INVESTIGATIONS ON THE MITOCHONDRIA OF THE HOUSEFLY, MUSCA DOMESTICA L.

III. Requirements for Oxidative Phosphorylation

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The mitochondria (sarcosomes) of flight muscle in the blowfly, *Phormia regina*, and housefly, *Musca domestica*, have been shown to contain high titres of cytochromes *a*, a_3, *b*, and *c*, as well as an active cytochrome *c* oxidase and catalase (Watanabe and Williams, 1951; Chance, 1952; Sacktor, 1952, 1953 *b*, 1953 *c*). Moreover, oxygen was consumed by sarcosome preparations in the presence of malic, pyruvic, α-glycerophosphoric, α-ketoglutaric, and oxaloacetic acids; and adenylate kinase activity was also found (Watanabe and Williams, 1951; Sacktor, 1953 *a*). Subsequently, the presence of malic dehydrogenase, $DPNH_2$ oxidase,[1] and malic and succinic-cytochrome *c* reductase activities was demonstrated (Sacktor, 1953 *c*).

These observations indicated the presence in the mitochondria of a complete oxidative system such as would be required for oxidative phosphorylation, but efforts to demonstrate such a process were inconclusive (Sacktor, 1953 *a*). Although the negative result could be attributed in part to the presence of an extremely active ATPase, further study showed that this could not be the sole cause, for it was established that an active hexokinase preparation was capable of competing successfully with ATPase for ATP generated from ADP and inorganic phosphate. Direct evidence for the occurrence of oxidative phosphorylation in an insect has now been obtained, and is presented in this paper, in which some of the necessary conditions are characterized.

EXPERIMENTAL

Isolation of Mitochondria

Houseflies, mixed sexes, 4 to 7 days old, were taken from a laboratory culture maintained as described previously (Sacktor, 1950). Their sarcosomes were

[1] The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, muscle adenylic acid; tris, tris-(hydroxymethyl) aminomethane; αkg, α-ketoglutaric acid; DPN, diphosphopyridine nucleotide; P, inorganic phosphate; P/O, the ratio of the uptake of oxygen (in microatoms) to the decrease of inorganic phosphate (in micromols).

isolated essentially by the same simple technique already detailed (Sacktor, 1953 *a*). Unless stated otherwise, this method was modified by isolating and washing the mitochondria in ice cold 0.25 M sucrose, instead of 0.2 M sucrose, followed by washing with 0.9 per cent KCl. Because of this modification in handling the preparation the diameter of the sarcosomes, previously reported as 3 to 8 micra (Sacktor, 1953 *a*), was changed to 2 to 3 micra. As seen from Table I, the abnormally large size (3 to 8 micra) was due to swelling of the mitochondria in the hypotonic extracting and washing solutions used earlier. By the method employed here, microscopic examination of the mitochondrial suspension revealed a practically pure preparation of sarcosomes.

In some experiments, "soluble" fractions and whole thoracic homogenates were utilized. These were made by the methods described earlier (Sacktor, 1953 *a*).

Materials

ATP was obtained from Pabst Laboratories; ADP, AMP, cytochrome *c*, and tris buffer from Sigma Chemical Company; αkg from Nutritional Biochemical Company; Folin-Ciocalteu phenol reagent and creatine from Fisher Scientific Company; *l*-arginine from H. M. Chemical Company; and crystalline bovine serum albumin from Armour and Company. All materials, when obtained in the form of Ba salts, were converted to their Na salts with Na_2SO_4. The solutions were then adjusted to pH 7.4. Deionized water was used to prepare all solutions.

Hexokinase was prepared from bakers' yeast by the method of Loomis (unpublished). This active preparation contained very little ATPase.

Methods

Oxygen consumption measurements were made in a conventional Warburg apparatus. After a few preliminary experiments, it was found expedient to use the mitochondria extracted from 50 thoraces per manometer cup. The temperature was 25°C. and the gas phase was air. All the constituents of the reaction mixture, except hexokinase, were localized in the main compartment of flasks kept in chipped ice. The hexokinase was placed in the side arm. As soon as the mitochondrial suspension was prepared, aliquots were added to the main compartment and the flasks were mounted on the water bath. The hexokinase was tipped 5 minutes later. After an additional 5 minute equilibration period the stopcocks were closed (zero time) and oxygen consumption readings were begun. The mitochondria were allowed to respire for 30 minutes, at which time the flasks were rapidly removed from the bath and placed in chipped ice. Immediately, 0.2 ml. of cold 50 per cent TCA was added. The precipitates were centrifuged down, discarded, and the supernatants analyzed for inorganic phosphate. The quantity of inorganic phosphate esterified was computed by subtracting the inorganic phosphate values at the end of the experiment from the value obtained on a corresponding zero time sample. Inorganic phosphate was assayed by the method of Sumner (1944) in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. In those experiments in which creatine or arginine was

a constituent of the reaction mixture, inorganic phosphate was determined by the less acidic technique of Lowry and Lopez (1946), as modified by Siekevitz and Potter (1953 *a*).

