INVESTIGATIONS ON THE MITOCHONDRIA OF THE HOUSE FLY, MUSCA DOMESTICA L.

I. Adenosinetriphosphatases

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In the course of studying synthetic processes associated with mitochondria of insects, it was found that the preparations contain enzymes which presumably could interfere with synthesis by breaking down ATP.[1] A systematic investigation of the dephosphorylating mechanisms was therefore undertaken in order to ascertain the significance of such opposing reactions.

With the possible exclusion of the enzymes found in mammalian muscles which split phosphate from ATP, the nature of this activity in tissues remains partially obscure. Dephosphorylation of ATP by liver mitochondria has been reported by Kielley and Kielley (1951) and Novikoff *et al.* (1952). Swanson (1951) characterized "soluble" ATPase obtained from the supernatant of liver fractions. A "soluble" ATPase from muscle has also been described by Kielley and Meyerhof (1948). Meyerhof and Ohlmeyer (1952) achieved a 50-fold purification of yeast ATPase. Pyro-pase activity has also been found in yeast (Bailey and Webb, 1944), in liver (Swanson, 1952), and in other tissues. These few data are admittedly insufficient for a satisfactory resolution of the mechanism of dephosphorylation in mammalian tissues, and in insects practically nothing is known of adenine nucleotide breakdown; in fact, the mere presence of ATP in insects has only recently been affirmed by Albaum and Kletzkin (1948) and Calaby (1951), while the dephosphorylation of ATP has been but briefly noted. Gilmour (1948) found a soluble enzyme derived from grasshopper myosin extracts which splits both labile phosphates from the ATP molecule. He also noted that grasshopper muscle contains an enzyme which hydrolyzes inorganic Pyro-P. An apyrase was observed in cockroach muscle homogenates by Barron and Tahmisian (1948) and Chin (1951). The latter claimed that his preparation was specific for ATP in so far as no phosphorus was split from H-P or Gly-P.

[1] The following abbreviations will be used: ATP, adenosinetriphosphate, ADP, adenosinediphosphate, AMP, muscle adenylic acid, Pyro-P, inorganic pyrophosphate, Gly-P, glycerol phosphate, H-P, hexose phosphate, G-1-P, glucose-1-phosphate, F-6-P, fructose-6-phosphate, F-1,6-DP, fructose-1,6-diphosphate, TRIS, tris-(hydroxymethyl) aminomethane, NMN, nicotinamide mononucleotide, DPN, diphosphopyridine nucleotide.

The high metabolic activity of mammalian mitochondria has become well established, but it is only recently that some of the biochemical properties of insect mitochondria have been investigated (Watanabe and Williams, 1951). In the thorax of flies are found large "indirect" flight muscles which are distinguishable from other types of skeletal muscle by several criteria, among which perhaps the most conspicuous are the relatively large diameter of the fibrils and the presence between them of closely packed rows of apparently extracellular mitochondria, known as sarcosomes. These bodies seem exceedingly well suited for studying the basic organization of enzyme systems because they are available in abundance, easy to isolate, of large size, and of high physiological activity. High titers of cytochromes *a*, a_3, *b*, and *c*, active cytochrome oxidase and catalase, and significant succinic, malic, pyruvic, and alpha-glycerophosphate dehydrogenase activities were found in the sarcosomes of the blow fly, *Phormia regina* (Watanabe and Williams, 1951). The mitochondria of the house fly, at least, will also oxidize alpha-ketoglutaric acid rapidly (Sacktor, unpublished data).

The abundance of oxidative enzymes leads to the presumption that these mitochondria should be capable of effecting oxidative phosphorylation, but efforts to demonstrate formation of ATP from inorganic phosphate plus ADP or AMP have been inconclusive. As suggested above, the failure of such experiments might be due to the presence of enzymes capable of dephosphorylating ATP. In this paper evidence will be presented which defines some of the factors concerned with a specific ATPase activity in the mitochondrial fraction of the house fly, *Musca domestica* L., and the distribution and nature of such activity in other fractions.

EXPERIMENTAL

Isolation of Mitochondria

House flies of mixed sexes, 4 to 6 days old, were taken from a laboratory culture, maintained as described previously (Sacktor, 1950). The sarcosomes or mitochondria were isolated by a slight modification of the technique of Watanabe and Williams (1951). A known number of flies, usually 200, were anesthetized with carbon dioxide and the thoraces obtained by removing heads and abdomens. The thoraces, as acquired, were placed in approximately 1.5 ml. of ice cold 0.2 M sucrose. All subsequent operations were carried out in the cold (0–3°C.). The thoraces were ground gently with mortar and pestle and the resulting brei filtered by suction through two surgical gauze pads previously moistened with sucrose solution. The pads were then washed with about 20 ml. of 0.2 M sucrose. On centrifuging the combined filtrates (5 minutes at 3,000 *g*), the mitochondria were sedimented as a red-brown mass. This precipitate was resuspended in 0.9 per cent KCl and centrifuged again. After two such washings the mitochondria were suspended in KCl solution to a final volume of approximately 2.0 ml. Microscopic examination of the suspension at this stage showed a practically pure preparation of well rounded sarcosomes, 3 to 8 micra in diameter.

