EFFECTS OF INHIBITORS ON FUMARATE REDUCTASE IN ANAEROBIC MITOCHONDRIA FROM *RANGIA CUNEATA* MANTLE

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ABSTRACT

Robert Earl Beasley. EFFECTS OF INHIBITORS ON FUMARATE REDUCTASE IN ANAEROBIC MITOCHONDRIA FROM RANGIA CUNEATA MANTLE. (Under the direction of Takeru Ito) Department of Biology, June 1979.

Light and electron micrographs of the mantle of Rangia cuneata, the common east and gulf coast clam, showed that distribution of mitochondria in this tissue appeared to be much lower than that in most aerobic organisms.

The NADH fumarate reductase, the electron transport components which catalyze anaerobic oxidation of NADH in the presence of the terminal electron acceptor fumarate, was present in the mantle mitochondria of this organism. The NADH fumarate reductase was sensitive to both rotenone and malonate, and thus considered to comprise at least NADH dehydrogenase and succinic dehydrogenase. All attempts to demonstrate coupled phosphorylation with the NADH fumarate reductase failed, which had been postulated to occur in considerable extent in many obligate and facultative anaerobes. The failure appeared to be due to the isolated mitochondria probably being in a highly uncoupled and disrupted state.

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INTRODUCTION

In the anaerobic environment some organisms seem able to satisfy their energy requirements, at least partially, by the degradation of glucose to succinate (Hochachka and Somero, 1973). Evidences indicate that in this anaerobic pathway, glucose is degraded by glycolysis through phosphoenolpyruvate (PEP), which is converted to oxaloacetate (OAA), malate, and ultimately to fumarate, which as a final electron acceptor, is reduced by NADH, the various sources of which have been postulated. This last step is thought to comprise a number of electron transfer components of mitochondria and is collectively known as the fumarate reductase reaction. Evidently this electron transport from NADH to fumarate in mitochondria is coupled to the formation of ATP. This process is an example of anaerobic oxidative phosphorylation. Some specific examples of oxidative phosphorylation associated with the reduction of fumarate by NADH catalyzed by NADH fumarate reductase include parasitic worms (Saz and Lescure, 1969; Saz, 1971; Fioravanti and Saz, 1978), free living annelids (Schroff and Schottler, 1977), some bivalves (Hochachka and Mustafa, 1972), and some bacteria (Peck and Reddy, 1978; Dorn et al., 1978). Many of these organisms sustain life during extended periods of anoxia.

The bivalve Rangia cuneata Gray was chosen for the present study because of the expected existence of this anaerobic energy producing mechanism which is considered as the first site of oxidative phosphorylation, and which would be an isolated model system for the studies of oxidative phosphorylation. Another reason for the choice was its ready availability. This work was concerned first with finding a

suitable tissue for the isolation of mitochondria from R. cuneata. This was aided by electron microscopy. Secondly, attempts were made to characterize the components of the NADH fumarate reductase with the aid of the classic inhibitors of electron transport, and to demonstrate coupled phosphorylation associated with the reductase in the isolated mitochondria.

REVIEW OF LITERATURE

Rangia cuneata has been established as a dominant bivalve in low salinity environments (less than 5 pp/thousand) of southern estuarine systems from Virginia to the gulf coast. It was first reported on the east coast in the 1950's, before that it was found in large numbers essentially on the gulf coast. However, from the 1950's to the 1960's it grew in numbers, and in 1967 was reported to have an average density of 275 individuals/m^2 in some low salinity areas of the Pamlico River (Tenore, 1971). Chen and Awapara (1969b) reported that R. cuneata survived up to three weeks in deoxygenated water without apparent harm, and was thus considered to be essentially an anaerobic organism.

