614-627) and may be calculated from the formula:

$$K = \frac{1-n}{n} \times \frac{[\text{substrate}]}{[\text{inhibitor}]}$$

$$\text{where } n = \frac{\text{reaction rate with inhibitor}}{\text{reaction rate without inhibitor}}$$

That the enzyme surface is saturated is shown by only a very slight increase in the rate of oxidation of 140 u

Figure 3. The stimulation of succinate oxidation by malonate. Succinate concentration in all flasks: 3 μ moles. Curve A, 15 μ moles malonate; curve B, 1.5 μ moles malonate; curve C, 3 μ moles succinate; curve D, endogenous. For other additions see figure 1. Protein concentration 33 mg./flask.

FIGURE 3

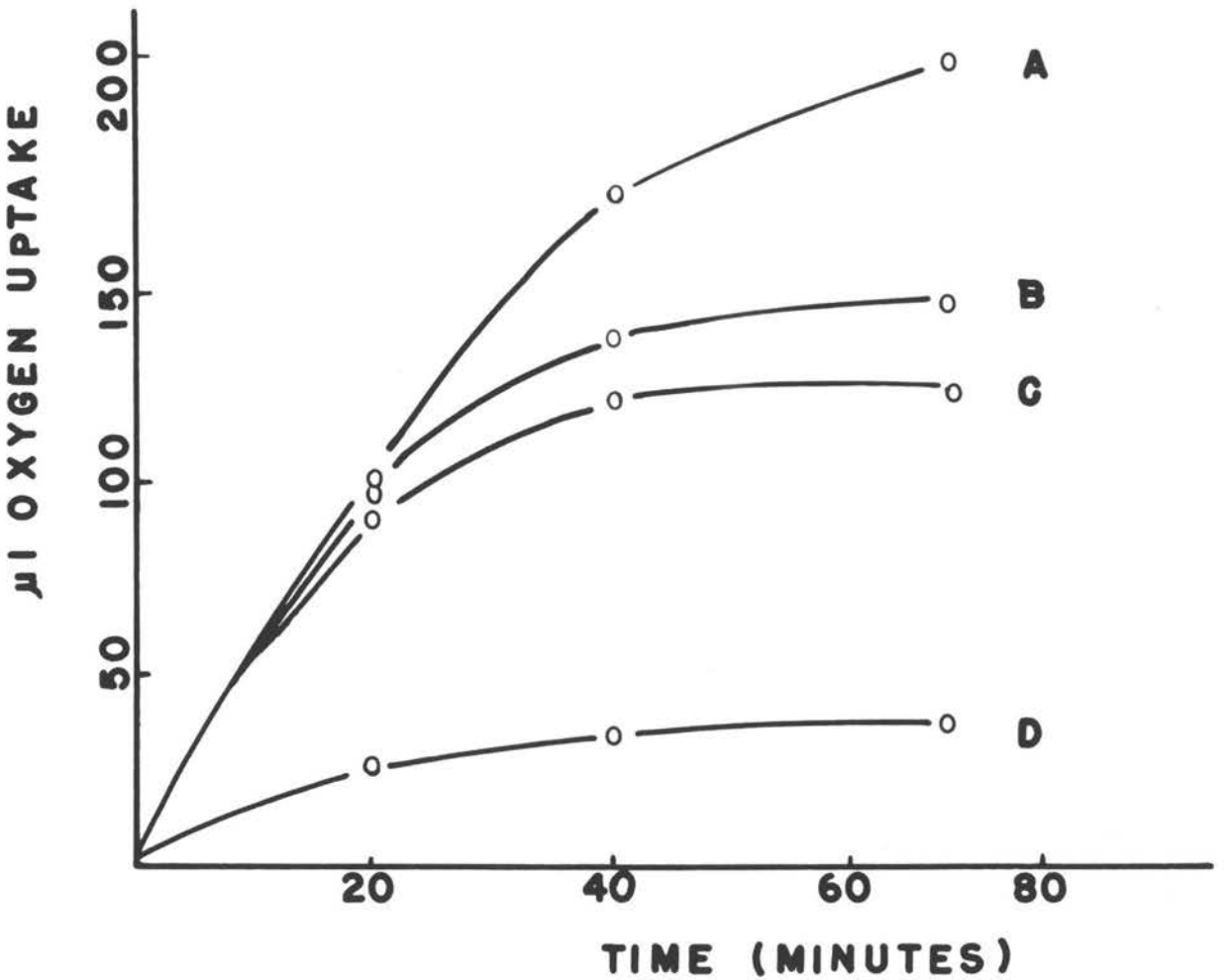


TABLE 2

Inhibition of Succinate Oxidation by Malonate in the Honey Bee

	Flask	µ l Oxygen uptake	n*	K*
1.	Endogenous	10	-	-
2.	100 µ moles Succinate	602	-	-
3.	140 µ moles Succinate	604	-	-
4.	100 µ moles Succinate 4 µ moles Malonate	462	0.77	7.5
5.	100 µ moles Succinate 8 µ moles Malonate	366	0.61	8.0
6.	100 µ moles Succinate 50 µ moles Malonate	188	0.31	4.4
7.	100 µ moles Succinate 80 µ moles Malonate	138	0.23	4.2

* See text for values of n and K.
The oxidations shown were measured manometrically at 37°C. for 30 minutes. Protein concentration: 10 mg./flask. For other additions to flask see figure 1.

moles of succinate over that of 100 μ moles. It can be seen that the K values determined fall into two groups, one about eight and the other about four. The higher set of values are reasonably close to the value of ten reported for mammalian tissue (12, p.616). One possible explanation for the lower values might be an inhibition of other enzymes systems, notably the oxidation of oxalacetic acid (21, pp.241-250), at higher malonate concentrations.