Other Methods of Fractionation

1. The ATPase activity of mitochondrial suspensions was compared with that of the "soluble" fraction and residue of whole thoracic homogenates. The homogenates were prepared by grinding 50 thoraces in 0.9 per cent KCl with a Potter-Elvehjem homogenizer for 1 minute. The brei was then filtered through several layers of cheese-cloth to remove pieces of cuticle. Thereafter, the filtrate was centrifuged as described above. The first supernatant was collected and used as the "soluble" fraction. Subsequently the residue was resuspended in KCl and centrifuged. After two washings the precipitate was suspended in KCl to a final volume of approximately 5.0 ml. Examination of this fraction under the microscope demonstrated the presence of mitochondria, muscle fibrils, and other large cellular components.

2. For reasons which will be discussed below, pure mitochondrial preparations, in KCl, were homogenized, centrifuged as previously indicated, and the supernatant separated from the precipitate. Microscopic examination of each fraction showed that most of the mitochondria had been fragmented, and that the larger particles were sedimented whereas the smaller particles had remained in the supernatant.

Materials

All substrates, when obtained in the form of Ba or Ca salts, were converted to their Na salts with Na_2SO_4, or by ion exchange according to the procedure of Kielley and Meyerhof (1948). The solutions were then adjusted to approximately pH 7.4. ATP, as the Na salt, was purchased from Pabst Laboratories; ADP, the Ba salt, and AMP, pure crystals, from Sigma; HDP, the Ca salt, from Schwarz; F-6-P, the Ba salt, from Nutritional Biochemical Co.; Gly-P (52 per cent alpha), the Na salt, from Eastman; and $Na_4P_2O_7 \cdot 10H_2O$, C.P. grade, from Baker. Tris-(hydroxymethyl) aminomethane, from Sigma, was used as the buffer. Deionized water was used to prepare all solutions.

Methods

In the present investigation all experiments were conducted at pH 7.4. The reaction mixtures were incubated at room temperature (22 to 25°C.). The final concentration of ingredients, patterned after the method of DuBois and Potter (1943), was (except where otherwise indicated): TRIS buffer, pH 7.4, 0.03 M; $MgCl_2$, 10^{-3} M; ATP, 0.05 per cent, and 0.03 ml. of enzyme preparation per milliliter of reaction mixture; and 0.9 per cent KCl to the desired final volume. Inorganic phosphate was assayed by the method of Sumner (1944) in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. Zero time was at the instant of addition of enzyme, and the value determined at this time was subtracted from all subsequent values.

In studies of the action of mitochondria on various phosphate-containing substrates, the final concentrations of these substrates were 0.003 M.

The adenylate kinase[2] activity of the mitochondria was determined by measuring their ability to promote the reaction of ADP with glucose in the presence of yeast hexokinase. Except where otherwise indicated the incubation mixtures contained

[2] The name adenylate kinase has been proposed by Colowick (1951) for the enzyme also known as myokinase and ADP phosphomutase.

0.2 ml. ADP (0.03 M); 0.4 ml. TRIS buffer, pH 7.4 (0.3 M); 0.1 ml. $MgCl_2$ (0.1 M); 0.1 ml. NaN_3 (0.4 M); 0.1 ml. glucose (1.0 M); 0.04 ml. hexokinase; 0.3 ml. mitochondrial preparation; and KCl (0.9 per cent) to a final volume of 4.0 ml. The hexokinase, prepared according to Berger *et al.* (1946) through step 5B, possessed an activity of 12,000 units per ml. The reaction mixtures were incubated for 1 hour at room temperature. At zero and 60 minutes aliquots of the mixtures were removed and the reaction stopped with 0.1 volume of 1 N HCl. After centrifugation, samples of the clear supernatants were then taken for determinations of ortho-P, acid-labile P (11 minutes' hydrolysis in 1.0 N H_2SO_4 at 100°C.), and AMP. P was measured by the method of Fiske and SubbaRow (1925) and AMP was measured by the method of Kalckar (1947) from the change in optical density at 265 mμ in the presence of AMP deaminase and citrate buffer, pH 6.4. The stock deaminase was prepared by the method of Nikiforuk and Colowick;[3] 0.03 ml. of a 1:10 dilution of this stock was used in a final volume of 3.0 ml. In these experiments the AMP was completely deaminated in approximately 3 minutes.

The dry weight of mitochondria was determined by drying an aliquot of the preparation in an oven at 110°C. for 24 hours. The weights were corrected for salt concentration.

RESULTS

1. Studies with Mitochondria

(*a*) *Effect of Time and Enzyme Concentration.*—The rate at which ATP is dephosphorylated is dependent upon the quantity of enzyme used relative to the amount of ATP. In a typical experiment, the progress of this liberation of orthophosphate for five different mitochondrial concentrations is shown in Fig. 1. It is obvious from the data that the rate at which phosphate is split is not constant. The decrease observed with time is similar to that found with liver homogenates (Novikoff *et al.*, 1952) and with a purified yeast ATPase (Meyerhof and Ohlmeyer, 1952).