As mentioned in the introduction, in the anaerobic energy producing mechanism of some invertebrates and microorganisms, glucose was degraded to phosphoenolpyruvate (PEP) by the well known process of glycolysis (see Figure 4, Appendix). PEP was then converted into oxaloacetate (OAA) by the cytosol enzyme PEP carboxykinase (PEPCK) instead of being converted to pyruvate by pyruvate kinase. Saz (1971) with his work on Ascaris and Hochochka and Mustafa (1973) with their work on oysters answered the question as to what controls the flow of PEP toward OAA during anaerobiosis and not to pyruvate. For instance Hochochka and Somero (1973) presented evidence, in the case of the oyster, that the two enzymes involved here, PEPCK and pyruvate kinase (PK), were not able to function at significant rates simultaneously

and in fact they were active on a reciprocal basis. During anaerobiosis the bivalve molluscs' tissues and fluids became acidic. Hochochka and Somero reported that this drop in pH played the pivotal role in causing PEP to go towards the PEPCK reaction and away from the PK reaction. The pH profile for PEPCK and PK were essentially non-overlapping, that is, PEPCK being active at an acidic pH and PK at an alkaline pH. Alanine, from transamination of pyruvate, formed later, also accumulated along with succinate under anaerobic conditions. Alanine greatly inhibited PK by increasing its Km for PEP and thereby decreased the calalytic rate. It also stimulated the activity of PEPCK. Thus, during anoxia, PEP was converted to OAA and a high energy phosphate compound (ITP or GTP) rather than pyruvate and ATP.

The OAA produced was reduced by the cytosol malate dehydrogenase to form malate. The reduction of OAA by NADH caused the regeneration of NAD^+ which then became available for the glyceraldehyde-3-phosphate dehydrogenase reaction. In the case of the parasitic worms (Saz,1971), malate then crossed the inner mitochondrial membrane (see Figure 5, Appendix). Saz stated that in these helminths one malate was oxidatively decarboxylated by the action of malic enzyme to form reducing power in the form of NADH and also pyruvate. The pyruvate was transaminated to alanine, moved back into the cytosol, and inhibited PEPCK. Saz also found in these worms that a second malate which entered the inner mitochondrial membrane was dehydrated to fumarate by a reversal of the fumarase reaction. The fumarate was then reduced to succinate by the previously produced NADH. The reduction of fumarate by NADH was catalyzed by a number of electron transfer components which were

collectively known as fumarate reductase. During this reaction one mole of ATP was produced per mole of succinate reduced in Ascaris according to the work by Saz.

In the case of the bivalve molluscs Hochachka and Somero (1973) reported that malate which was produced from the reduction of OAA by cytosol malate dehydrogenase did not enter the mitochondrial inner membrane (see Figure 6, Appendix). Instead one malate was oxidatively decarboxylated by cytosol malic enzyme to produce NADPH (instead of NADH) and pyruvate. The pyruvate entered the mitochondria and was transaminated to alanine which diffused into the cytosol and inhibited the PEPCK reaction as mentioned previously. These investigators also reported that a second malate was reduced by cytosol (or membrane bound) fumarase to form fumarate. They then postulated that, in bivalves, fumarate entered the mitochondrion and was reduced to succinate by NADH via the enzyme complex fumarate reductase. Hochochka and Somero reported that this enzyme complex was selected for in these anaerobic energy producing organisms because of its high affinity for fumarate and relatively low affinity for succinate.

The reducing power, NADH, was produced indirectly from the transamination of pyruvate. In bivalves pyruvate, the product of cytosol malic enzyme, crossed the inner mitochondrial membrane, and was transaminated with glutamate to form alanine and α-ketoglutarate. It was noted that in most organisms, the α-ketoglutarate produced during glutamate-alanine transamination was reconverted to glutamate by glutamate dehydrogenase (GDH), a reaction which requires NADH. However, Hochocka and Somero (1973) noted that in the bivalves the

specific activity of GDH was very low while α-ketoglutarate dehydrogenase (α-KG-DH) activities were realtively high. Thus the α-KG-DH reaction would probably outcompete the GDH reaction for the common substrate α-ketoglutarate. The α-ketoglutarate was oxidized via α-KG-DH to form the reducing power, NADH, and succinyl CoA. This NADH produced then reduces fumarate to succinate via the fumarate reductase reaction which was presumably coupled to form ATP. Succinyl CoA, the product of α-KG-DH, was the source of a second route to succinate production. In this reaction succinyl CoA was converted to succinate with simultaneous formation of GTP by succinyl CoA synthetase which was another source of anaerobic energy production.

Kluytman et al., (1975) also postulated a pathway in his work with sea mussel which was quite similar to the one in oysters studied by Hochachka and Somero.

