OXIDATIVE ENZYME SYSTEMS OF THE HONEY BEE, APIS MELLIFERA L.*

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The ultimate pathways of carbohydrate metabolism in most tissues investigated to date are included within the scope of three major metabolic pathways, namely the tricarboxylic acid (TCA) cycle, glycolysis, and the pentose cycle (hexosemonophosphate "shunt"). The TCA cycle has been demonstrated in several insects and the enzymatic activity has been shown to be associated with the mitochondria (Watanabe and Williams, 1951; Sacktor, 1953). Barron and Tahmisian (1948) reported glycolytic activity in cockroach muscle, and the pentose cycle has been demonstrated in the pea aphid (Newburgh and Cheldelin, 1955) and the housefly (Chefurka, 1955). The purpose of this communication is to explore further the patterns of carbohydrate oxidation in insects. The honey bee was chosen for this because of its year round availability, its relative ease of handling under laboratory conditions, and its generally high metabolic activity.

Direct evidence was obtained for the presence of the TCA cycle enzymes and the acetate-activating enzyme in the mitochondria. The presence of the pentose cycle in the soluble portion of a thoracic homogenate has been indicated. Pecularities were observed in malonate inhibition in this organism, as well as in the stability of the mitochondria.

Materials and Methods

Analytical Methods.—A conventional Warburg apparatus was used for the manometric measurements, a Beckman Model B spectrophotometer for the studies of pyridine nucleotide reductions at 340 mμ, and a Bausch and Lomb spectronic 20 colorimeter for the colorimetric measurements. α-Ketoglutaric acid was measured by precipitation as the 2,4-dinitrophenylhydrazone and the amount present deter-

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mined by the method of Brummond and Burris (1953). Acetyl CoA[1] was determined by the method of Lipmann and Tuttle (1945) with the exception that the incubation period was extended to 2 hours. Citric acid was assayed by the method of Saffran and Dendstedt (1948). Protein was measured by the method of Weichselbaum (1946).

Preparation of Mitochondria and Soluble Enzymes from Honey Bee Thoraces.—Mitochondria were prepared from the thoracic segments. Approximately three hundred caged bees were anesthetized with carbon dioxide, transferred into a beaker, and washed several times with distilled water. Heads and abdomens were removed and the thoraces ground in a cold mortar with 0.9 per cent KCl–0.01 M EDTA solution. The resultant brei was strained through eight layers of cheesecloth and centrifuged at 500 × *g* for 5 minutes to remove whole cells. The supernatant fluid was then centrifuged at 10,000 × *g* for 10 minutes to obtain the mitochondrial fraction. This particulate matter was resuspended twice in the KCl–EDTA solution and recentrifuged at the higher speed. The mitochondria thus obtained absorbed Janus green B stain and appeared homogeneous when observed under a microscope. All operations were performed at 4°C. The procedure for preparing soluble material from fresh thoracic homogenates was the same as that for mitochondria, except that the centrifugation was maintained at 10,000 × *g* for 45 minutes and the supernatant fraction was collected.

Solubilization of Honey Bee Thoracic Mitochondria Enzymes.—For the characterization of certain enzymic reactions associated with the thoracic particulate or mitochondria, a solubilization procedure had to be followed. The one adopted employed alternate freezing and thawing, either slowly at −20°C. in a deep freeze or more rapidly with a chloroform–dry ice mixture. The slower process proved to be generally superior, since gelatinization occurred during rapid freezing.

Materials.—The following chemicals were all commercial preparations: AMP, CoA, cytochrome *c*, TPN, DPN, and G-6-P (Sigma Chemical Company), EDTA (Versenes, Inc.); DL-isocitric acid (California Foundation for Biochemical Research); α-ketoglutaric acid (Krishell Laboratories, Inc.); TPP (S. A. F. Hoffmann-La Roche, Inc.); and Armour's coenzyme concentrate (containing about 2.5 per cent CoA, 7 per cent DPN, 4 per cent TPN, and 0.25 per cent pyridoxal phosphate plus pyridoxamine phosphate). 6-PGA was prepared by the method of Seegmiller and Horecker (1951).