It is also apparent from Fig. 1 that with prolonged incubation periods more orthophosphate is liberated than is accountable for by the terminal phosphate. However, evidence will be presented later in this paper to prove that the additional phosphate is not derived from the activity of adenylpyrophosphatase (as defined by Novikoff *et al.*) nor of non-specific phosphatase, but is due to the action of adenylate kinase.

Further, it is evident that the rate at which inorganic phosphate is produced is not linearly related to the enzyme concentration when a high proportion of the available ATP-phosphate is split. This is shown in Fig. 2. These relationships are manifest even when ATP concentration is increased threefold, as shown in Fig. 3.

(*b*) *Effect of Substrate Concentration.*—Comparison of Figs. 1 and 3 reveals that the amount of phosphate split from ATP is also dependent upon the sub-

[3] Nikiforuk, G., and Colowick, S. P., in preparation.

strate concentration. This relationship was therefore examined more closely, with results which are shown in Fig. 4 as a Lineweaver-Burk (1934) plot. As

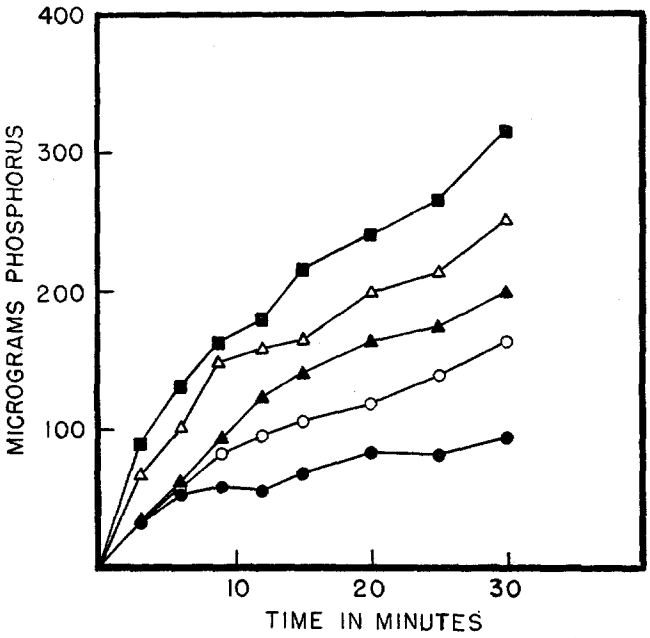

FIG. 1. ATPase activity as a function of time for different mitochondrial concentrations. The following symbols: ■, △, ▲, ○, and ● designate the orthophosphate liberation in the presence of 0.7 ml., 0.5 ml., 0.3 ml., 0.2 ml., and 0.1 ml. respectively, of the mitochondrial preparation (9.3 mg. dry weight/ml.). Reaction mixture was as follows: 1.0 ml. TRIS buffer, pH 7.4 (0.03 M); 0.5 ml. $MgCl_2$ (5×10^{-3} M); 0.5 ml. ATP (stock contained 10 mg./ml.). Final volume made to 10.0 ml. with 0.9 per cent KCl. Room temperature. Labile P = 500 micrograms.

determined by the method of least squares, the experimental points fit very closely the line described by the equation

$$1/v = 0.009 + 0.015527(1/(S)) \quad (1)$$

The Michaelis-Menten constant $K_s = 2.78 \times 10^{-3}$ M, and $V_{max.} = 76$ micrograms P/min./mg. dry weight of mitochondria.

(*c*) *Effect of Activating Ions.*—It has been found that bivalent cations have a considerable effect on ATPase activity of mammalian and yeast preparations. In Fig. 5 are presented data on the ATPase activity of insect mitochondria in the presence of various concentrations of Mg^{++}, Mn^{++}, and Ca^{++}. Each point on the graph is the average of two to four determinations. It is evident that under these experimental conditions the ATPase is activated by

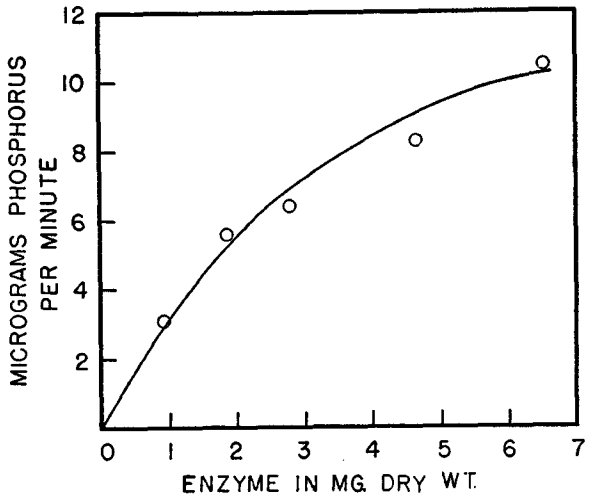

FIG. 2. Rate of production of inorganic phosphate as a function of the mitochondrial concentration. Incubation mixture same as shown in Fig. 1. Time 30 minutes.