Schottler (1977b) in his recent study of the free living helminth, Tubifex indicated that intramitochondrial malate under anaerobic conditions might not only be acted upon by malic enzyme and mitochondrial fumarase. He pointed out that other pathways for mitochondrial oxidation of malate with concurrent generation of NADH was likely, such as malate dehydrogenase, isocitrate dehydrogenase, and pyruvate dehydrogenase.

Work by several investigators (Saz, 1971; Schottler, 1977a; Schroff and Schottler, 1977; Peck and Reddy, 1978) suggested that the anaerobic reduction of fumarate to succinate was not catalyzed by a single fumarate reductase, but through a complex sequence of electron

transfer components. Through the use of certain specific inhibitors of electron transport the components of the fumarate reductase in different invertebrates and bacteria were determined. In most cases there was complete agreement on the complex enzyme system which makes up fumarate reductase; and in fact this pathway from NADH to fumarate with concurrent ATP formation consisted in most cases of complex I and complex II (Hatefi et al., 1962) of the electron transport chain which encompassed site one phosphorylation (see Figure 7, Appendix). Thus, the electrons were passed from NADH to NADH dehydrogenase (fp_1) to an ubiquinone (Coenzyme Q) to succinic dehydrogenase (fp_s) and finally to fumarate. One ATP was produced at the site one phosphorylation (between NADH and Coenzyme Q) for each pair of electrons transferred to fumarate. When this anaerobic fumarate reductase pathway was compared to the overall aerobic NADH oxidation through the electron transport chain to oxygen (see Figure 8, Appendix), it was clear that only the first part or site one phosphorylation was used.

This proposed pathway seemed to be accepted by most investigators of parasitic and free living helminths. However, Peck and Reddy (1978) and Dorn et al., (1978), who worked with the energy producing anaerobic reduction of fumarate in bacteria, showed through the use of spectrophotometric data that cytochrome b, as well as the previously mentioned components, was included in the fumarate reductase pathway.

This anaerobic energy producing mechanism of fumarate reduction via NADH was also shown in mammalian submitochondrial particles. Both in beef heart particles (Sanadi and Fluharty, 1963) and in rat heart

particles (Wilson and Cascarano, 1970) the anaerobic oxidation of NADH by fumarate had the same response to inhibitors of electron transport as was seen in the completely anaerobic or facultatively anaerobic invertebrates. An accumulation of succinate as an end product as well as production of ATP was also seen. The involvement of NADH dehydrogenase and succinic dehydrogenase in fumarate reductase was evident from the response of the electron transport process to the inhibitors rotenone and malonate (Sanadi and Fluharty, 1963). In addition there was an evidence of involvement of cytochrome b (Wilson and Cascarano, 1970). Although those authors suggested this reaction could serve as an important mechanism for generation of extra energy during periods of anoxia, the primary energy producing pathway in these mammals was known to be the aerobic electron transport chain. In contrast to the aerobic organisms the major route of energy production in strictly anaerobic and facultatively anaerobic organisms was considered to be via fumarate reductase. These organisms could exist for extended periods of time and some even indefinitely from the energy derived from this fumarate reductase pathway. This, however, could not be considered as the case in mammals.

Work previously done on R. cuneata (Chen and Awapara, 1969a) agreed with Hochochka and Somero (1973) that all of the enzymes catalyzing the overall flow of carbon from glucose to succinate were located in the cytosol except for fumarate reductase. This was in contrast to the helminths which contained fumarase and malic enzyme as well as fumarate reductase in the mitochondria. However, one inconsistency in the overall catabolic scheme as presented by the two

groups of bivalve investigators was seen in the action of malic enzyme. Chen and Awapara (1969a) reported that malic enzyme oxidatively decarboxylates malate to produce NADH; whereas Hochachka and Mustafa (1973) reported that NADPH was produced. This difference was indeed minor and perhaps of no consequence since in bivalves the malic enzyme was located in the cytosol, and thus the reducing power produced by this enzyme (NADH or NADPH) did not enter the mitochondria for reduction of fumarate without the aid of a mitochondrial membrane-bound transhydrogenase (see Figure 6, Appendix).

Although data on distribution and action of enzymes in R. cuneata were available (Chen and Awapara, 1969a), there have been no data indicating the nature of the components of fumarate reductase presumably consisting of complex I and complex II, nor any data showing phosphorylation associated with the fumarate reductase in this organism, other than a mere demonstration of NADH oxidation with simultaneous fumarate reduction in anaerobically incubated mitochondrial fractions. The nature of the components of fumarate reductase was obscure in oysters and other bivalves even though there was an evidence of coupled phosphorylation with this process.