RESULTS

Complete oxidation of TCA cycle members to carbon dioxide and water occurred only when EDTA was used for cell-free preparations (optimum concentration = 0.01 M). Oxygen consumption was slight in the absence of this reagent. These results are contrary to those found by Sacktor (quoted by Watanabe and Williams, 1953) but in agreement with those of Lewis and

[1] The following abbreviations are used throughout this paper: AMP, adenosine-5-phosphate; ATP, adenosinetriphosphate; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; EDTA, disodium ethylene diamine tetraacetic acid ("Versene"); G-6-P; glucose-6-PO_4; 6-PGA, 6-phosphogluconic acid; TCA, tricarboxylic acid cycle, TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate; and tris, trimethylolaminomethane.

Slater (1954). In experiments with the blowfly, McGinnis *et al.* (1956) were able to obtain consistently more active TCA cycle oxidations when the mitochondria were prepared with EDTA.

TCA Cycle Enzymes Associated with Honey Bee Mitochondria.—Before demonstrating the individual enzymes involved, an attempt was made to show the complete oxidation of compounds directly associated with the TCA cycle. Table I shows that with the exception of oxalacetate and α-ketoglutarate, all intermediates were completely oxidized. The oxidation of DL-isocitrate, based on the utilization of only the L-isomer, was considerably above theoretical.

TABLE I

The Oxidation of TCA Cycle Members and Related Compounds by Honey Bee Mitochondria

The complete system consisted of: 14 μM phosphate buffer, pH 7.2, 4 μM Mg^{++}, 10 μM AMP, 1 mg. cytochrome *c*, 1 mg. Armour's coenzyme concentrate, substrate as noted, and 60 mg. protein per flask. Total volume 3.0 ml. Temperature 37°C. Gas, oxygen.

Substrate	Micromoles added	Microatoms oxygen consumed (80 min.)	
		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 the basis of utilization of only the L-isomer.

A reaction characteristic of the TCA cycle is the competitive inhibition of succinate oxidation by malonate. This reaction displayed unusual features when quantities of succinate were used which did not saturate the surface of the succinic dehydrogenase enzyme. Added malonate had the effect of increasing the rate of oxidation of the substrate, as illustrated in Fig. 1. It was thought at first that malonate itself might be oxidized, possibly by a preliminary decarboxylation; however, neither oxidation of malonate alone nor decarboxylation was demonstrated.

Further work on this problem with a fully saturated enzyme established malonate as a potent inhibitor of succinate oxidation. This is shown in Table II, in which K represents the ratio of succinate to malonate which will give 50 per cent inhibition of the oxidation of succinate alone. This value was derived by Krebs *et al.* (1952) and may be calculated from the formula:

$$K = \frac{1-n}{n} \times \frac{\text{(Substrate)}}{\text{(Inhibitor)}} \text{ in which } n = \frac{\text{Reaction rate with inhibitor}}{\text{Reaction rate without inhibitor}}$$

That the enzyme surface is saturated is shown by the equal rates of oxidation of 100 and 140 μM of succinate. It can be seen that the K values determined fall into two groups, one at eight and the other at four. The higher values approach the figure of ten reported for mammalian liver by Krebs *et al.* (1952).

In early experiments it was found that the oxidation of α-ketoglutarate could be inhibited by arsenite. Since 65 mg. of protein were used in each flask it was necessary to use large amounts of arsenite. With 50 μM arsenite, 38 per cent inhibition of α-ketoglutarate oxidation occurred; with 250 μM, 65 per cent inhibition; and with 500 μM, complete inhibition. The formation of α-