An attempt was next made to measure some of the individual reactions of the cycle. The formation of α-ketoglutarate from citric and isocitric acids was first investigated. It was found that added sodium arsenite inhibited the rate of oxidation of α-ketoglutarate and that this inhibition was proportional to the amount of arsenite added. Considerable protein, about 65 mg. per flask, was used in these experiments, so a proportionally high concentration of arsenite was required. Fifty μ moles of arsenite gave 38% inhibition, 250 μ moles of arsenite gave 65% inhibition, and with 500 μ moles the inhibition was complete. Several attempts were made to isolate α-ketoglutaric acid as the 2,4 dinitrophenyl hydrazone from citrate and isocitrate by use of this arsenite block. All were unsuccessful and it was assumed that arsenite in these high concentrations had interfered with the action

of some enzyme prior to α-ketoglutaric dehydrogenase.

The problem of α-ketoglutarate formation from citrate and isocitrate was solved by use of an anaerobic system and substrate quantities of pyridine nucleotides. Between citric and isocitric acids and α-ketoglutarate, there is but one oxidative step, namely, the dehydrogenation of isocitric acid. After added pyridine nucleotide had been reduced, no further oxidative reactions, (e.g., the oxidative decarboxylation of α-ketoglutarate), could occur. The reaction would, therefore, stop at this point. This procedure worked quite well as can be seen from the data given in Table 3. These data indicate, in addition to the formation of α-ketoglutarate, that TPN is specifically required by isocitric dehydrogenase. No explanation can be given for the smaller formation of α-ketoglutarate from isocitrate than from citrate. This is especially puzzling in light of the high value for oxidation of isocitrate as shown in Table 1.

Activity for isocitric dehydrogenase was demonstrated by following the reduction of TPN at 340 mμ. This is shown in Figure 4. The TPN specificity of isocitric dehydrogenase as shown by this method is in agreement with the data obtained by formation of α-ketoglutarate from isocitrate.

An effort was next made to establish cofactor

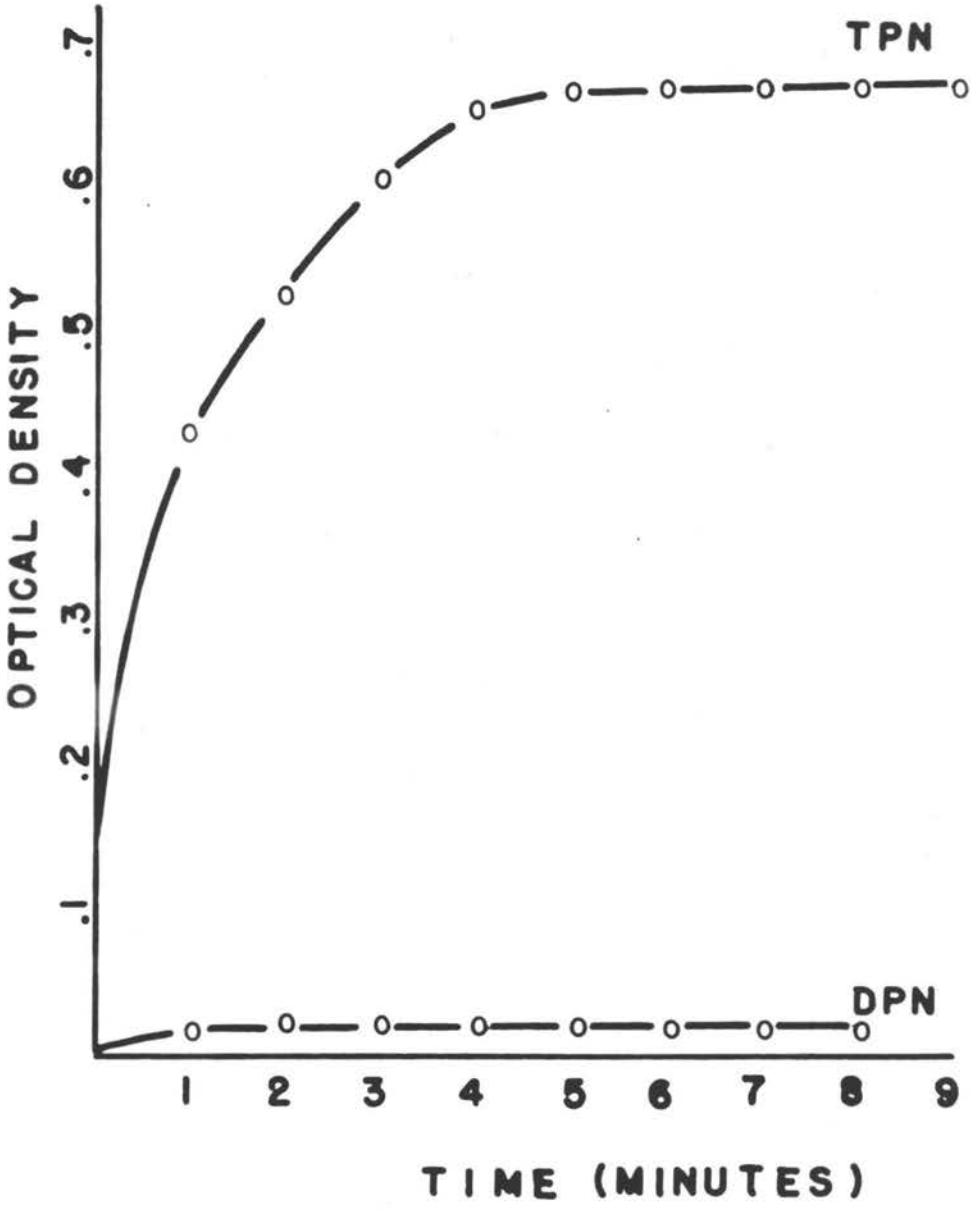
TABLE 3

The Formation of α-Ketoglutaric Acid
from Citric and Isocitric Acids
by the Honey Bee