MATERIALS AND METHODS

Isolation of Rangia cuneata Mitochondria

Rangia cuneata were obtained from the Pungo River and Bath Creek in North Carolina. The mitochondrial isolation method is a variation of the method used by Scott, Storey, and Lee (1978) on isolation of rabbit skeletal muscle mitochondria. All operations were carried out under refrigeration or in ice bath unless impractical. All solutions were ice cold.

The mantle from four clams was excised and placed on ice in 0.15 M KCl buffered with 20 mM Tris-Cl at pH 7.4. The tissue (8-10g) was washed four to five times in this solution and then ground for 25 seconds in a Waring blender containing 250 ml of medium consisting of 120 mM KCl, 50 mM Tris-Cl pH 7.4, 3 mM $MgSO_4$, and 1 mM EDTA (KCl-medium). Nagarse (a bacterial protease from Sigma, 1 mg/g tissue) was added (Chance and Hagihara, 1961) to the ground tissue, that was homogenized for one minute with a Potter-Elvehjem glass homogenizer and a motor driven Teflon pestle. The homogenate was filtered through four, then eight layers of bleached cheesecloth and centrifuged at 700 x g for five minutes to remove coarse debris and unbroken cells. The supernatant was diluted 1:1 with BSA-medium, which contained 0.8% Bovine serum albumin (BSA) in the KCl-medium, then centrifuged at 9500 x g for ten minutes. The pellet was resuspended in 50 ml of the BSA medium, then followed by centrifugation at 800 x g for ten minutes. The resulting supernatant was centrifuged at 7700 x g for ten minutes, the pellet was washed and resuspended in a medium containing 0.25 M sucrose, 10 mM Tris-Cl pH 7.4 (the sucrose-medium). This suspension

was centrifuged at 7700 x g for ten minutes and the resulting mitochondrial pellet was resuspended in 0.5 ml distilled water at 4° C for five minutes (Lehninger, 1951). Addition of 0.1 ml of 1.5 M sucrose, 60 mM Tris-Cl pH 7.4 was necessary to bring the suspension back to the previous isotonic condition. The hypotonic treatment of the mitochondrial fraction was thought to be necessary to allow the passage of NADH, rotenone, and other impermeable substances across the mitochondrial membrane. I had hoped to show as Lehninger (1951) had, that even though the mitochondrial membrane had been disrupted by hypotonic treatment, phosphorylation could still be measured.

Isolation of Rat Liver Mitochondria

Rat liver mitochondrial fractions were also isolated (Ito and Johnson, 1964), and as in the previously described isolation procedure all operations were carried out under refrigeration or in ice bath unless impractical. All solutions were ice cold. The liver from a rat was quickly excised and placed in a beaker containing preparation medium (0.25 M sucrose, 1 mM EDTA pH 7.5). After washing twice with the preparation medium, the liver was quickly homogenized in about 30 - 35 ml preparation medium with a Potter-Elvehjem glass homogenizer and motor driven Teflon pestle. The homogenate was diluted to 120 - 160 ml with the preparation medium and centrifuged at 600 x g for ten minutes. The supernatant was recentrifuged at 8700 x g for ten minutes, and the resulting pellet was uniformly dispersed into 120 - 160 ml of preparation medium. This suspension was centrifuged again at 8700 x g for ten minutes and the mitochondrial pellet uniformly suspended in 5 - 10 ml of preparation medium.

NADH Oxidation

NADH oxidation was determined by following decrease in absorbance at 340 nm with a Coleman 124 double beam spectrophotometer and a Coleman 165 recorder. The reaction mixture consisted of 1.5 ml 0.20 M phosphate buffer pH 7.4, 0.3 ml 0.1 M Na fumarate, 0.1 ml 4.5 mM NADH, 0.3 - 0.9 ml H_2O, and depending upon the assay conditions 0.3 ml 0.1 M KCN, 1 µl 0.15 mM rotenone in ethanol, 0.3 ml 0.5 M malonate, or 0.3 ml 0.5 M oxaloacetate. The reaction was started with the addition of 0.1 - 0.2 ml mitochondrial suspension. Total volume under all assay conditions was 3.0 ml.