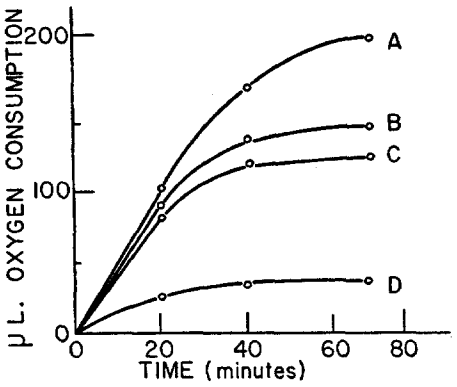

FIG. 1

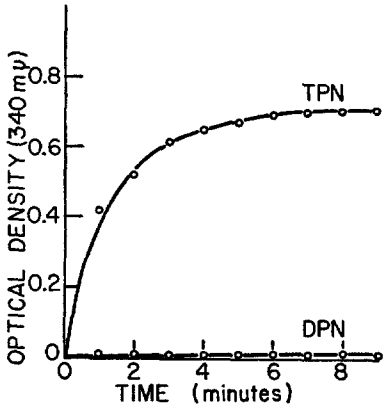

FIG. 2

FIG. 1. The stimulation of succinate oxidation by malonate. The complete system and experimental conditions were the same as those for Table I. The concentration of succinate in each experiment was 3 μM while the concentration of malonate was as follows: curve A, 15 μM, curve B, 1.5 μM, curve C, no malonate, curve D, no succinate or malonate. Protein concentration was 33 mg. per flask.

FIG. 2. The reduction of TPN by isocitric dehydrogenase from honey bee mitochondria. The complete system contained: 4 μM DL-isocitrate, 0.5 μM pyridine nucleotide (TPN or DPN), and 50 μM tris buffer, pH 7.5. Protein concentration, 3 mg. per cuvette. Total volume 2.5 ml. Controls without substrate, enzyme, or pyridine nucleotide were included.

ketoglutarate from citrate and isocitrate was determined in an anaerobic system, using substrate amounts of pyridine nucleotides. Since α-ketoglutarate was not further metabolized, it was possible to measure its formation. The results are shown in Table III. Activity for isocitric dehydrogenase was also demonstrated by spectrophotometric methods and the results are shown in Fig. 2. The presence of TPNH oxidase was not sought, and therefore it is not known whether the incomplete reduction of TPN in this system may have been the result of this enzyme. These data indicate the TPN specificity of the mitochondrial isocitric dehydrogenase.

α-Ketoglutaric acid dehydrogenase was also demonstrated using a frozen and thawed mitochondrial system. The reduction of DPN can be seen from Fig. 3 to be dependent on Mg^{++}, TPP, and CoA. TPN was not active in this system.

TABLE II

Inhibition of Succinate Oxidation by Malonate in the Honey Bee

The complete system and experimental consitions were the same as those in Table I. The protein concentration was 10 mg. per flask. The oxygen consumption was measured for 30 minutes.

Substrate		Oxygen consumption	n*	K*
Succinate	Malonate			
μM	μM	μl.		
0	0	10		
100	0	602		
140	0	604		
100	4	462	0.77	7.5
100	8	366	0.61	8.0
100	50	188	0.31	4.4
100	80	138	0.23	4.2

* See text for values of n and K.

TABLE III

The Formation of α-Ketoglutarate from Citric and Isocitric Acids by Honey Bee Mitochondria

The complete system consisted of: 6 μM $KHCO_3$, 10 μM nicotinamide, 12 μM $MnCl_2$, 15 μM TPN or DPN as indicated, and 55 mg. of protein per flask. Total volume 2.2 ml. Temperature 37°C. Gas 95 per cent N_2, 5 per cent CO_2.

Substrate	Cofactor	Substrate added	α-Ketoglutarate formed
		μM	μM
Citrate	TPN	10	6.3
Isocitrate (DL)	TPN	20	3.1
Citrate	DPN	10	0.7
Isocitrate (DL)	DPN	20	0.1

The actetate-activating enzyme and the condensing enzyme were studied in an extract from the frozen and thawed mitochondrial preparation. The formation of the hydroxamic acid derivative with time is shown in Fig. 4. In order to determine the cofactor requirements it was necessary to dialyze the preparation against frequent changes of 0.02 M tris buffer, pH 8.1, for 16 to 24 hours. These requirements are shown in Table IV. This table also indicates the presence of the condensing enzyme, which was demonstrated by citrate formation from acetate and oxalacetate.