Substrate	Cofactor	μ moles Substrate added	μ moles α-ketoglutarate formed
Citrate	TPN	10	6.3
Isocitrate (DL)	TPN	20	3.1
Citrate	DPN	10	0.7
Isocitrate	DPN	20	0.1

Figure 4. The reduction of TPN by isocitric dehydrogenase from a frozen and thawed mitochondrial system. The complete system contained 4 μ moles DL isocitrate, 0.5 μ moles pyridine nucleotide, and 50 μ moles TRIS buffer, pH 7.5. Appropriate controls were included for the complete system less substrate, enzyme, or pyridine nucleotide.

FIGURE 4



requirements as well as activity for several enzyme systems associated with the Krebs cycle. One of these studied was the enzyme α-ketoglutaric dehydrogenase. The activity of this enzyme was studied by following the reduction of DPN on the Beckman spectrophotometer using a frozen and thawed mitochondrial system. The reduction of DPN can be seen from figure 5 to be dependent on Mg^{++}, thiamine pyrophosphate and coenzyme A. This is to be expected if Mg^{++} and thiamine pyrophosphate were required for an initial decarboxylation prior to the reduction of DPN. TPN is not reduced by this enzyme system. Each cuvette contained approximately 3 mg. of protein.

The conversion of acetate to acetyl CoA and the cofactor requirements for this reaction were next studied by the hydroxamic acid method of Lipmann and Tuttle (16, pp.21-28). Several attempts were made to show this activation of acetate by use of an intact mitochondrial preparation but, here again, it was found necessary to solubilize the enzyme system by alternate freezing and thawing. Initial work was designed to show that acetyl CoA could be formed from acetate, coenzyme A, and ATP. Mg^{++} was an obligatory cofactor. The results of this experiment are shown in figure 6 where the formation of acetyl coenzyme A is plotted as a function of time.

In an attempt to establish cofactor requirements

Figure 5. α-Ketoglutaric dehydrogenase activity from a frozen and thawed mitochondrial system of the Honey Bee illustrating dependency upon DPN, CoA, Mg^{++}, and TPP. The complete system contained 0.4 μ moles DPN, 10 μ moles Mg^{++}, 0.1 mg. TPP, 50 Lipmann units CoA, and 70 μ moles TRIS buffer, pH 7.5. Enzyme concentration, 3.0 mg./cuvette.
1 u mole -Ketoglutarate was added at time zero and where indicated.