Succinic Dehydrogenase Activity

Succinic dehydrogenase was determined by following decrease in absorbance at 400 nm of $K_3Fe(CN)_6$ reduction using succinate as substrate (Bonner et al., 1955) with the same instrument used for the determination of NADH oxidation. The reaction mixture consisted of 0.3 ml 0.1 M KCN, 0.3 ml 0.01 M $K_3Fe(CN)_6$, 0.2 ml 0.2 M Na succinate, 1.5 ml 0.20 M phosphate buffer pH 7.4, 0.2 - 0.5 ml H_2O, and depending upon the assay conditions 0.3 ml 0.5 M malonate or 1 µl 0.15 mM rotenone in ethanol. The reaction was started with the addition of 0.2 ml mitochondrial suspension. Total volume under all assay conditions was 3.0 ml.

NADH Oxidation with Concurrent Phosphorylation as Measured by ^{32}Pi Uptake

NADH oxidation was determined as described above except that the reaction mixture consisted of 1.0 ml 0.20 M phosphate buffer pH 7.4,

0.1 ml 4.5 mM NADH, 0.3 ml 0.1 M Na fumarate, 0.3 ml 0.1 M KCN, 10 ul $H_3{}^{32}PO_4$ (1 mCi/ml), 0.05 ml 1% hexokinase (Sigma, Type IV) in 0.1% glucose, 1.0 ml (3 mM ATP (Sigma), 15 mM $MgCl_2$, 150 mM glucose, 3 mM EDTA) pH 7.0, 0.04 ml H_2O, and depending upon the assay conditions 1 µl 0.15 mM rotenone in ethanol. The reaction was started with the addition of 0.2 ml mitochondrial suspension.

To determine ^{32}Pi incorporation into organic ^{32}P a 0.3 ml aliquot of the reaction mixture was taken at time zero and at 15 minutes after the reaction had begun. The 0.3 ml aliquot, at both times, was immediately added to 2.7 ml 5% TCA. In order to eliminate any possible quenching errors which might occur during scintillation counting, a blank reaction mixture was additionally prepared by mixing all of the assay reactants except for $H_3{}^{32}PO_4$. A 0.3 ml aliquot was taken from the blank at time zero and also added to 2.7 ml 5% TCA.

The TCA mixture for the blank, the time zero sample, and the 15 minute sample was centrifuged at 3000 x g for 15 minutes to remove precipitated protein. After centrifugation the entire supernatant of the blank and of the two sample mixtures were separately poured into three separatory funnels.

In each supernatant of the samples and the blank the inorganic phosphate was separated from the organic phosphate by the method of Martin and Doty (1949). The 3 ml deproteinized supernatant was mixed with 1.0 ml of 10% ammonium molybdate in 5 M H_2SO_4 and 5.0 ml of isobutanol-benzene mixture (equal parts). This mixture was then shaken for 15 seconds.

After separation of the phases from each sample (zero time or 15 minute time) a 0.02 ml aliquot was taken from the organic layer (containing Pi) and mixed with a 0.02 ml aliquot taken from the aqueous layer of the blank. A 0.02 ml aliquot was taken from the sample aqueous layer (containing organic phosphate if any) and mixed with a 0.02 ml aliquot taken from the organic layer of the blank. The blank thus is expected to reduce quenching errors by establishing conditions as near identical as possible among all counting samples. The only difference, of course, is that the blank lacks the extremely small volume of ^{32}Pi added to the phosphorylation samples.

Five milliliters of scintillation cocktail (7.5 gm PPO and 0.3 gm POPOP in 1 liter toluene mixed 2:1 with Triton X-100) was added to the sample and blank aliquot mixtures. Radioactivity was determined by a Beckman LS-233 Liquid Scintillation System. Since no ^{32}Pi uptake was observed (i. e., there was no increase of ^{32}Pi counts in the aqueous layer at 15 minutes time over time zero) total phosphate was not analyzed.

Protein Determination

Protein determination was performed according to Lowry et al., (1951).

