Pyruvate Oxidation and the Permeability of Mitochondria from Blowfly Flight Muscle

Abstract. *The rate of pyruvate oxidation by mitochondria from blowfly flight muscle decreased in the presence of tris (hydroxymethyl) aminomethane (Tris). An increase in the rate of mitochondrial swelling was concomitant with the loss of pyruvate oxidation. These changes were prevented by bovine serum albumin, adenosine triphosphate, and magnesium ions, factors required for mitochondrial contraction. Proline, but not glutamate or malate, restored the rate of pyruvate oxidation to original values. These findings suggest that mitochondrial swelling leads to leakage of intramitochondrial intermediates of the Krebs cycle, accounting for the decrease in the rate of pyruvate oxidation. Exogenous proline penetrates the mitochondrial membrane and is rapidly oxidized, via glutamate, forming intramitochondrial precursors of oxaloacetate. Malate and glutamate were ineffective because of the selective permeability of the mitochondrial membrane.*

Partially oxidized intermediates do not accumulate appreciably during a long-term flight of the blowfly (*1*). This finding indicates that during continuous flight, carbohydrates, which are the main energy reserve for flies (*1, 2*), are oxidized completely, the rate of pyruvate utilization via the citric acid cycle being sufficiently rapid to account for essentially five-sixths of the total oxygen uptake, as suggested by Van den Bergh and Slater (*3*). A similar conclusion was reached in a study of the locust during flight (*4*). Studies on the rate of oxidation of pyruvate by isolated flight-muscle mitochondria in vitro are conflicting, however. It is generally agreed that intact mitochondria of flies oxidize α-glycerophosphate at a high rate but do not oxidize exogenous tri- and dicarboxylic acids of the Krebs cycle at significant rates. In some studies, the rate of oxidation of pyruvate was found to be as low as those of members of the Krebs cycle (*5, 6*) and, in others, pyruvate was metabolized at a rate at least half that of α-glycerophosphate oxidation (*3, 4, 7*). Studies with Van den Bergh and Gregg showed that procedural variations markedly affected the rate and stability of pyruvate oxidation, although the nature of the critical variant was not discovered in these preliminary experiments (*8*). We have reexamined the importance of the isolation and suspension media for the maintenance of the metabolic and structural integrity of mitochondria from blowfly flight muscle.

Blowflies, *Phormia regina*, were reared and maintained in laboratory culture (*9*). Mitochondria from flight muscle were isolated as described previously (*10*). Respiration was measured polarographically with the Clark oxygen electrode. We used the biuret method to assay mitochondrial preparations for protein (*11*). Bovine serum albumin (BSA) was used as the standard protein. The Tris [tris (hydroxymethyl) aminomethane] buffer preparations used in these studies were "Sigma 7-9," recrystallized from H_2O; an older preparation of "Sigma 121," with and without recrystallization; and reagent grade "Trizma Base," obtained as a gift from Sigma Chemical Co., St. Louis, Mo. All buffer preparations were adjusted to *p*H 7.4 with HCl.

Media containing sucrose, ethylenediaminetetraacetate (EDTA), and Tris buffer have been used in this laboratory and in others for the isolation and suspension of mitochondria from different tissues. Under certain conditions, however, the oxidation of pyruvate, but not that of other substrates, by mitochondria of blowfly flight muscle was inhibited by Tris buffer. The rate of pyruvate oxidation decreased as the time of exposure of the mitochondria, at 0°C, to solutions containing Tris increased (Fig. 1). Inhibition was more severe in the less purified and older preparations of buffer and in higher concentrations of Tris. Pyruvate oxidation was essentially abolished within a few minutes after the mitochondria were resuspended in media prepared with Tris of grade "Sigma 121." The use of this buffer in earlier experiments can account for the low rate of oxidation of pyruvate obtained (*5*). Recrystallization of this Tris base only partially eliminated the inhibition (*12*). The decline in the rate of pyruvate oxidation could be completely prevented by the addition of bovine serum albumin (BSA), adenosine triphosphate (ATP) and Mg^{2+} to the various suspension media. Neither BSA nor ATP was as effective as a combination of the two. After the rate of oxidation of pyruvate had decreased to a negligible value in mitochondria exposed for 40 minutes at 0°C to a medium containing Tris of grade "Sigma 7-9," addition of proline to the reaction mixture restored the rate to its initial value. Glutamate or malate could not be substituted for proline. When the mitochondria were exposed to preparations of "Sigma 121" the inhibition was more general, proline could no longer restore pyruvate oxidation, and the oxida-