FIGURE 5

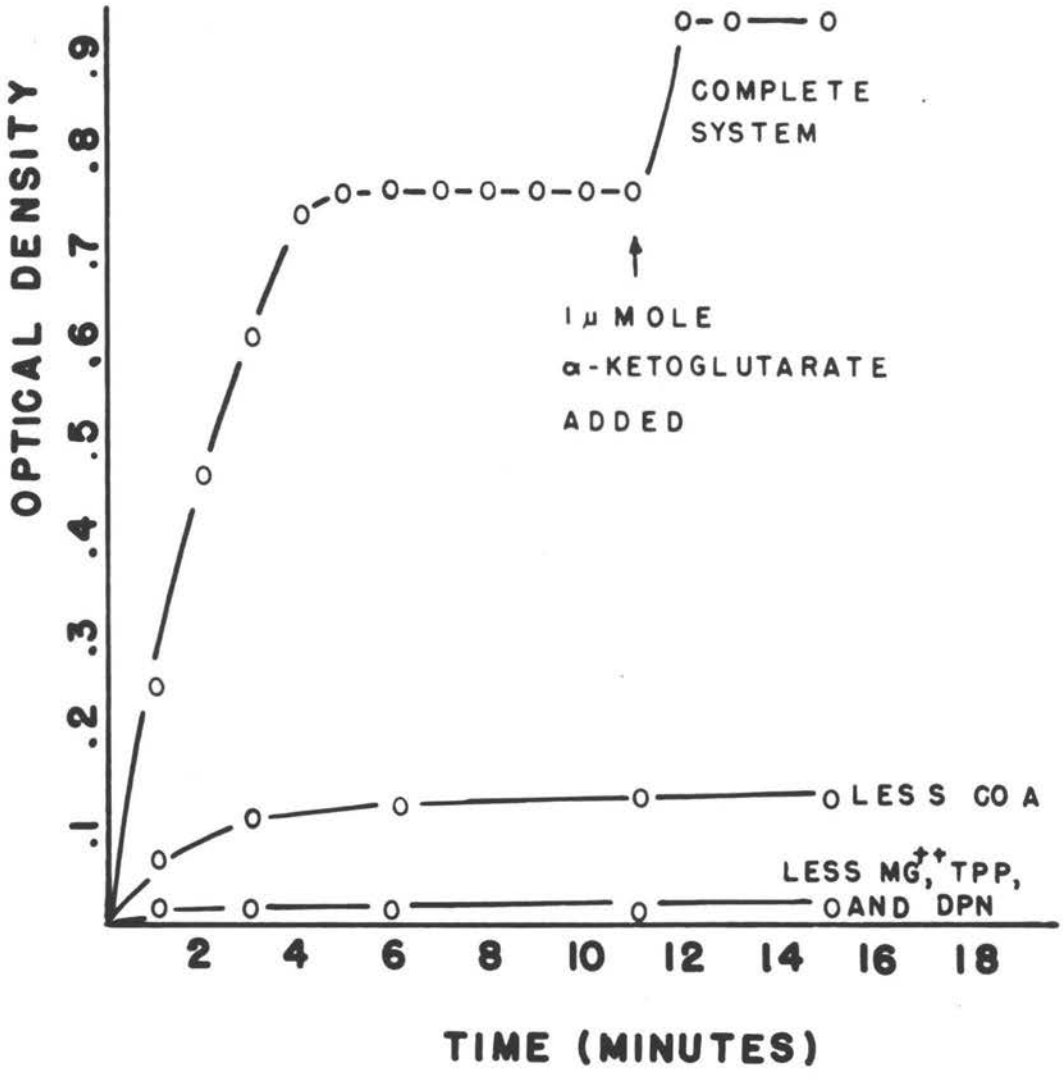
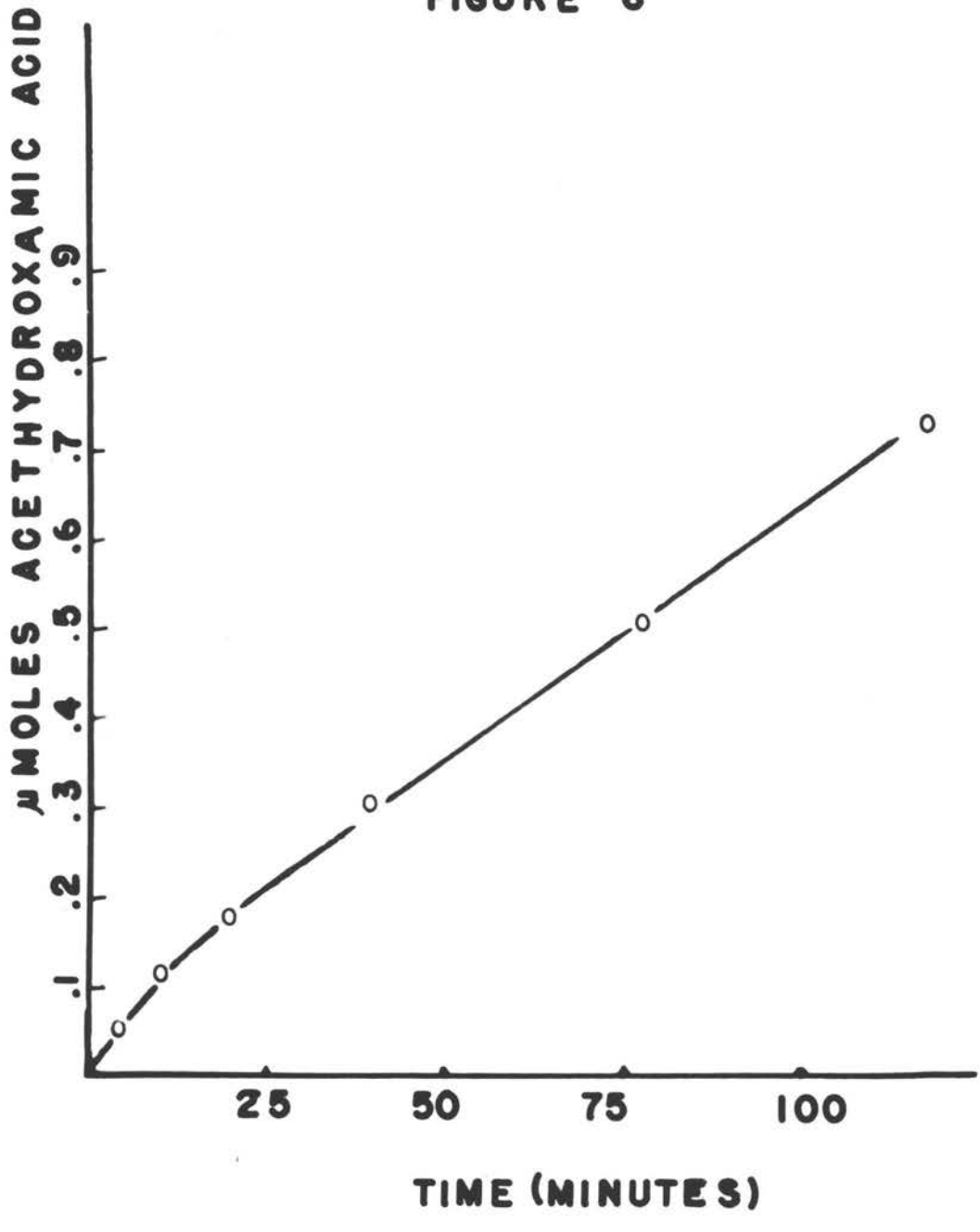

OPTICAL DENSITY
.1
.2
.3
.4
.5
.6
.7
.8
.9
COMPLETE SYSTEM
1 μ MOLE α-KETOGLUTARATE ADDED
LESS CO A
LESS Mg^{++}, TPP, AND DPN
2
4
6
8
10
12
14
16
18
TIME (MINUTES)

Figure 6. The formation of acethydroxamic acid by a frozen and thawed mitochondrial system of the Honey Bee. This system contained 100 μ moles acetate, 50 Lipmann units (35%) coenzyme A, 400 μ moles hydroxylamine, 10 μ moles Mg^{++}, 10 μ moles ATP[1], 100 μ moles TRIS buffer, pH 8.1, and 10 μ moles glutathione. All acidic materials were neutralized with KOH. Protein concentration, 42 mg./ml.