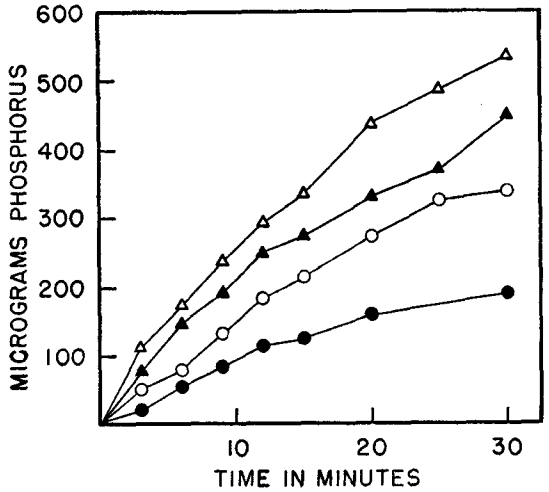

FIG. 3. ATPase activity with increased substrate for different mitochondrial concentrations. The following symbols: △, ▲, ○, and ● designate the orthophosphate liberated in the presence of 0.20 ml., 0.15 ml., 0.10 ml., and 0.05 ml., respectively, of the mitochondrial preparation (13.8 mg. dry weight/ml.). Reaction mixture same as in Fig. 1 except 1.5 ml. ATP stock.

Mg^{++} and Mn^{++}, whereas it is not activated by Ca^{++}. Further, the optimal concentration of Mg^{++} or Mn^{++} is approximately 0.6×10^{-3} M, at which level the activation is about 60 per cent. Concentrations of these ions or Ca^{++} higher than 6×10^{-3} M inhibit. Moreover, experiments with combinations of

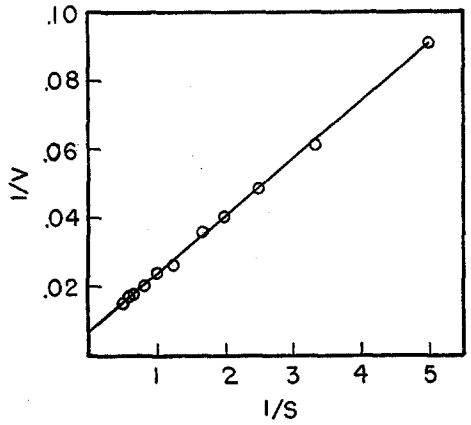

FIG. 4. Lineweaver-Burk plot to show the effect of substrate concentration. *S* expressed as milligrams of ATP. *V* expressed as micrograms P produced in 8 minutes. Incubation mixture is as follows: 0.1 ml. TRIS buffer pH 7.4 (0.03 M); 0.06 ml. $MgCl_2$ (6×10^{-4}); 0.03 ml. enzyme (equivalent to 0.408 mg. dry weight of mitochondria). Final volume made to 1.0 ml. with 0.9 per cent KCl. Room temperature.

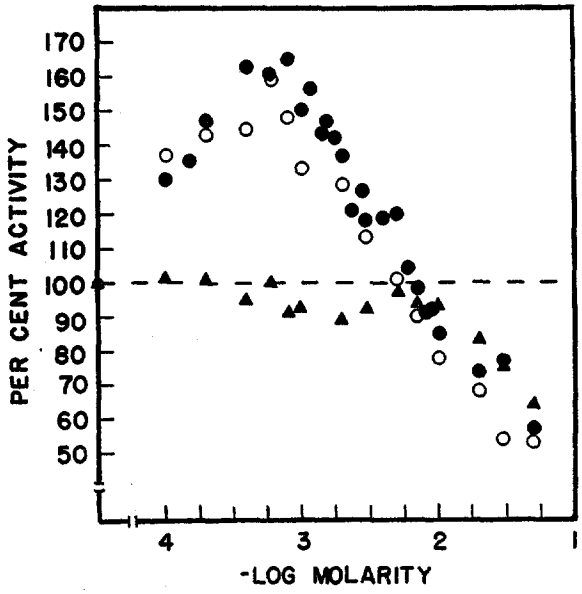

FIG. 5. The effect of bivalent ions on ATPase activity. The following symbols: ●, ○, and ▲, designate Mg, Mn, and Ca, respectively. Incubation at room temperature for 15 minutes. TRIS buffer pH 7.4, ATP 0.05 ml., 0.03 ml. enzyme, and 0.9 per cent KCl to final volume of 1.0 ml. Activity in the absence of bivalent ions expressed as 100 per cent.

Mg^{++} and Mn^{++} show that either ion can replace the other and that the effects are additive, depending solely on the final concentration of the combination.