ATPase activity of mitochondrial preparations was assayed by the previously described method (Sacktor *et al.*, 1953) The incubation mixtures contained: 0.1 ml. tris (0.3 M), pH 7.4; 0.06 ml. $MgCl_2$ (0.01 M); 0.03 ml. ATP (0.05 M); 0.03 ml. mitochondrial preparation; and 0.78 ml. 0.9 per cent KCl. Reaction time was 10 minutes.

Protein, in mitochondrial suspensions and other fractions, was determined by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the standard protein.

TABLE I

Effect of Isolation Media on Oxidative Phosphorylation

Medium	Morphology of mitochondria	ΔO	ΔP	P/O	O_2/mg. Protein/ hr.	ATPase P/mg. protein/ 10 min.
		μatoms	*μM*		*c.mm.*	*μM*
Distilled H_2O	Lysed	0.9	+2.3	0.0	4.6	1.81
0.9 per cent KCl	Considerably swollen but intact	5.3	6.0	1.1	23.3	2.21
0.25 M sucrose	Normal (2–3 micra)	5.4	8.5	1.6	17.8	1.67
0.25 M sucrose and washed in 0.9 per cent KCl	Moderately swollen but intact	5.8	6.7	1.2	20.6	1.88
0.8 M sucrose	Shrunken	1.1	+0.4	0.0	8.0	2.35

The reaction mixture was as follows: phosphate buffer, pH 7.4, 30 μM; $MgCl_2$, 20 μM; αkg, 30 μM; glucose, 100 μM; ATP, 5 μM; 0.25 ml. serum albumin (20 per cent); 0.3 ml. hexokinase solution; 0.5 ml. mitochondrial suspension; 0.15 ml. NaOH (5 N) in center well; 0.9 per cent KCl to a final volume of 2.5 ml. Reaction time was 30 minutes.

All data reported in this paper are based on at least four separate determinations, each of which was made on a different day with a different mitochondrial preparation.

RESULTS

1. The Influence of the Isolation Medium.—By the use of different isolation media, alterations in the morphology of the sarcosomes; *i.e.*, changes in the form and density, can be produced. These mitochondrial configurations have been related to the spatial organization of the enzymes within the mitochondria and, in turn, to the metabolic capacity of these cellular constituents (Harman and Feigelson, 1952). Therefore, it seemed logical to explore the effect of various isolation media on the ability of insect sarcosomes to effect oxidative phosphorylation. In addition to this coupling phenomenon, the role of the physical state of the mitochondria with respect to oxidation, phosphorylation, and ATPase activity could be evaluated. The results of these experiments are shown in Table I.

As seen from this table, the molarity of the isolation medium had considerable influence on the configuration of insect mitochondria. These effects were rapid. Within 30 minutes of beginning the preparation, morphological alterations could be observed. In 0.25 M sucrose, the sarcosomes were well rounded, dense, and 2 to 3 micra in diameter. When isolated, or subsequently washed, in 0.9 per cent KCl, the sarcosomes became swollen and much paler. In 0.8 M sucrose, the mitochondria appeared shrunken, and irregular in outline. Distilled water completely disrupted the normal morphology of the mitochondria; many empty "ghosts" and minute mitochondrial constituents were observed. Similar morphological observations on the effect of these and other media on sarcosomes of *Phormia* and *Drosophila* were made by Watanabe and Williams (1953).

Associated with these physical aberrations, the enzymatic capacity of the mitochondria was markedly altered (Table I). The maximum coupling ability was found to occur with mitochondria isolated and washed in 0.25 M sucrose (P/O = 1.6). Sarcosomes, isolated either in distilled water or hypertonic sucrose, could no longer effect oxidative phosphorylation. Mitochondria, isolated or washed in saline, exhibited reduced phosphorylation, which resulted in lowered P/O.

It was also noted that the molarity of the medium affected the oxidation and the phosphorylation of the mitochondria to different extents. The fall in phosphorylation exceeded the drop in oxidation in those mitochondria which were prepared in water or hypertonic sucrose. In these experiments there was no net uptake of phosphate; in fact, more inorganic phosphate was found at the end of the incubation period than had been placed in the reaction mixture. This excess phosphate was produced by the dephosphorylation of added ATP. In contrast, when saline was used, oxidation, calculated on a protein basis, was normal or even slightly stimulated. Phosphorylation, however, was curtailed.