FIGURE 6


μMOLES ACETHYDROXAMIC ACID
.1
.2
.3
.4
.5
.6
.7
.8
.9
25
50
75
100
TIME (MINUTES)

for the acetate activating system, the enzymes were dialyzed for varying periods of time against the resin Dowex 50. This had been reported by earlier investigators (11, p.237) to completely remove all cofactors. In the honey bee, however, extended dialysis against this resin served to inactivate the enzyme system. This problem was resolved by dialysis with frequent changes of 0.02 M TRIS buffer, pH 8.1, over periods ranging from 16 to 24 hours. The results of these experiments are seen in Table 4 and clearly show the requirements of this system for ATP, Mg ion, and Co A. Table 4 includes also data to show the condensation of the acetyl coenzyme A formed with oxalacetate to form citrate. Spirites (26, p.25) had previously shown the presence of the condensing enzyme in insects but with a less well defined system. The present demonstration is believed to be the first one for the acetate activating system in insect tissue.

A variation of the hydroxamic acid method for measuring acetate activation was used to study pyruvic oxidase. In these experiments, hydroxylamine was omitted and citrate formation in the presence of oxalacetate was used as a measure of cofactor requirements. These data are summarized in Table 5. This experiment clearly shows the formation of citrate but fails to give clear cut data for the cofactor requirements. The reduced citrate

TABLE 4

Acetate Activating and Condensing
Enzyme Systems and Cofactor Requirements
in the Honey Bee

Experiment	Conditions	μ moles Acetyl CoA Formed
1	Complete System	1.5
2	Complete System	0.9
2	ATP omitted	0.1
2	Mg^{++} omitted	0.1
3	Complete System	0.6
3	Co A omitted	0.1

Experiment	Conditions	μ moles Citrate Formed
4	Complete System 10 μ moles oxal-acetate (less H_2NOH)	1.11
4	Complete System 5 μ moles oxal-acetate (less H_2NOH)	0.87

For the complete system involved, see figure 6.
Experiment 4 contained no hydroxylamine.

TABLE 5

Cofactor Requirements for the

Conversion of Pyruvate and Oxalacetate

to Citrate in the Honey Bee

Tube	Conditions	μ moles Citrate Formed
1	Complete System less GSH	1.57
2	Complete System less CoA	1.12
3	Complete System less Mg	2.25
4	Complete System less lipoic acid	2.25
5	Complete System less DPN	1.79
6	Complete System less TPP	2.18
7	Complete System	2.25
8	Complete System	2.25

The system employed was identical with that given for experiment 4, figure 6 with the exception that 40 μ moles of pyruvate are used instead of acetate. The system contained 4.6 mg. protein/ml.

formation shown in the absence of coenzyme A and DPN can be considered significant but the negative values for Mg^{++}, lipoic acid, and TPP could easily represent a failure to remove these cofactors under the dialysis conditions used. Of these three, lipoic acid is known to be tightly bound to the enzymes with which it is associated. In an attempt to remove the other cofactors, various methods were used, but all resulted in an inactivation of the enzymes involved.

An unsuccessful attempt was made to follow the reduction of DPN by malic acid in the presence of malic dehydrogenase using a soluble system from frozen and thawed mitochondria. A possible explanation for this failure might be the presence of oxalacetic acid, which has been reported (27, p.261) to inhibit, even in minute quantities, the action of malic dehydrogenase.

The Pentose Cycle

Characterization of pentose cycle activity encompassed the following reactions: the reduction of TTZ by certain substrates associated with this cycle, the pyridine nucleotide specifity of these reductions, the formation of sedoheptulose from ribose-5-phosphate, and the spectrophotometric detection of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase.

Acetone powders of both the whole bee and abdomens alone were used in the TTZ reductions. Table 6 shows the extent of reduction and pyridine nucleotide specificity when 1 μ mole of substrate is oxidized by the enzymes present in an extract from whole bee acetone powders. Parallel experiments with an extract from abdomen acetone powders showed only very slight activity for these substrates. The anaerobic reduction of TPN rather than DPN suggests the reduction of TTZ by enzyme systems other than glycolysis. Later experiments with TTZ using soluble enzyme preparations from a thoracic homogenate by centrifugation demonstrated oxidation of a α-glycerol phosphate, α-ketoglutarate, and succinate in addition to those compounds listed in Table 6.

As further evidence for pentose cycle activity, the activity of the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were followed by the reduction of TPN at 340 mμ on the Beckman Spectrophotometer. These reductions are shown in figures 7 and 8. Neither of these enzymes possessed activity in the presence of DPN.