With respect to optimal concentration and extent of activation, the effects of Mg and Mn ions in these experiments are very similar to those obtained by Meyerhof and Ohlmeyer with crude yeast ATPase. The influence of these ions has also been studied by Swanson (1951), DuBois and Potter (1943), and others. Swanson found that the optimal concentration of Mg^{++} was 5×10^{-3} M, and some activation was still observed even with concentrations as high as 0.1 M. The failure of Ca^{++} to activate insect mitochondrial ATPase is in agreement with the results of Novikoff *et al.* with the ATPase of liver mitochondria and with those of Meyerhof and Ohlmeyer with ATPase from yeast. DuBois

TABLE I

Rate of Splitting Various Phosphate Compounds by House Fly Mitochondria

Substrate	Final concentration	Control	With Mg^{++} 0.001 M	With Ca^{++} 0.001 M
	M × 10^{-3}	*μg. P/hr./mg. dry weight*		
ATP	0.8	238	381	226
ADP	3.0	8	62	11
AMP	3.0	0	0	0
F-6-P	3.0	0	0	0
F-1,6-DP	3.0	0	0	0
G-1-P	3.0	0	0	0
Gly-P	3.0	0	0	0
Inorganic Pyro-P	3.0	0	0	0

The samples with ATP were incubated for 15 minutes; the values obtained were multiplied by 4. Incubations were at room temperature, TRIS buffer, pH 7.4. Control values represent micrograms P liberated in the absence of added bivalent ions.

and Potter, and Swanson, reported activation by Ca^{++} in liver homogenates, although this activation was not so great as with Mg^{++}. In contrast, the specific activation of ATPase from skeletal muscle by Ca^{++} is well known (Bailey, 1942).

(*d*) *The Action of Mitochondria on Various Substrates.*—The specificity of the dephosphorylating mechanism in insect mitochondria is demonstrated in Table I, from which it may be seen that the only substrates utilized by this system are ATP and, to a lesser extent, ADP. It is also clear that Mg^{++} has a profound effect on the rate at which phosphate is liberated from ADP, for in its absence only small amounts of ADP are dephosphorylated.

The course of liberation of orthophosphate from ADP, in the presence of Mg ions, is illustrated in Fig. 6. Each point represents the average of three determinations. The slow initial rate indicates a period of lag before maximum activity; and this observation, plus the necessity of Mg^{++} suggested that the

ADP was not being dephosphorylated by an ADPase, but that adenylate kinase might be present in these mitochondria. This enzyme, previously demonstrated in mammalian mitochondria by Kielley and Kielley (1951), Barkulis and Lehninger (1951), and Novikoff *et al.* (1952), converts 2 molecules of ADP to 1 molecule each of ATP and AMP. The inorganic phosphate found in our preparations when ADP was supplied as substrate might then have resulted from the action of adenylate kinase followed by that of a specific ATPase.

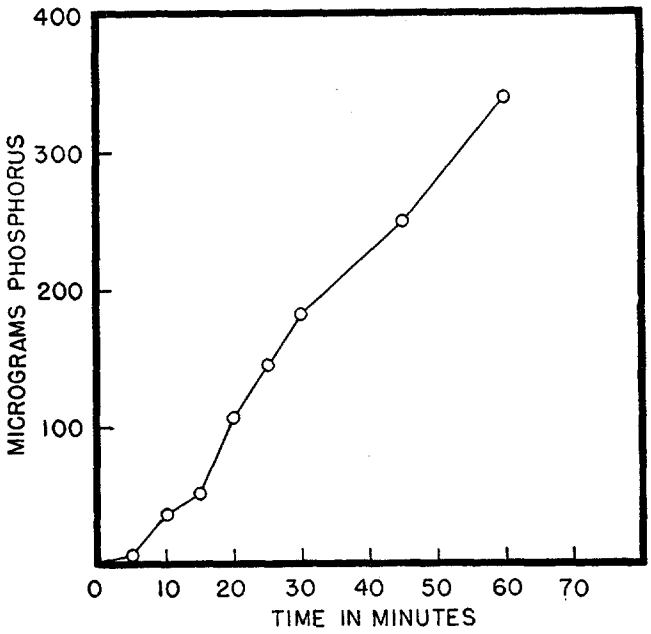

FIG. 6. Orthophosphate liberation from ADP in the presence of Mg ions. 0.3 ml. ADP (0.003 M), 0.1 ml. $MgCl_2$ (1×10^{-2} M), 1.0 ml. TRIS buffer, pH 7.4 (0.03 M), 0.3 ml. enzyme, final volume made to 10.0 ml. with 0.9 per cent KCl. Room temperature.

In order to establish whether insect mitochondria possess adenylate kinase activity and to confirm the specificity of the ATPase, the ADP-mitochondrial system was supplemented with glucose and hexokinase and tested as described above. The results of such experiments are given in Table II. These data show that AMP and ATP are formed from ADP and, when glucose-hexokinase is present to produce glucose phosphate from ATP, no orthophosphate is liberated. Further, the decrease in acid-labile phosphate is approximately equivalent to the AMP produced. These results prove the presence of adenylate kinase activity in insect mitochondria. Moreover, the data establish that ADP is not dephosphorylated directly, but that, by means of the adenylate kinase

reaction, ATP is formed which can then be dephosphorylated by the specific ATPase present. When azide was added to inhibit the ATPase, a pronounced effect of hexokinase on AMP formation and labile P disappearance was observed (Table II, Experiment 1).