Electron and Light Microscopy

Rangia cuneata mantle was fixed with 3% glutaraldehyde in 1:1, sea water: 0.2 M sodium cacodylate buffer pH 7.2 for 30 minutes. The mantle was then washed four times for 15 minutes each with 0.1 M

sodium cacodylate pH 7.2 in sea water. The sample was further fixed by 2% OsO_4 in the 0.1 M sodium cacodylate sea water buffer. The tissue was dehydrated in a graded ethanol series and embedded in Aradilite 6005. Sections (600 - 900 Å thick) were double stained with lead and uranium salts and observed with a Hitashi HS-8 transmission electron microscope.

For light microscopy, the tissue was identically fixed, dehydrated, and embedded. Sections (0.5 µm thick) were stained with Tolouidine Blue and observed under bright field with a Zeiss W L Research Light Microscope.

Anaerobic Environment

Rangia cuneata was on one occasion kept in a cylindrical anaerobic chamber (about 30 cm tall and 10 cm in diameter) for periods up to six days.

RESULTS AND DISCUSSION

Failure to produce an active mitochondrial preparation from the entire clam after several attempts necessitated brief electron microscopic examination of the adductor muscle, mantle, and foot, which indicated that the great majority of mitochondria was located in the mantle. It was thus decided to undertake light and electron microscopy of the mantle to try to determine the relative amount and distribution of the mitochondria in more detail.

From a light micrograph showing the clam mantle cut in cross section, three distinct layers were noted (see Figure 1, Appendix). The outer surface contained a conspicious outer layer of microvilli, which faced the interior of the bivalve. The inner darker stained surface was that part of the mantle which lay closest to the shell. The extremely large middle layer of the mantle was found to be virtually devoid of mitochondria, and largely appeared to be composed of collagen, muscle fibers, and lipids.

A higher magnification (see Figure 2, Appendix) of the outer mantle layer showed large numbers of mitochondria, especially located near the cell junctions. Numerous glycogen granules were intimately associated with the mitochondria. This electron micrograph was taken from the section just below the uniformly distributed microvilli layer. A higher magnification of the inner mantle layer also showed large numbers of mitochondria in intimate association with numerous glycogen granules (see Figure 3, Appendix). Numerous cilia as well as microvilli were noted. This was the layer of mantle that was pulled away from the shell during excision.

It seemed logical that more mitochondria would be seen in the mantle than in any other part of the clam since it is known to be the actively metabolizing part of the organism which produces the shell. Although mitochondria were found in sections of Rangia mantle, the distribution of the mitochondria shown in these electron micrographs in this study was clearly much lower than that of well-documented liver and muscle mitochondria of obligate aerobes. By far the greatest concentration of mitochondria was found in the outer and inner mantle layers, and in fact the middle layer was composed almost entirely of a dense connective tissue collagen matrix. For these reasons it was decided to use the bacterial protease Nagarse for approximately ten minutes hopefully to free the mitochondria from the denser collagen and muscle tissue as described in the Materials and Methods section (Chance and Hagihara, 1961). The mitochondria thus isolated were used for the subsequent studies described below.

Succinic dehydrogenase activity was assayed since it has generally been believed to be a major component of the NADH fumarate reductase. Indeed, this activity was found to occur in R. cuneata mitochondrial fractions (see Table 1, Appendix). The succinic dehydrogenase activity in rat liver mitochondrial fractions, however, was six times greater than that seen in R. cuneata mantle. The result appeared to indicate that the R. cuneata mitochondrial fraction contained fewer mitochondria and more extraneous tissue debris than the rat liver mitochondrial fraction. It was a somewhat expected result since the low distribution of mitochondria in Rangia tissues had already been seen in the electron microscopy study.

The addition of malonate did in fact cause a 50% inhibition of the activity in the mantle mitochondrial fraction under the condition. The inhibition by malonate was 83% in the rat liver mitochondrial fraction.

For beef heart mitochondria the v_I/v ratio (see Appendix, p. 46, equation 4) gave 0.08 under the same condition used here (the substrate concentration, 0.013 M; the malonate concentration, 0.05 M) with Km and K_I being 2.9×10^{-5} M (Slater and Bonner, 1952) and 10^{-5} M (Thorn, 1953) respectively. The malonate inhibition of the succinic dehydrogenase of beef heart mitochondria under the condition would have been, therefore, 92%, which was comparable with the one found here in rat liver mitochondria.