The formation of sedoheptulose from ribose-5-phosphate was also demonstrated. This is shown graphically in figure 9. An attempt was made to measure hexose formation also but an exceptionally high hexose endogenous

TABLE 6

Reduction of TTZ by Phosphorylated Hexoses and Pentoses in the Honey Bee

Substrate	Cofactor	μ moles TTZ Reduced
G-6-P	TPN	0.47
G-6-P	DPN	0.10
HDP	TPN	0.20
HDP	DPN	0.00
F-6-P[1]	TPN	0.55
F-6-P	DPN	0.10
R-5-P[1]	TPN	0.60
R-5-P	DPN	0.15

A complete system for these reductions contained 3.2 mg. TTZ, 1 μ mole substrate, 0.1 μ moles pyridine nucleotide, and 0.1 ml stock solution. Stock solution contained 5 parts phosphate buffer, pH 7.2, 0.1 M, 1 part Mg^{++}, 0.1 M, 1 part Armour coenzyme concentrate, 10 mg./ml, and 1 part TPP, 1 mg./ml.

Figure 7. The reduction of TPN by glucose-6-phosphate dehydrogenase from a soluble enzyme preparation of the Honey Bee. This system contained, in addition to enzyme solution, 2 μ moles glucose-6-phosphate, 0.5 μ moles pyridine nucleotide, and 50 μ moles TRIS buffer, pH 7.5. Protein concentration was 4.0 mg./cuvette. Appropriate controls were run as before.

FIGURE 7

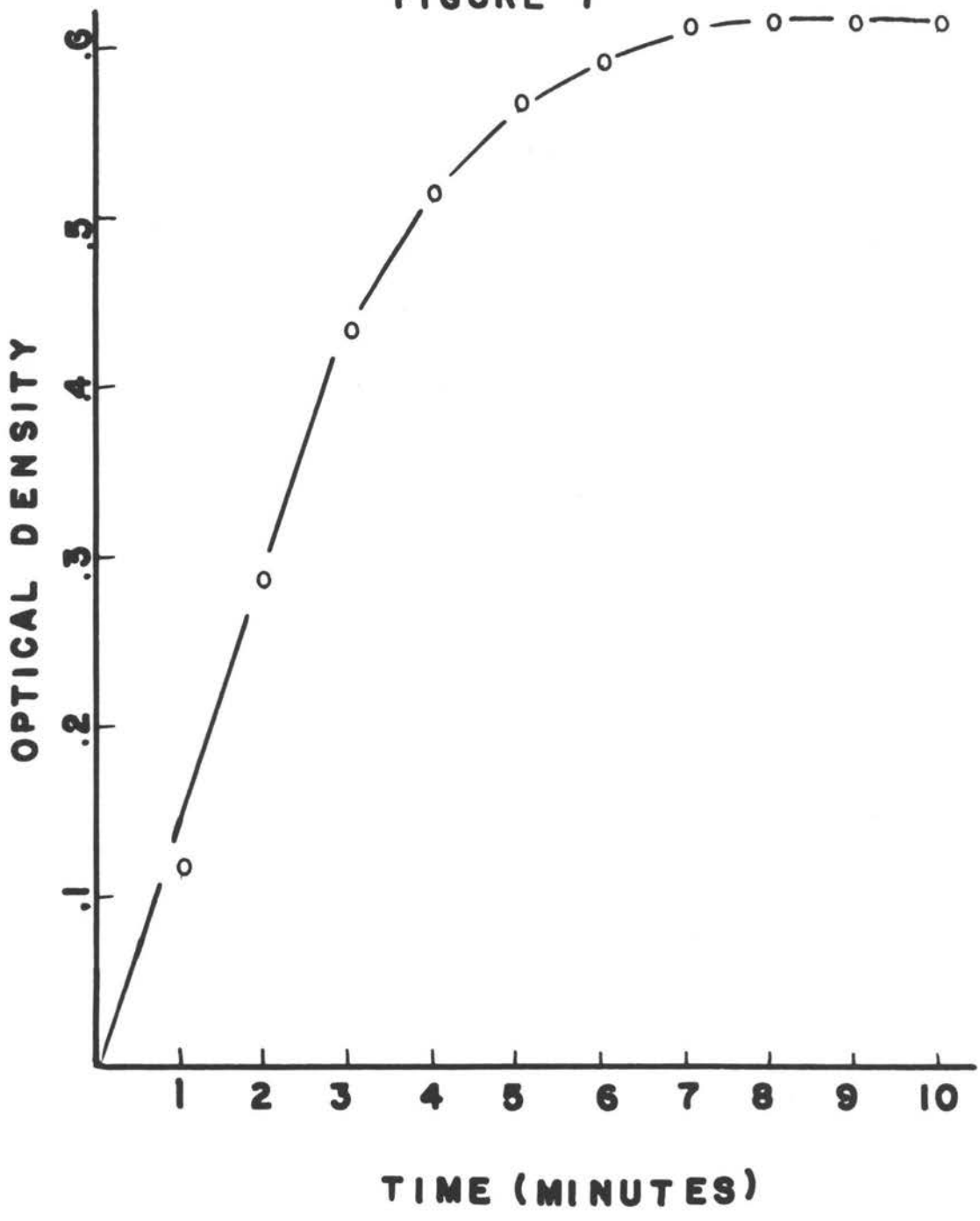


Figure 8. The reduction of TPN by 6-phosphogluconic dehydrogenase from a soluble enzyme preparation of the Honey Bee. The system contained 2 μ moles 6-phosphogluconic acid and 4.0 mg. protein/cuvette. For other details of the system see figure 7.