These results also indicate the presence of a "latent" ATPase in insect sarcosomes, for it was shown that mitochondria extracted in isotonic sucrose had the greatest phosphorylating activity and this coincided with the least ATPase activity. This inverse relationship occurred with the other isolation media. A similar association between oxidative phosphorylation and ATPase activity had been observed by Kielley and Kielley (1951) and others in liver mitochondria. An apparent exception to this rule was noted in the present experiments when distilled water was used. This caused the mitochondria to lyse; and, when lysed, some of the constituents of the sarcosomes remained in the supernatant fraction during the isolation procedure (Sacktor, 1953 *a*). Furthermore, this fraction possessed considerable ATPase activity. Therefore, the low ATPase activity noted in the distilled water experiments of the present series was probably due to the loss during preparation of an active ATPase portion of the mitochondria.

2. The Effect of Serum Albumin.—The preceding section has demonstrated the important relationship existing between the physical state of the mitochondria and their ability to effect oxidative phosphorylation. Moreover, it was found that the mitochondria needed to be further stabilized by the addition of an inactive protein to the reaction mixture even though an optimal concentration of sucrose was used as the isolation medium. In the absence of a supplementary protein, degenerative changes of the mitochondria began almost immediately and lysis was complete within 30 minutes. A satisfactory environment was provided by adding crystalline bovine serum albumin to the reaction mixture, into which the mitochondria were pipetted as soon as they had been isolated. The effects of different concentrations of this protein are shown in Table II.

It was apparent from the data that the maintenance of a critical environment throughout the incubation period, and not merely during the isolation

TABLE II

Effect of Serum Albumin

Final concentration	ΔO	ΔP	P/O
per cent	*μatoms*	*μM*	
0.0	4.4	+4.3	0.0
0.5	5.9	6.9	1.2
1.0	5.5	7.9	1.4
1.5	5.7	8.8	1.5
2.0	6.2	10.5	1.7

Reaction mixture same as that in Table I.

procedure, was necessary for oxidative phosphorylation. In the absence of serum albumin, not only was the uptake of phosphate completely abolished, but deleterious effects on the oxidative capacity of the mitochondria were observed. On the other hand, no significant changes in oxygen consumption occurred when serum albumin concentration was varied from 0.5 to 2.0 per cent, but marked reductions in phosphorylation were noted in the lower serum albumin concentrations.

3. Time Course of Oxidative Phosphorylation.—One of the first requisites in a systematic investigation of oxidative phosphorylation in insects was to determine the linearity with respect to time of the oxygen consumption and the uptake of inorganic phosphate. The results of such a study are shown in Table III. The data demonstrated that during the observed periods both oxygen uptake and the loss of inorganic phosphate proceeded at approximately constant rates; therefore, P/O remained the same throughout the experiments. These measurements of oxygen and phosphate uptake were begun at the same instant. This, as described in a previous section, was 10 minutes after the addition of the

mitochondria. In some experiments with mammalian preparations, measurements for the disappearance of inorganic phosphate were begun upon addition of mitochondria, but determinations of oxygen consumption were started after thermal equilibration. This latter procedure would result in higher P/O.

4. The Effect of Substrate Concentration.—In all the experiments reported in this paper only one substrate, α-ketoglutaric acid, was used. The effects of

TABLE III

Time Course of Oxidative Phosphorylation

Time	ΔO	ΔP	P/O
min.	*μatoms*	*μM*	
15	2.1	4.0	1.9
20	3.1	6.2	2.0
25	3.7	7.0	1.9
30	4.5	8.0	1.8

The reaction mixture was as follows: phosphate buffer, pH 7.4, 50 μM; $MgCl_2$, 20 μM; αkg, 30 μM; cytochrome *c*, 0.026 μM; glucose, 100 μM; ATP, 5 μM, 0.25 ml. serum albumin (10 per cent); 0.3 ml. hexokinase solution; 0.5 ml. mitochondrial suspension; 0.15 ml. NaOH (5 N) in center well; 0.9 per cent KCl to a final volume of 2.5 ml.

TABLE IV

Effect of Substrate Concentration

Substrate	ΔO	ΔP	P/O
μM	*μatoms*	*μM*	
0	3.1	0.3	0.1
3	3.3	7.4	2.2
6	4.2	8.0	1.9
12	4.7	7.7	1.6
18	4.3	7.2	1.7
24	4.4	6.8	1.5
30	4.7	8.4	1.8

The reaction mixture was the same as that reported in Table III, except that no cytochrome *c* was added.

different concentrations of this metabolite are shown in Table IV. The data revealed the necessity of a suitable oxidizable substrate for, when αkg was not added to the reaction mixture, phosphorylation did not occur. Furthermore, it was evident that, with the observed oxidative rate and the 30 minute incubation period, the substrate concentration could be varied from 3 to 30 μM without any marked effect on the disappearance of inorganic phosphate. When the αkg concentration was reduced to but 3 μM, the rate of oxidation was also lowered.