A variation of the hydroxamic acid assay method was used to study pyru-

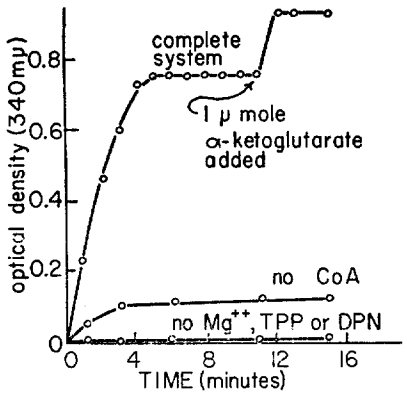

FIG. 3

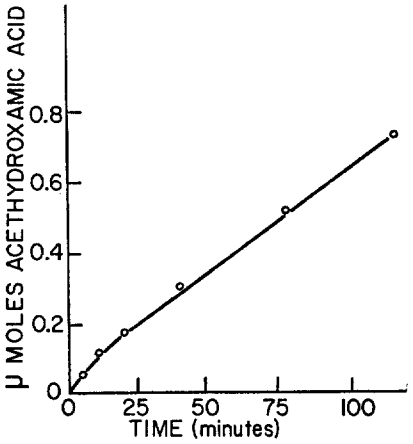

FIG. 4

FIG. 3. α-Ketoglutaric acid dehydrogenase from a soluble enzyme system from honey bee mitochondria, illustrating cofactor dependency. The complete system consisted of: 0.4 μM DPN, 10 μM Mg^{++}, 0.1 μM TPP, 100 γ CoA, 70 μM tris buffer, pH 7.5, and 1 μM α-ketoglutarate. Protein concentration, 3.0 mg. per cuvette. Total volume 2.4 ml. α-Ketoglutarate was added as indicated, also at time zero.

FIG. 4. The formation of acethydroxamic acid by a soluble preparation from honey bee mitochondria. The complete system contained: 100 μM acetate, 100 γ CoA, 400 μM hydroxylamine, 10 μM Mg^{++}, 10 μM ATP, 100 μM tris buffer, pH 8.1, and 10 μM glutathione. All acidic materials were adjusted to pH 7.5 with KOH. Protein concentration, 4.2 mg. per ml. Volume, 2.4 ml.

TABLE IV

Acetate-Activating and Condensing Enzyme Systems from a Soluble Mitochondrial Preparation from the Honey Bee

Additions were the same as in Fig. 4 except as noted. Reaction time, 2 hours.

Experiment	Conditions	Acetyl CoA formed	Citrate formed	Protein conc.
		μM	μM	mg./ml.
1	Complete system	1.5		4.2
2	Complete system	0.9		3.8
2	No ATP	0.1		
2	No Mg	0.1		
3	Complete system	0.6		4.0
3	No CoA	0.1		
4	Complete system plus 10 μM oxalacetate less hydroxylamine		1.11	4.0
5	Complete system plus 5 μM oxalacetate less hydroxylamine		0.87	4.0

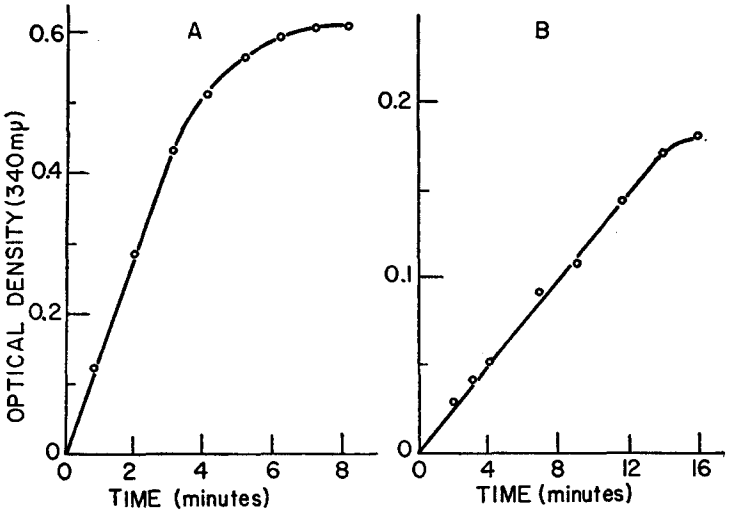

FIG. 5. The reduction of TPN by glucose-6-phosphate and 6-phosphogluconic acid in a soluble enzyme fraction from honey bee thoraces. The complete system consisted of: 2 μM substrate, 0.5 μM TPN, 50 μM tris buffer, pH 7.5. Total volume, 2.5 ml. Protein concentration was 4.0 mg. per cuvette. A, substrate was G-6-P; B, substrate 6-PGA.