FIGURE 8

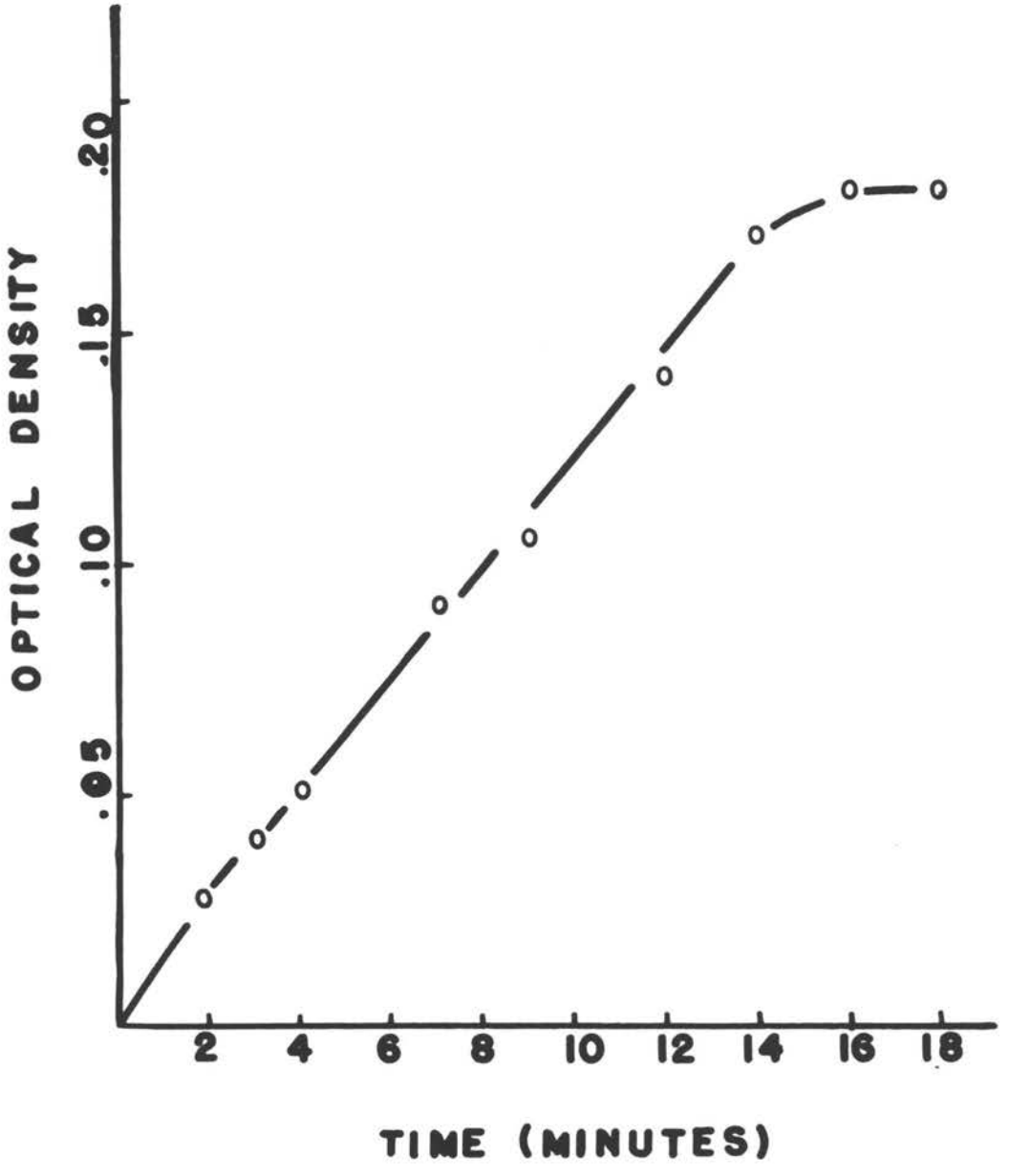
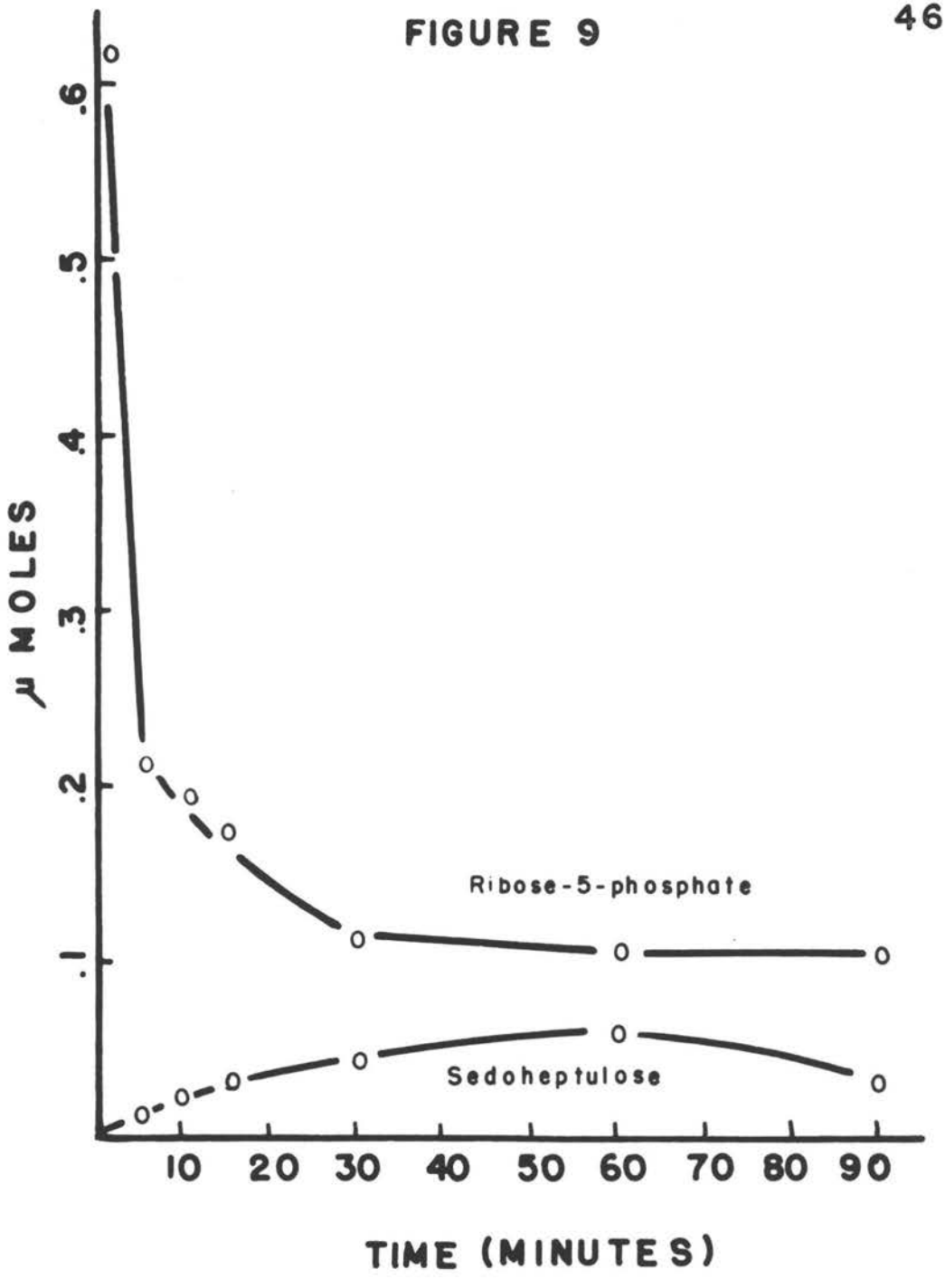