In addition, this table shows that, even in the absence of added substrate, considerable oxygen uptake occurred. When no substrate was added, there was practically no oxygen consumption at the beginning of the reaction but during the course of the experiment the rate of oxygen uptake increased so that almost a normal rate was obtained at the end. In contrast, in the presence of substrate, oxidation proceeded linearly with respect to time throughout the experimental period. A comparison of the rate of oxygen uptake in the presence and absence of αkg is shown in Fig. 1. It was also observed that the sharp increase in the rate of oxidation, in the absence of substrate, coincided with abnormal changes in the mitochondria. After 10 minutes the mitochondria had ruptured completely. Following this disorganization of the mitochondria, oxida-

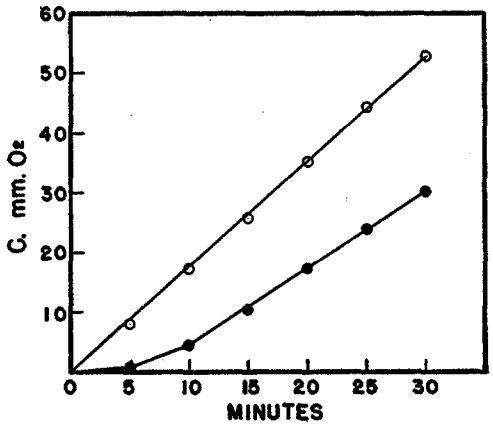

FIG. 1. A comparison of the rate of oxygen consumption in the presence (open circles) and absence (closed circles) of substrate. The reaction mixture was the same as reported in Table IV.

tion continued at an increased rate not greatly different from that which was found when substrate was present. These observations indicate the necessity of available substrate for preservation of mitochondrial structure, and suggest also that the mitochondria possess metabolites or precursors, capable of being oxidized, that are not available to the oxidizing enzyme systems in the spatially organized or intact mitochondria.

Because in the absence of added substrate the mitochondria were ruptured and, following this disorganization, marked increases in oxygen uptake were noted, a satisfactory evaluation of the endogenous respiration of intact mitochondria could not be obtained. Therefore, no corrections for the endogenous respiration were made in the other experiments in this paper. Since no corrections were made, a higher oxygen consumption would be indicated than would have resulted actually from αkg oxidation; this had the effect of lowering

P/O. The inability to make this correction in the oxygen uptake data, however, might not be significant in view of the low oxidative rate when the mitochondria were still intact, as shown in the first 5 minutes in Fig. 1.

5. The Effect of Inorganic Phosphate.—Although the question whether phosphate is an obligatory component for citric acid cycle oxidations has yet to be settled, the regulatory role of phosphate in metabolic reactions has been studied by many investigators (*cf.* Hunter, 1951). The data shown in Table V demonstrate that in general no significant differences in respiration of insect mitochondria were found in the presence or absence of added inorganic phosphate. Furthermore, inorganic phosphate, varied from 20 to 50 μM, had no considerable effect on phosphate uptake. An exception to this indifference towards phosphate was noted when but 10 μM was added to the Warburg flasks. In this case, it was observed that oxygen consumption was increased while essentially

TABLE V

Effect of Inorganic Phosphate

Phosphate	ΔO	ΔP	P/O
μM	*μatoms*	μM	
0	5.0	—	—
10	6.7	9.9	1.5
20	5.1	9.3	1.8
30	6.0	10.7	1.8
40	5.6	11.4	2.0
50	5.2	8.7	1.7

The reaction mixture was the same as that reported in Table III, except that no cytochrome *c* was added. 0.1 M tris buffer, pH 7.4, replaced the phosphate buffer.

complete phosphorylation was obtained. The stimulation in oxidative rate as well as the unavailability of additional inorganic phosphate resulted in a reduced P/O ratio.

The lack of an effect on respiration in the absence of added inorganic phosphate was similar to the results of Crane and Lipmann (1953) with liver mitochondria; but Lardy and Wellman (1952) had shown that when most of the original phosphate had been esterified, a new, slower rate of oxidation then occurred and that the addition of phosphate before the respiratory rate had dropped permitted a high rate of oxidation to be maintained. This apparent discrepancy can be partially reconciled by other studies on the ATPase activities of the various preparations. Whereas the liver mitochondria used by Lardy and Wellman possessed little ATPase activity, housefly mitochondria were extremely active in hydrolyzing added ATP. Thus, in our experiments, although inorganic phosphate was not added to the reaction mixture, some was undoubtedly present due to the dephosphorylation of ATP. No satisfactory

explanation can be offered at present for the observed stimulation in oxygen consumption when only 10 μM of inorganic phosphate was added. Further studies, including nucleotide determinations for phosphate acceptors, may aid in clarifying this point.