(*e*) *Inhibition of Mitochondrial ATPase.*—The effect of some previously reported inhibitors of ATPase on the enzyme in insect mitochondria is shown in Table III. From these results it is evident that NaF, at the concentrations used, does not inhibit the ATPase activity of this preparation. Activity is inhibited, however, by NaN_3 and chloromercuribenzoate. The degree of inhibition depends on the concentration of the agent. Rather similar results were obtained with liver mitochondria by Novikoff *et al.* (1952), and inhibition of yeast ATPase by azide was reported by Meyerhof and Ohlmeyer (1952).

TABLE II

Adenylate Kinase Activity in Insect Mitochondria

Experiment	System	Formation of		Decrease in labile P
		AMP	Ortho P	
			mM	
1A	Complete	3.78	0.12	4.20
1B	" minus glucosehexokinase	1.62	1.87	2.32
1C	" minus mitochondria	0.00	0.10	0.79
2A	Same as 1A, minus azide	3.67	0.00	4.38
2B	" " 1B " "	2.54	4.27	4.82
2C	" " 1C " "	0.00	0.10	0.93

Incubation mixture and details of the experiment are described in the text.
Initial ADP = 6.04 micromoles labile P.

2. *Comparison of Mitochondria with Other Fractions*

The foregoing experiments have partially characterized the ATPase activity of insect mitochondria with respect to enzyme kinetics, and have delineated the effects upon this activity of bivalent ions and some inhibitors. The following section will be devoted to a comparison of the dephosphorylating activity found in mitochondria with such activity in other isolated fractions of the house fly thorax.

(*a*) *Distribution of ATP-Dephosphorylating Activity.*—Since it is known that mammalian skeletal muscle contains an ATPase associated with contractile processes, as well as a "soluble" ATPase, analogous fractions were procured, as described above, from the thorax of flies. The distribution of ATP-dephosphorylating activity in these isolated components was examined, with results shown in Table IV. These data demonstrate that inorganic phosphorus is liberated from ATP by enzymes present in the supernatants and residues of whole thorax homogenates as well as by isolated mitochondria. Moreover, as

TABLE III

Inhibition of Mitochondrial ATPase

Inhibitor	Concentration	P liberated	Inhibition
		μg.	*per cent*
None		28.2	
Na F	1×10^{-2} M	27.3	3
	5×10^{-3} M	27.2	4
	1×10^{-3} M	28.2	0
	5×10^{-4} M	28.5	0
NaN_3	1×10^{-2} M	4.0	86
	5×10^{-3} M	6.2	78
	1×10^{-3} M	6.8	76
	1×10^{-4} M	16.0	47
Chloromercuribenzoate	1×10^{-3} M	12.0	57
	5×10^{-4} M	17.0	40
	1×10^{-4} M	28.8	0
	5×10^{-5} M	28.2	

Incubation mixture was as follows: 0.1 ml. TRIS buffer, pH 7.4 (0.03 M); 0.1 ml. inhibitor (neutralized); 0.06 ml. $MgCl_2$ (0.6×10^{-3} M); 0.05 ml. ATP (stock contained 10 mg./ml.); 0.03 ml. enzyme (equivalent to 0.312 mg. dry weight of mitochondria); final volume made to 1.0 ml. with 0.9 per cent KCl. Incubation time 8 minutes; room temperature.

TABLE IV

Distribution of ATP-Dephosphorylating Activity

Fraction	Control	Mg^{++}	Ca^{++}
		0.6×10^{-3} M	1.0×10^{-3} M
	μg. P/min./mg. dry weight		
Residue of homogenate	5.5	10.9	10.9
Supernatant of homogenate	3.9	11.9	3.2
Mitochondria	10.2	18.7	9.9

Incubation mixture is as described in Table III. Control values obtained in absence of added bivalent ions. Time 8 minutes.

in mitochondria, it is apparent that the "soluble" enzymes are activated by Mg^{++} but not by Ca^{++}. This is in agreement with results on the "soluble" ATPase of mammalian muscle, as reported by Kielley and Meyerhof. In contrast, the residue contains enzymes which are stimulated by both Mg^{++} and Ca^{++}. The residue, however, consists of mitochondria and muscle fibrils; thus, the activation by Mg^{++} can be attributed to the ATPase in these mitochondria,

whereas it becomes evident that insect muscle fibrils, like mammalian muscle, must possess a Ca^{++}-activated ATPase.

(*b*) *Distribution of Inorganic Pyro-Pase Activity.*—It has been shown in Table I that, under the conditions of these experiments, inorganic Pyro-P is not hydrolyzed by insect mitochondria. Swanson (1951) reported, however, that the "soluble" fractions of liver can split Pyro-P. The results of similar tests with the various insect fractions are given in Table V. These show that the residue of the whole thorax homogenate is like the isolated mitochondria in the inability to hydrolyze inorganic Pyro-P. It is noteworthy, however, that the "soluble" fraction can utilize this substrate and that Mg ions are necessary for this activity. According to Swanson (1952), the optimal concentration of Mg^{++} for this reaction is 10^{-2} M. The Pyro-pase of the "soluble" fraction from