Figure 9. The formation of sedoheptulose from ribose-5-phosphate in a soluble enzyme system from the Honey Bee. The completed system contained 32 mg. protein, 100 μ moles TRIS buffer, pH 8.0, 30 μ moles Mg^{++}, 30 μ moles TPP, 8 μ moles R-5-P. 1.0 ml. samples were taken at the times indicated and the reaction stopped with 1.0 ml. of 10% trichloroacetic acid.

FIGURE 9


μ MOLES
.6
.5
.4
.3
.2
.1
Ribose-5-phosphate
Sedoheptulose
10 20 30 40 50 60 70 80 90
TIME (MINUTES)

system made these data meaningless.

Glycolysis

Glycolytic activity was measured by the method of LePage (29, p.144). The enzymes were obtained by centrifugation of a thoracic homogenate for 45 minutes at 10,000 x g. Measurements were made manometrically at 37°C. The complete system contained 6 μ moles HDP, 75 μ moles bicarbonate, 7 μ moles phosphate, 12 μ moles $MgCl_2$, 12 μ moles ATP, 0.6 μ moles DPN, 60 μ moles nicotinamide, 30 μ moles KF, and 15 μ moles pyruvate. This system produced 1.82 u moles CO_2 by acid displacement from the added bicarbonate and 1.4 μ moles lactate as measured by Barker and Summerson and described in Umbreit (29, p.192). All of the added HDP disappeared during the course of the experiment. While the stoichiometry of these conversions is poor, there is evidence for the anaerobic reduction of DPN and the formation of an acid liberating CO_2 from bicarbonate. These reactions are indicative of a glycolytic pathway.

SUMMARY AND CONCLUSION

A study has been made of carbohydrate metabolism in the honey bee, Apis mellifica. Three principal pathways have been investigated with considerable emphasis placed on the tricarboxylic acid cycle. For this particular pathway, information was obtained concerning the following: the oxidation of directly and peripherally associated substrates and the effect of EDTA upon these oxidations; the dual role of malonate as both a stimulator and inhibitor of succinate oxidation, depending upon the concentration of succinate; the inhibition of respiration by arsenite; the anaerobic formation of α-ketoglutaric acid from citrate and isocitrate; the cofactor requirements for α-ketoglutaric dehydrogenase and isocitric dehydrogenase as followed on the Beckman spectrophotometer; the conversion of acetate to citrate in the presence of oxalacetate and the cofactor requirements for this reaction; the conversion of pyruvate to citrate in the presence of oxalacetate, and finally limited information on the cofactor requirements for this reaction.

Information on pentose cycle activity was obtained concerning the oxidation of phosphorylated sugars and the pyridine nucleotide specificity for these oxidations, the enzymatic conversion of ribose-5-phosphate to

sedoheptulose, and, finally, activity for the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase. Information on a glycolytic pathway was limited to a measurement of lactic acid formation and CO_2 evolution during an anaerobic manometric experiment. Lactate was presumably formed by reduction of added pyruvate with DPNH produced in the oxidation of glyceraldehyde-3-phosphate. The acid produced by this reaction, 3-phosphoglyceric, displaced CO_2 from the bicarbonate buffer.

The experimental data presented here leave little doubt that the tricarboxylic acid cycle is operative in the honey bee, Apis mellifica. The data are also suggestive of the presence of glycolysis and the pentose cycle in this organism. All three pathways, as far as investigated, show only slight variations from the corresponding pathways in other tissue.

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