6. The Role of Magnesium in Oxidative Phosphorylation.—It has been established (Cross *et al.*, 1949) that Mg ions are essential for optimal oxidative phosphorylation in mammalian mitochondria. The data presented in Table VI demonstrate that Mg is required in insect sarcosomes too. In the absence of added Mg not only was phosphorylation curtailed but a reduction in oxidation was also observed. By varying Mg from 5 to 20 μM, it was found that, although respiration was not influenced, the disappearance of inorganic phosphate was accentuated with the higher concentrations. The data show further that neither Ca nor Mn could replace Mg. In fact, the presence of Ca or Mn

TABLE VI

The Role of Magnesium in Oxidative Phosphorylation

Cation	Concentration	ΔO	ΔP	P/O
	μM	*μatoms*	μM	
None	—	5.1	5.2	1.0
Mg	5	5.6	7.0	1.3
Mg	10	5.5	8.0	1.5
Mg	20	5.5	8.8	1.6
Mn	20	2.7	0.0	0.0
Ca	20	3.5	0.0	0.0

The reaction mixture was the same as that reported in Table I, except that 0.25 ml. serum albumin (10 per cent) was used.

completely abolished phosphorylation and caused a considerable inhibition of oxygen uptake; this inhibition was even greater than that obtained in the absence of Mg. These results suggested the possibility that Ca and Mn, in addition to their effect *per se*, might also be competing with the endogenous Mg present in the mitochondria. This possibility is partially ruled out by subsequent experiments wherein various combinations of Mg and Ca were added simultaneously. It was found that either 10 or 20 μM Ca, when in combination with either 10 or 20 μM Mg, caused the same detrimental effect to both oxygen and phosphate uptakes.

In the course of these experiments with Ca, it was noticed that Ca precipitated some of the phosphate which was also added to the reaction mixture. The removal of phosphate should not result in any marked decrease in oxygen consumption since, as previously shown in Table V, the absence of inorganic phosphate had little effect on respiration. However, further to eliminate this possibility 10 μM of Ca was added to flasks also containing from zero to 90

μM phosphate. The results showed that, regardless of the phosphate concentration, Ca still had the same adverse action.

The inhibitory effect of Ca ions on oxidative phosphorylation in mammalian mitochondria had been noted by Lehninger (1949) and Slater (1952). In addition, Slater was able to stabilize oxidative phosphorylation in heart muscle sarcosomes with the use of a calcium-chelating agent, versene (ethylene diaminetetraacetate). Because of the adverse effects of Ca in insect mitochondria, several experiments were conducted in which housefly sarcosomes were isolated in 0.25 M sucrose containing 0.01 M versene. In contrast to Slater's results, it was found, here, that versene completely inhibited both respiration and phosphate uptake. Some insight into this phenomenon was obtained from the observations of Watanabe and Williams (1953), who found that versene destroyed the normal configuration of blowfly mitochondria. Other differences between insect and mammalian mitochondria are apparent. In contrast to the marked inhibition of oxygen consumption in the presence of Ca, Siekevitz and Potter (1953 *b*) found that low concentrations (3×10^{-4} M) of Ca stimulated whereas higher concentrations (8×10^{-4} M) depressed the respiration of liver mitochondria. This was attributed to the effect of Ca in activating the ATPase of their preparation. This cannot be a satisfactory explanation of the present results since it has been shown previously (Sacktor, 1953 *a*) that Ca does not activate the ATPase or adenylate kinase activities in housefly mitochondria.

7. The Effect of Cytochrome c.—Exogenous cytochrome *c* is often added to reaction mixtures for the study of oxidative phosphorylation with mammalian mitochondria. Furthermore, in a previous study on the oxidation of reduced DPN with insect sarcosomes (Sacktor, 1953 *c*) it was found that the addition of cytochrome *c* produced a three- to fourfold increase in the rate of oxidation. In view of these facts, the effect of cytochrome *c* on oxidative phosphorylation with insect mitochondria was investigated. The results of this study show that the addition of cytochrome *c* had no effect on either respiration or phosphorylation. This implies that insect sarcosomes have ample endogenous cytochrome *c*; the concentration within the mitochondria was not limiting the over-all oxidation of αkg. These results support the findings of Chance (1952), who found that insect sarcosomes had even more endogenous cytochrome *c* than did heart muscle preparations. Moreover, since exogenous cytochrome *c* increased the rate of $DPNH_2$ oxidation whereas it had no effect on αkg oxidation, and since DPN is a constituent of the αkg oxidase system (Sanadi and Littlefield, 1953), these results suggest that, in insect mitochondria at least, the reduction of DPN is the limiting factor in the over-all αkg oxidase system.