TABLE V

Cofactor Requirements for the Conversion of Pyruvate and Oxalacetate to Citrate by a Soluble Preparation from Honey Bee Mitochondria

The complete system contained: 40 μM pyruvate, 100 γ CoA, 10 μM Mg^{++}, 100 μM tris buffer, pH 8.1, 10 μM glutathione. All other materials were adjusted to pH 7.5 with KOH. Protein concentration, 4.6 mg. per ml. Total volume 2.4 ml. Reaction time, 2 hours. Temperature 37°C.

Conditions	Citrate formed
	μM
Complete	2.25
No GSH	1.57
No CoA	1.12
No Mg^{++}	2.25
No lipoic acid	2.25
No DPN	1.79
No TPP	2.18

vate oxidation. In these experiments, hydroxylamine was omitted and citrate formation was used as a measure both of cofactor requirements and the enzymes associated with the formation of citrate. The results are summarized in Table V. This experiment clearly shows the formation of citrate but fails

to give clear cut data for cofactor requirements. The reduced citrate formation in the absence of CoA and DPN can be considered significant, but the negative results for Mg, lipoic acid, and TPP could be due to failure to remove these cofactors from the enzyme. Various methods were used in an attempt to remove them, but resulted in inactivation of the system.

Soluble Enzymes.—The presence of G-6-P and 6-PGA dehydrogenases was demonstrated in the *soluble* portion of the thoracic homogenate by following the reduction of pyridine nucleotides at 340 mμ in the presence of these substrates. The results are shown in Fig. 5. DPN was inactive in these systems. Some evidence was obtained for the formation of sedoheptulose from ribose-5-phosphate but the high amounts of carbohydrate present which would interfere with the colorimetric tests precluded the obtaining of good stoichiometric data for ribose disappearance or sedoheptulose and hexose formation. The presence of these enzymes in the "soluble" cell fractions has also been reported for mammalian tissues (Newburgh and Cheldelin, 1956).

DISCUSSION

The presence of the TCA cycle in the honey bee has been demonstrated and this system of enzymes has been shown to be associated with the mitochondrial fraction from a thoracic homogenate. It is of interest that in order to obtain consistently active mitochondrial preparations it was necessary to include in the homogenizing media a relatively large concentration of EDTA. Since it is known that in other systems calcium has a deleterious effect on the oxidations associated with mitochondria (Slater and Cleland, 1952), it seems likely that one function of EDTA may be to remove calcium ions from the mitochondria, resulting in consistently active preparations.

The formation of more α-ketoglutarate from citrate than from isocitrate is not easily understood. The simplest explanation would be that the D-isomer is slightly inhibitory toward the isocitric dehydrogenase of bee mitochondria.

One explanation for the increased succinate oxidation in the presence of malonate when the enzyme is not saturated with substrate may be an uncoupling of oxidative phosphorylation, as was shown to occur in other systems (Lehninger, 1951). The lower K value for the inhibition of succinate oxidation by malonate when the enzyme is saturated with substrate may likely be the result of inhibition of oxalacetate oxidation by malonate (Pardee and Potter, 1949). It is interesting to note that while the higher value is not equal to that found by Krebs for other systems it does approach it, indicating the similarity of the bee enzyme to those of other tissues.

These experiments constitute the first direct demonstration of the acetate-activating enzyme in insects. From the cofactor requirements this enzyme appears to be similar to that found in other organisms. Although pyruvic oxidase was not demonstrated directly, the evidence indicates its presence

since it was possible to demonstrate the formation of citrate from pyruvate and oxalacetate. The failure to observe a requirement for lipoic acid in these reactions may be the result of inability to remove these cofactors from the enzyme without inactivation. Lipoic acid is known to be tightly bound to enzymes with which it is associated.

SUMMARY

1. Oxidative dissimilation has been studied in enzymes from the honey bee. Using mitochondria isolated from the thoraces, complete oxidation of most of the TCA cycle members has been shown.

2. The presence of the acetate-activating enzyme, citrate-condensing enzyme, isocitric dehydrogenase, α-ketoglutarate dehydrogenase, glucose-6-phosphate, and 6-phosphogluconic dehydrogenase has been demonstrated and the cofactor requirements established.

3. The oxidation of isocitric acid has been shown to be either non-specific for the D- or L-isomer, or the presence of a racemase is indicated.

4. The presence of the pentose cycle is indicated in the soluble portion of the thoracic homogenate.

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