TABLE V

Distribution of Inorganic Pyro-Pase Activity

Fraction	Bivalent ion concentration	Control	Mg	Ca
		μg. P/min./mg. dry weight		
Residue of homogenate	1×10^{-3}	0.0	0.0	0.0
Supernatant of homogenate	1×10^{-3}	0.3	4.1	0.0
	1×10^{-2}	0.3	20.0	0.0
Mitochondria	1×10^{-3}	0.0	0.0	0.0

Incubation mixture as described in Table III except that the concentration of inorganic Pyro-P was 3×10^{-3} M. Control values obtained in absence of added bivalent ions. Time 8 minutes.

house flies was therefore tested at this concentration. The results in Table V reveal that there was a fivefold stimulation of enzyme activity in the presence of Mg^{++} (10^{-2} M) as compared with Mg^{++} (10^{-3} M). However, concentrations of Ca^{++} as high as 10^{-2} M still did not elicit any Pyro-pase activity from the supernatant.

(*c*) *Effect of Fluoride on Mg-Activated Inorganic Pyro-Pase Activity.*—The data in Table VI establish that F^- (0.01 M) completely inhibits the Mg-activated inorganic Pyro-pase activity in the supernatant from whole thorax homogenates. This is in accord with Swanson's (1952) report of a similar effect of F^- on the inorganic Pyro-pase activity of liver. Our results show further that even with Mg^{++} (10^{-2} M) no orthophosphate or at most an insignificant amount is liberated from inorganic pyrophosphate by house fly mitochondria or by the residues of whole thorax homogenates.

(*d*) *The Nature of the ATPase Activity in the "Soluble" Fraction.*—The "soluble" fraction of the house fly thorax is distinguished from the other prep-

arations by the presence of considerable inorganic Pyro-pase activity in association with the ability to dephosphorylate ATP. It is possible, therefore, that orthophosphate liberated from ATP by the "soluble" fraction has been produced *via* the preliminary formation of inorganic pyrophosphate, perhaps in a manner resembling that described by Zeller (1950) or Kornberg (1950), followed by hydrolysis of the pyrophosphate to orthophosphate.

Some support for such an hypothesis has been obtained through the use of fluoride. It was shown above (Table III) that F^- does not affect the specific ATPase found in house fly mitochondria, whereas 0.01 M F^- inhibits completely the Pyro-pase activity of the supernatant fraction from whole thoraces (Table VI).

TABLE VI

Effect of Fluoride on Mg-Activated Inorganic Pyro-Pase Activity

Fraction	Mg^{++} concentration	μg. P/min./mg. dry weight
Residue of homogenate	10^{-3}	0.0
	10^{-2}	0.0
	10^{-2} plus F^-(10^{-2} M)	0.0
Supernatant of homogenate	10^{-3}	2.2
	10^{-3} plus F^-(10^{-2} M)	0.0
	10^{-2}	29.5
	10^{-2} plus F^-(10^{-2} M)	0.4
Mitochondria	10^{-3}	0.0
	10^{-2}	0.7
	10^{-2} plus F^-(10^{-2} M)	0.5

Incubation mixture as described in Table III. Concentration of inorganic Pyro-P was 3×10^{-3} M. Time 8 minutes.

Accordingly, 0.8×10^{-3} M ATP and 10^{-2} M Mg^{++} in the standard assay system were incubated for 8 minutes with the "soluble" fraction in both the presence and absence of F^-. The following results were obtained:

	μg. P/min./mg. dry weight
Control	9.9
F^- (0.01 M)	3.6

These data show that five-eighths of the ATP-dephosphorylating activity of the "soluble" preparation is inhibited by F^-. The implications of this observation are discussed below.

As for the fluoride-insensitive activity remaining in the supernatant, it was suspected that this might be due to specific ATPase derived from fragmented mitochondria. As mentioned above, such particles fail to sediment during the

isolation procedure. To test the supposition, a pure preparation of mitochondria was fragmented as previously described and separated into two fractions, one including intact mitochondria and the larger particles, the other, smaller particles only. Comparison of the ATPase activities of these two particulate fractions yielded the following information:

	μg. P/min./mg. dry weight
Small mitochondrial fragments	49.0
Larger particles and intact mitochondria	17.8

In addition F^- has no effect on these activities. These facts do indeed suggest that the fluoride-insensitive ATPase activity in the "soluble" fraction of whole thorax homogenates is associated with the small particles of fragmented mitochondria likely to be present in such preparations.

Of even greater interest, however, is the observation that the finely dispersed mitochondrial constituents have much greater ATPase activity, in proportion to their weight, than does the fraction composed of larger fragments and intact sarcosomes.

DISCUSSION

The data presented demonstrate that in the isolated mitochondria of the house fly there is a magnesium-activated, specific ATPase. An assay for this enzymatic reaction has been achieved by observing the precautions noted by DuBois and Potter, namely, short incubation periods and low enzyme concentration. Under these conditions, it has been shown that ATP is the only substrate which is dephosphorylated. A high level of adenylate kinase activity also was found in these mitochondria. It has been demonstrated that orthophosphate liberated when ADP is the substrate results from conversion of ADP to ATP and AMP, followed by ATPase action.