8. The Effects of Phosphate Acceptors and Trapping System.—In the preceding experiments, ATP and the glucose-hexokinase trapping system were used. The following sections are concerned with the influence of the three adenine nucleotides and an effective trapping system on oxidative phosphorylation with insect mitochondria.

The requirement of a nucleotide for phosphate uptake was well known from previous work with mammalian mitochondria. The data shown in Table VII demonstrate the necessity of a nucleotide for oxidative phosphorylation in insect mitochondria too. In the absence of ATP, or ATP plus hexokinase, no phosphorylation occurred. The failure to obtain any phosphate uptake in the presence of hexokinase, but in the absence of ATP, suggests that, if these mitochondria possess endogenous nucleotides, these nucleotides are unable under these conditions to effect phosphorylation or to react with added hexokinase at an appreciable rate. It was also noted that, when ATP was omitted from the reaction mixture, there was no significant effect on respiration. It is difficult to relate this latter observation to the results of similar experiments with mammalian preparations. Lehninger (1949) reported that the rate of β-hydroxybutyrate oxidation was not diminished by omitting adenylate

TABLE VII

Effects of Nucleotide and Trapping System

Experiment	ΔO	ΔP	P/O
	μatoms	*μM*	
Hexokinase, no ATP	6.0	0.0	0.0
No hexokinase nor ATP	6.9	0.0	0.0
ATP (5 μM) + hexokinase	6.5	11.2	1.7
ATP (5 μM)	6.3	+3.1	0.0
ADP (5 μM) + hexokinase	6.5	9.9	1.5
ADP (30 μM)	4.2	+3.9	0.0
AMP (5 μM) + hexokinase	6.6	9.6	1.5
AMP (30 μM)	5.3	0.3	0.1

The reaction mixture was the same as that reported in Table I.

whereas Green (1949) found that αkg oxidation was retarded about 60 per cent in the absence of adenylate. To further confuse the situation, Lardy and Wellman (1952) described experiments in which curtailments, stimulations, or no differences of respiration were obtained when a comparison was made between the absence or presence of ATP in the reaction mixture. It is obvious that additional experiments are needed to settle this question.

It was evident from Table VII that ADP and AMP, as well as ATP, were capable of participating in oxidative phosphorylation. The data also demonstrated the necessity of a trapping system, for only when ATPase activity was curtailed by the use of hexokinase could a net phosphorylation be shown. In fact, when ATP or ADP were used and hexokinase was not added, more inorganic phosphate was found at the end of the incubation period than had been placed in the reaction mixture. This was due to ATPase and adenylate kinase activities in these sarcosomes (Sacktor, 1953 *a*). The fact that no excess of inorganic phosphate was found when AMP was added showed that AMP

cannot be dephosphorylated, as was shown also by previous experiments (Sacktor, 1953 *a*).

When hexokinase was added to the reaction mixture (Table VII), a slight but significant increase in phosphorylation was found with ATP as compared with ADP or AMP. Irrespective of the adenine nucleotide, however, respiration was the same. Without the use of a trapping system this was not the case. Here, oxygen uptake was greater in the presence of ATP than when AMP or ADP was added. Thus, by contrasting oxygen consumption with each of the nucleotides in the presence or absence of hexokinase, it can be seen that the hexokinase phosphate acceptor system did not cause a marked stimulation of respiration when ATP was the nucleotide, but did when ADP or AMP was used, although in the presence of hexokinase the final level was the same with each. A marked increase in oxygen consumption on addition of hexokinase was also found with all 3 nucleotides by other investigators (Rabinovitz *et al.*, 1951; Lardy and Wellman, 1952; and Siekevitz and Potter, 1953 *b*). This stimulation had been attributed by them to the action of hexokinase in accelerating the conversion of ATP to ADP and thus providing phosphate acceptors. Since insect mitochondria dephosphorylate ATP to ADP rather easily, a ready supply of ADP must have been available. On the other hand, this possibility is partially refuted by the data in Table VII where it is shown that in the absence of nucleotides respiration was essentially unaffected. Also, Harman and Feigelson (1952) showed that oxidation was accelerated in the presence of ATP but not with AMP when hexokinase was used. These results are directly opposed to those reported here. Harman and Feigelson (1952) also found that the addition of hexokinase to the reaction mixture was associated with degenerative alterations of the mitochondria. All this emphasizes the complexity of the situation, for it is apparent that the activity of the mitochondria, isolated from each tissue, is regulated by a delicate balance of mutually interrelated factors such as structural integrity, phosphate acceptors, labile phosphate, and perhaps other conditions. Considerable additional information is yet required before a satisfactory explanation can be evolved.