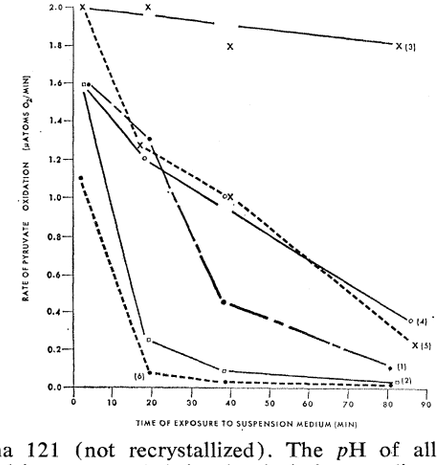

Fig. 1. The effect of suspension media upon the rate of pyruvate oxidation. Thoraxes of blowflies were homogenized and mitochondria isolated in a solution containing 0.25*M* sucrose and 1 m*M* EDTA. Equal portions of mitochondria were resuspended in various media and assayed at 32°C in the standard reaction medium containing 0.25*M* sucrose, 5 m*M* $MgCl_2$, 20 m*M* phosphate, 2.5 m*M* ADP, 1 m*M* pyruvate, *p*H 7.4, in a final volume of 2.0 ml. Each assay contained 1.74 mg of mitochondrial protein. Suspension media contained, in addition to 0.25*M* sucrose: (1) 10 m*M* Tris (Sigma 7-9); (2) 50 m*M* Tris (Sigma 7-9); (3) 50 m*M* Tris (Sigma 7-9); 0.5 percent BSA, 5 m*M* ATP, 1 m*M* $MgCl_2$ (complete medium); (4) complete medium without BSA; (5) complete medium without ATP; and (6) 50 m*M* Sigma 121 (not recrystallized). The *p*H of all media was 7.4. The activity of mitochondria resuspended in the isolation medium containing sucrose and EDTA, in the absence of Tris, decreased slowly during 90 minutes of exposure at 0°C, although the activity was not as stable as that for mitochondria resuspended in the complete medium (condition 3). Essentially the same results were obtained with reagent grade "Trizma base" as with "Sigma 7-9," recrystallized from H_2O. Crystallized bovine serum albumin (BSA) was obtained from Pentex, Inc. ATP, "A Grade," was purchased from Calbiochem.

Table 1. Qo_2 values for several substrates. Mitochondria were isolated in the medium described in Fig. 3 and assayed at 30°C in the standard reaction medium. The oxygen consumption (Qo_2) was measured in microliters of oxygen per milligram of protein per hour.

Substrate	Qo_2
Pyruvate	664
α-Glycerophosphate	1390
Proline	125
Glutamate	25
Malate	30
Fumarate	35
α-Ketoglutarate	35
Citrate	10

tion of α-glycerophosphate or proline was inhibited as much as 50 percent.

The decrease in rate of pyruvate oxidation induced by Tris was concomitant with an increase in the rate of mitochondrial swelling (Fig. 2). The decrease in absorbancy was most rapid in mitochondria suspended in the less pure preparation of Tris. Additions of BSA, ATP, and Mg^{2+} to the media prevented swelling.

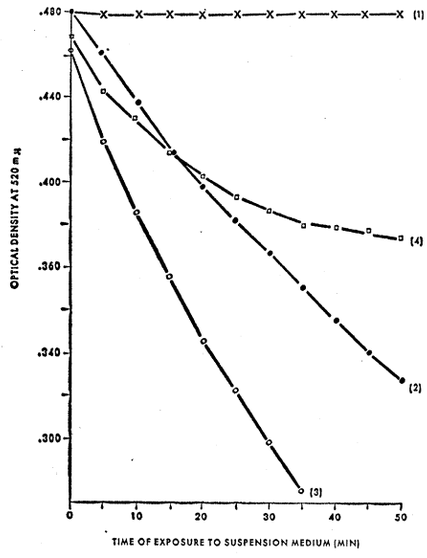

Fig. 2. The effect of suspension media upon the rate of spontaneous mitochondrial swelling. The experiment was carried out as described in Fig. 1. Immediately after the suspension of the mitochondria in the respective media, 0.05 ml of the suspension was diluted with the same media to 1.0 ml in a cuvette, and absorbancy measurements were made at 520 mμ. Each cuvette contained 0.76 mg of mitochondrial protein. Suspension media contained, in addition to 0.25*M* sucrose: (1) 50 m*M* Trizma-HCl, 0.5 percent BSA, 5 m*M* ATP, 1 m*M* $MgCl_2$; (2) 50 m*M* Trizma-HCl; (3) 50 m*M* Sigma 121 (not recrystallized); and (4) sucrose alone. The *p*H of all media was 7.4.