Moreover, it is evident that the hexokinase reaction can serve effectively to trap newly synthesized ATP. This was established through the use of an active hexokinase preparation which competed successfully with ATPase for the ATP generated from added ADP. The failure thus far to observe oxidative phosphorylation in insect mitochondria supplemented with hexokinase cannot therefore be attributed to splitting of generated ATP by ATPase. Further, since it has been shown that glucose and hexokinase are capable of reacting with mitochondrial constituents, it is obvious that the reactants must have come into close juxtaposition. This fact suggests that the finding of Williams and Watanabe (1952), that the mitochondrial membrane is impermeable to glucose and proteins, may have little metabolic significance.

Kielley and Kielley have observed a latent ATPase in liver mitochondria. Their determinations of this ATPase activity indicated that the freshest and least damaged preparations were always the least active. Ageing the enzyme

caused relatively enormous increases. This disclosure can be correlated with the present finding that finely dispersed mitochondrial particles have much greater ATPase activity than the intact or large particulate fraction, which suggests that the effect of ageing reported for liver mitochondria might well be due to lysis that resulted in the formation of small-sized, active particles. The important influence of the physical state of the enzyme preparation on the mutual accessibility of the components of the system has been demonstrated clearly by Keilin and Hartree (1949).

Our results with isolated fractions of the insect thorax establish the presence of several different enzymes capable of dephosphorylating ATP. In the mitochondria, there occurs a specific ATPase activated by Mg^{++} or Mn^{++}, but not by Ca^{++}. The muscle fibrils, in contrast, contain a calcium-activated ATPase.

In the supernatant fraction, however, the "soluble" ATPase is also Mg^{++}-activated. Associated with this ATPase is an active inorganic pyrophosphatase, which is completely inhibited by F^-. Moreover, F^- (0.01 M) can prevent approximately 60 per cent of the ATP dephosphorylation in this "soluble" fraction although it is without inhibitory effect on the specific ATPase of the mitochondria.

It is tempting to speculate concerning the nature of the fluoride-sensitive reaction. Several alternate pathways, or combinations of these, seem logical:

1. There may be present in the supernatant a magnesium-activated ATPase which differs from the one occurring in the mitochondria by being water-soluble as well as by being inhibited by fluoride. These properties would render it similar to the enzyme described by Kielley and Meyerhof from muscle.

2. Other hypotheses involve the preliminary production of inorganic pyrophosphate, (*a*) as found in snake venom by Zeller (1950),

$$\text{ATP} \rightarrow \text{AMP} + \text{P-P};$$

or (*b*), as described by Kornberg (1950),

$$\text{NMN} + \text{ATP} \rightarrow \text{DPN} + \text{P-P}.$$

The inorganic pyrophosphate thus produced would then be hydrolyzed to orthophosphate by the inorganic Pyro-pase present in this fraction. Further investigation from these various points of view will be required before a satisfactory interpretation of the actual mechanism can be reached.

The 40 per cent of ATP dephosphorylation not inhibited by fluoride may, at least for the present, be attributed to fragments of mitochondria remaining in the supernatant fraction.

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SUMMARY

1. The ATPase activity of insect mitochondria has been investigated. A comparison was made to determine the distribution and nature of such activity in other isolated fractions of the house fly, *Musca domestica* L.

2. The ATPase in insect mitochondria is specific in that orthophosphate can be cleaved only from ATP. The Michaelis-Menten constant $K_s = 2.78 \times 10^{-3}$ M and $V_{max.} = 76$ micrograms P min.$^{-1}$ mg.$^{-1}$ dry weight.

3. Mg^{++} and Mn^{++} activate this enzymatic reaction in mitochondria, but Ca^{++} does not. The extent of activation is 60 per cent with the optimal concentration 6×10^{-4} M. Experiments with combinations of Mg^{++} and Mn^{++} show that either ion can replace the other and that the effects are additive, depending solely on the final concentration of the combination. Concentrations of Mg, Mn, or Ca ions higher than 6×10^{-3} M inhibit the enzyme.

4. Fluoride does not inhibit the ATPase of insect mitochondria, whereas azide and chloromercuribenzoate do. The per cent inhibition depends on the concentration of inhibitor.

5. Finely dispersed mitochondrial particles have much greater ATPase activity than intact mitochondria. The possible relationship of this observation to latent ATPase is considered.

6. A magnesium-activated adenylate kinase is present in these mitochondria. The liberated orthophosphate, derived from ADP, is the result of the activity of adenylate kinase followed by the specific ATPase.

7. ATP can be dephosphorylated by enzymes found in the muscle fibrils, and in a "soluble" fraction, as well as in mitochondria. The fibrillar ATPase is Ca^{++}-activated. The "soluble" fraction, however, like the mitochondria, is Mg^{++}-activated. The "soluble" ATP dephosphorylation mechanism is distinguished from the mitochondrial ATPase in that it is inhibited by fluoride.

8. The "soluble" fraction also contains a magnesium-activated inorganic pyrophosphatase. Fluoride completely inhibits this enzymatic reaction.

9. The possible mechanism of ATP dephosphorylation in the "soluble" fraction is discussed.

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