9. The Effects of Fluoride.—The preceding experiments have shown that the three adenine nucleotides, when supplemented by the hexokinase system, could effect oxidative phosphorylation. It had been suggested (Barkulis and Lehninger, 1951; Kielley and Kielley, 1951) that ADP was the primary phosphate acceptor and that the phosphorylation of AMP was a secondary process dependent on its prior conversion to ADP by means of the adenylate kinase reaction. Since it was also known that fluoride inhibited adenylate kinase activity (Barkulis and Lehninger, 1951; Siekevitz and Potter, 1953 *a*) and that insect mitochondria possessed considerable adenylate kinase activity (Sacktor, 1953 *a*), fluoride might be a useful tool in differentiating the possible pathways of AMP phosphorylation here. As a control to those experiments in which

fluoride was added to flasks containing AMP, fluoride was also added to those with ATP. The results of these experiments are shown in Table VIII.

When AMP was the nucleotide, it was apparent (Table VIII) that fluoride caused a marked reduction in P/O, despite the observation that fluoride also inhibited oxygen consumption. This indicates that fluoride has a greater effect on phosphate uptake. These results were similar to those reported for mammalian tissues (Barkulis and Lehninger, 1951; Kielley and Kielley, 1951) and support their contention for the role of adenylate kinase in the phosphorylation of AMP. But, in view of the unspecific action of fluoride and the objections to this hypothesis (Lindberg and Ernster, 1952), the actual mechanism of the way AMP was brought into the reaction has yet to be settled. However, since these latter investigators had suggested that AMP was required for the maintenance of the coupling capacity of the system, 5 μM AMP plus 5 μM ATP was

TABLE VIII

Effects of Fluoride

Experiment	ΔO	ΔP	P/O
	μatoms	*μM*	
ATP	6.8	10.9	1.6
ATP + F^- (0.01 M)	5.8	10.7	1.8
ATP + F^- (0.03 M)	5.2	5.3	1.0
AMP	6.7	9.2	1.4
AMP + F^- (0.01 M)	5.6	5.7	1.0
AMP + F^- (0.03 M)	4.6	2.4	0.5

The reaction mixture was the same as that reported in Table I. When indicated 5 μM of AMP was used.

compared with 10 μM ATP in our system. No differences in phosphorylation were found; this further indicates that the actual role of AMP is still obscure.

In contrast to the inhibitory effect of fluoride in a system containing AMP, when ATP was used notable differences were found (Table VIII). Here, as in the case with AMP, oxygen consumption was inhibited to the same extent. But, when just 0.01 M fluoride was added there was no appreciable depression of phosphorylation; this resulted in a small but significant increase in P/O. This increase in P/O with the use of fluoride had been reported by many investigators, who had attributed this effect to the inhibition of ATPase by fluoride. This cannot satisfactorily explain the present results since it was shown previously that fluoride did not inhibit the ATPase of insect sarcosomes (Sacktor, 1953 *a*). It is then apparent that fluoride must participate in an, as yet, hypothetical reaction to prevent a leakage of inorganic phosphate back into the reaction mixture. Although according to the phosphorylating scheme proposed by Lindberg and Ernster (1952) inorganic pyrophosphate was one of the

products of phosphorylation and fluoride inhibited inorganic pyrophosphatase (Sacktor, 1953 *a*), this cannot be involved here either since insect mitochondria do not possess inorganic pyrophosphatase. When 0.03 M fluoride was used, phosphorylation as well as respiration was inhibited, which resulted in reduced P/O. This again indicates the diversified action of fluoride as well as the delicate balance of the entire phosphorylating scheme.

10. The Effect of Other Trapping Systems.—It had been shown that the availability of a phosphate acceptor system, glucose-hexokinase, was necessary for the demonstration of oxidative phosphorylation. Since hexokinase is a non-physiological acceptor and since these mitochondria are isolated from flight muscle, it was desirable to determine whether other acceptor systems might be applicable. These might be the creatine and arginine transphosphorylase systems. The former had been proven to be a suitable acceptor with mammalian heart muscle mitochondria (Harman and Feigelson, 1952). Since Meyerhof and Lohmann (1928) found that, in invertebrates, arginine replaced creatine and since, moreover, arginine phosphate was actually found in blowfly flight muscle (Baldwin and Needham, 1934), arginine was also tried as a possible acceptor. From the results obtained it is evident that neither creatine nor arginine can replace the glucose-hexokinase system, at least under these conditions.