Although the addition of BSA and ATP to the suspension medium of mitochondria isolated in a medium containing sucrose and EDTA was necessary for maintenance of pyruvate oxidation, the incorporation of 0.5 percent BSA into all media used in homogenization, isolation, as well as suspension of the mitochondria yielded stable preparations having good respiratory control and high ADP/O ratios with pyruvate as substrate (Fig. 3). These values, indicative of tightly coupled mitochondria, are in general agreement with those obtained manometrically by Gregg *et al.* (*7*) and Van den Bergh and Slater (*3*).

Values of Qo_2 for various substrates with mitochondria isolated in media containing BSA are shown in Table 1. The rate of pyruvate oxidation is in accord with that reported by Van den Bergh and Slater (*3*) and in contrast with that reported by Chance and Sacktor (*5*). Mitochondria isolated in 154 m*M* KCl plus 1 m*M* EDTA, *p*H 7.4, oxidized pyruvate at about the same rate as that reported in Table 1. However, mitochondria isolated in the saline medium had significant losses of cytochrome *c* (*13*), as well as increased rates of spontaneous mitochondrial swelling. It appears, therefore, that the saline medium is less desirable than the medium containing sucrose and BSA.

The low rates of oxidation of most Krebs cycle substrates and glutamate are attributable to the unusual phenomenon that the mitochondria are not readily permeable to these intermediates (*3*). Respiratory rates for these substrates increased markedly after the mitochondria had been disrupted with high-frequency sound or repeatedly frozen and thawed (*14*). The rate of proline oxidation was several-fold greater than that of glutamate oxidation or oxidation of substrates of the citric acid cycle (Table 1). Values of Qo_2 for proline were not increased when mitochondria were sonically disrupted or frozen and thawed. These findings plus that of rapid utilization of proline during flight (*1, 15*) suggest that proline, as well as α-glycerophosphate and pyruvate (*3*), is a normal substrate for flight-muscle mitochondria and readily penetrates the mitochondrial membrane.

The selective permeability of flight-muscle mitochondria is demonstrated further by the experiments illustrated in Fig. 4. We found that pyruvate was oxidized at the maximum rate only when added to the reaction medium before the addition of mitochondria. If pyruvate was added after the mitochondria, its rate of oxidation rapidly decreased as the elapsed time between the addition of mitochondria and of pyruvate increased. [The effect of order of addition on the rate of oxidation of pyruvate is a contributing factor to the low rate noted in earlier experiments (*5*).] We could prevent the loss of pyruvate oxidation by including BSA and ATP in the reaction medium or by adding proline to it. Results of experiments with labeled pyruvate or proline showed that the increase in oxygen uptake resulted from an enhancement of pyruvate oxidation rather than from an increase in proline oxidation (*14*). Malate and glutamate were mostly ineffective in restoring pyruvate oxidation. Van den Bergh (*16*) reported that exogen-

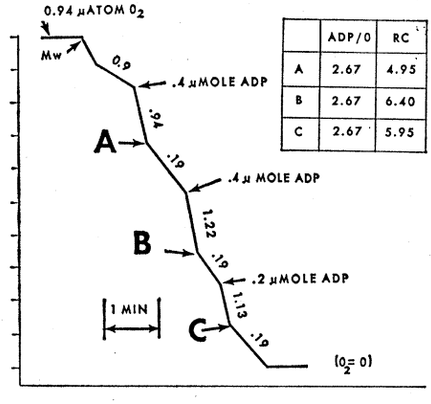

Fig. 3. Respiratory control during pyruvate oxidation. Mitochondrial isolation and suspension medium contained 0.25 *M* sucrose; 1 m*M* EDTA; 0.5 percent BSA; and 10 m*M* Tris (Sigma 7-9), *p*H 7.4. The reaction was conducted at 30°C in the standard reaction medium minus ADP, described in Fig. 1. Mitochondria (*Mw*) contained 1.70 mg of protein. Additions of mitochondria and ADP are shown by arrows. *ADP/O* is defined as the ratio of the micromoles of ADP added to the microatoms of oxygen utilized, induced by the addition of ADP. Respiratory control (*RC*) is defined as the ratio of the rate of oxygen uptake in the presence of added ADP to the rate of respiration after the added ADP has been completely utilized. *A, B,* and *C* represent changes in respiratory rates following three successive additions of ADP. Ordinate is in units of microatoms of oxygen; the initial and final oxygen contents of the reaction medium, 0.94 and 0 μatom, respectively, are indicated. Abscissa is in units of time; 1 minute is indicated. The rates of oxygen consumption in microatoms of oxygen per minute are indicated along the curve.