It was possible that either creatine or arginine was unsuccessful as a trapping system because the necessary transphosphorylating enzymes were in another fraction of the insect muscle. This hypothesis was suggested by previous findings that the several cellular fractions of insect flight muscle have specific metabolic functions (Sacktor, 1953 *a*, *c*). Therefore a "soluble" fraction and the whole thoracic homogenate were added to supplement the mitochondria. It was found that the addition of these other fractions still did not permit the phosphorylation of creatine or arginine. This problem will be discussed again later.

These experiments also demonstrated that, by themselves, neither the "soluble" fraction nor the whole homogenate, as prepared, could effect oxidative phosphorylation. In agreement with the previous finding that the "soluble" fraction lacked some of the respiratory enzymes (Sacktor, 1953 *c*), practically no oxygen uptake was observed with this fraction alone. Moreover, the addition of these fractions to mitochondrial systems supplemented with hexokinase had no appreciable effect on either respiration or phosphorylation. This is in contrast with the marked stimulation of oxidative phosphorylation obtained by supplementing liver mitochondria with nuclei (Johnson and Ackermann, 1953).

DISCUSSION

The data presented demonstrate that mitochondria isolated from the flight muscle of the housefly, can synthesize energy-rich phosphate bonds at the expense of energy released in the oxidation of a respiratory substrate. It can be

postulated that these high energy compounds produced by oxidative phosphorylation are available for and may serve to provide the energy necessary for the contractile processes as well as for other metabolic reactions which take place in the muscle. From the standpoint of comparative physiology, the evidence suggests that insects, like other organisms, utilize ATP as an energy-transporting mechanism. Furthermore, insect ATP is not different from the ATP of other organisms, as shown by Albaum and Kletzkin (1948) and Calaby (1951).

The principles of oxidative phosphorylation in insects thus resemble the analogous systems in other animals. True, some dissimilarities exist, such as the sensitivity to the environmental situation as well as some of the responses to the phosphate acceptor systems. Whether these differences are due to specific properties inherent in the insect mitochondria or are caused by variations in the experimental procedures used is still uncertain. However this may be, further investigations are needed, which may lead finally to the establishment of an over-all scheme for phosphorylation.

It is disconcerting that the phosphorylation of arginine could not be demonstrated. Phosphoarginine was found in blowfly muscle by Baldwin and Needham (1934). In crab muscle, in which phosphoarginine was also found, its breakdown during contraction and resynthesis upon recovery were demonstrated (Meyerhof and Lohmann, 1928). If in the housefly phosphoarginine should have the same role as it has in crab muscle, or as phosphocreatine has in vertebrate muscle, the contractile processes in insects and these other animals would be comparable. But since we failed to demonstrate phosphorylation of arginine, a serious gap in our knowledge of muscular physiology in insects is apparent. Lardy and Wellman (1952) and Harman and Feigelson (1952) have pointed out, however, that the phosphorylation of glucose by ATP is a much more exergonic reaction than the phosphorylation of creatine, and that mitochondrial systems containing glucose and hexokinase probably maintain a lower ATP:ADP ratio than do those containing creatine as labile phosphate acceptor. This may be the same for arginine. Should this be the case, the high ATPase activity in insect mitochondria may have overshadowed the phosphorylation of arginine. On the other hand, the failure to phosphorylate arginine may have been due to as yet unknown factors. Further investigations are obviously required.

SUMMARY

It has been found that mitochondria isolated from the flight muscle of the housefly, *Musca domestica*, are capable of effecting oxidative phosphorylation. A systematic investigation of the factors which regulate this coupling was undertaken. It was found:

1. The molarity of the isolation medium had considerable influence on the

morphology of the mitochondria. These physical alterations were associated with changes in oxidation, phosphorylation, and ATPase activity.

2. In addition to an optimum isolation medium, the normal morphology of the mitochondria needed to be further stabilized by serum albumin.

3. A "latent" ATPase activity in insect mitochondria was demonstrated. An inverse relationship was found between oxidative phosphorylation and ATPase activity.

4. Oxygen consumption and the uptake of phosphate were linear with respect to time.

5. A respiratory substrate was necessary for phosphorylation and for maintenance of spatially organized mitochondria.

6. No differences in oxygen uptake were found in the presence or absence of inorganic phosphate.

7. Magnesium was required for optimal oxidative phosphorylation. Calcium and manganese inhibited both respiration and phosphorylation.

8. The addition of cytochrome *c* had no effect on either oxygen or phosphate uptake.

9. ATP, ADP, or AMP were capable of participating in oxidative phosphorylation, but the glucose-hexokinase trapping system was necessary.

10. Fluoride inhibited the phosphorylation of AMP, but increased P/O when ATP was used. This stimulation was not due to the inhibition of ATPase.

11. Neither arginine nor creatine was phosphorylated.

12. The addition of other isolated fractions of flight muscle to the mitochondrial system had no appreciable effect on respiration or phosphorylation.

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