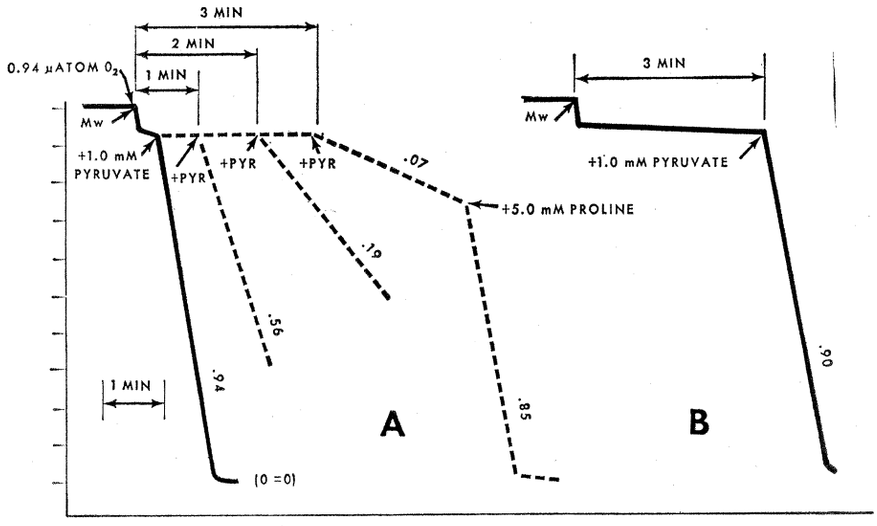

Fig. 4. Loss of pyruvate oxidation when added after the mitochondria. Mitochondrial isolation and suspension media are the same as described in Fig. 3. *A*, assayed at 30°C in the standard reaction medium containing 1.07 mg of mitochrondrial protein. *B*, assayed in standard reaction medium containing, in addition, 0.5 percent BSA, 5 m*M* ATP. Additions of mitochondria (*Mw*), pyruvate, and proline are shown by arrows. Ordinate is in units of microatoms of oxygen; the initial and final oxygen contents of the reaction medium, 0.94 and 0 μatom, respectively, are indicated. Abscissa is in units of time; 1 minute is indicated. The rates of oxygen consumption in microatoms of oxygen per minute are indicated along the curves.

ous α-ketoglutarate failed to prevent a decline in the rate of oxidation of pyruvate during long-term manometric reactions. Our finding that glutamate is also inadequate differs from his observation.

The correlation between the decrease in the rate of pyruvate oxidation and the increase in that of mitochondrial swelling suggests a functional relationship between the two. The protective effects of BSA, ATP, and Mg^{2+}, factors required for active mitochondrial contraction (*17*), support the view that inhibition of respiration and uptake of water may have resulted from a release of fatty acids from the mitochondria which was accelerated by the presence of Tris buffer. Swelling apparently leads to leakage of intramitochondrial precursors of oxaloacetate, thus accounting for the decline in the rate of pyruvate oxidation, as well as for the ability of proline to restore the original rate. Exogenous proline penetrates the mitochondria and is rapidly oxidized, via glutamate, to form intramitochondrial intermediates of the Krebs cycle (*14, 18*). Because they enter intact mitochondria very slowly, malate and glutamate were mostly ineffective in restoring pyruvate oxidation. The failure of added malate and glutamate to prime the oxidation of pyruvate also suggests that conditions which promote the leakage of endogenous substrates from the mitochondria do not necessarily increase the penetration of exogenous intermediates of the citric acid cycle into the mitochondria.

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Tetrachloroanisol: A Source of Musty Taste in Eggs and Broilers

Abstract. *Ingestion by hens and broilers of specific chloroanisols present in some wood shavings used in poultry cages can result in a musty taste in poultry products.*

A musty taste may occur in eggs and broilers from various commercial sources. This taste was noted in fresh eggs laid by hens at this institute. The hens had been fed a typical all-mash laying ration, ground oyster shell, and tap water. Changes of ration failed to eliminate the objectionable taste.

After wood shavings used in the bottoms of the cages to prevent egg damage were removed, the mustiness gradually disappeared. Within 2 to 3 weeks, the fresh eggs were normal in taste.

When 5 percent ground wood shavings from the batch previously used in the cages was added to the ration, hens produced musty eggs within 1 week. Japanese quail laid musty eggs within a few days, and the meat of broilers developed a similar taste.

To determine whether wood shavings, in general, contained a factor which caused the objectionable taste, samples of ten lots of wood shavings consisting of 130 bales were added to the diet (5 percent) as ground wood. None of the 130 samples contained the factor.

Twenty-five varieties of wood were similarly tested and two, limba (*Terminalia superba*) and obeche (*Triplochiton nigericum*), contained the factor. However, only 2 of 18 samples of limba caused the production of musty eggs. No further samples of obeche wood were tested.

The factor was found only in the superficial layer of the wood. This finding indicated that an outside influence, such as fungal growth on the wood or treatment of it with a wood preservative, might be responsible for the taste.