Since the malonate inhibition of the Rangia succinic dehydrogenase under the condition was 50%, Km was estimated to be approximately one fourth of K_I (see Appendix, p. 46, equation 4). This appeared to indicate that the succinic dehydrogenase of this anaerobe was structurally and functionally quite different from the enzyme in the aerobes. As expected (see Table 1, Appendix) succinic dehydrogenase of the Rangia mitochondrial fraction was insensitive to rotenone, an electron transport inhibitor of NADH dehydrogenase (see Figure 7, Appendix). Thus at least qualitatively the malonate-sensitive rotenone-insensitive Rangia succinic dehydrogenase was a typical one similar to the one found in many aerobic organisms, the lower malonate inhibition of the Rangia enzyme notwithstanding.

Since it was felt that anaerobic NADH oxidation by the fumarate reductase might also be high in clams living in anaerobic conditions, it was decided to determine if there was any difference in anaerobic NADH oxidation between the mitochondrial fractions isolated from clams living in anaerobic or aerobic environments (see Table 2, Appendix). For this determination, five clams were placed in brackish water with 1 mM fumarate for five days in an anaerobic chamber, and another five clams in simply brackish water for five days in another anaerobic chamber. In both cases an 80% survival rate was observed. In all cases the anaerobic assay condition for the NADH fumarate reductase of the subsequently isolated mitochondrial fractions was satisfied by the addition of KCN to the reaction cuvette (see Figure 8, Appendix). As can be seen (see Table 2, Appendix), there was very little difference in the anaerobic NADH oxidation rates among the mitochondrial fractions isolated from the clams living in the anaerobic chambers. Moreover, a comparison of anaerobic NADH oxidation between clams living aerobically or anaerobically showed only a slightly higher oxidation rate in the mitochondrial fractions from the clams living in oxygen. In the subsequent experiments, therefore, the clams living in an aerated chamber were used for the NADH fumarate reductase assay.

Rotenone inhibited anaerobic NADH oxidation by the mitochondrial fractions regardless of their previous environment (see Table 2, Appendix). The inhibition was 70% in clams living simply in anoxia, 78% in clams living in anoxia with 1 mM fumarate, and 91% inhibition in mitochondria isolated from clams living in the presence of oxygen.

The difference in the amount of inhibition was probably not significant. However, the NADH fumarate reducatse in Rangia was indeed rotenone-sensitive, and thus appeared to be composed of a NADH dehydrogenase of a typical mitochondria of aerobic organisms.

The endogenous anaerobic NADH oxidation (i. e., without fumarate) by the mitochondrial fractions isolated from the three clam sources was basically the same (see Table 2, Appendix). This provided evidence that each clam had essentially the same amounts of endogenous fumarate or perhaps other endogenous electron acceptors.

The higher rate of NADH oxidation in the absence of KCN by mitochondria isolated from the clams living in oxygen (see Table 2, Appendix) seemed to indicate that NADH was oxidized not only through the fumarate reductase system (see Figure 7, Appendix), but also through the cytochromes and oxygen, though to a rather small extent (see Figure 8, Appendix). The mere increase of 20% in the NADH oxidation rate did suggest that not the cytochrome system but the fumarate reductase played a dominant role in the NADH oxidation in Rangia. This was in agreement with the general belief that in many invertebrates either the cytochrome oxidase was missing or the fumarate reductase was the major electron transport system in their mitochondria (Saz, 1971; Schroff and Schottler, 1977).

A higher rate of anaerobic NADH oxidation by mitochondria isolated from rat liver (see Table 2, Appendix) than Rangia was again probably due to higher purity of the rat liver mitochondrial fraction than the Rangia's. An explanation as to why there was no inhibition of

NADH oxidation by rotenone and as to why the endogenous anaerobic NADH oxidation was somewhat lower than the other two conditions appeared to require postulating the presence of a rotenone-insensitive NADH dehydrogenase, that would link to the fumarate reductase, in addition to a typical rotenone-sensitive NADH dehydrogenase. However, this unusual result warranted more studies.

As mentioned previously succinic dehydrogenase was thought to be an integral part of the NADH fumarate reductase. In order to test this possibility in Rangia mitochondria, effects of malonate and oxaloacetate, which were known to be potent competitive inhibitors of succinic dehydrogenase (Lehninger, 1975), were examined (see Table 3, Appendix). The malonate inhibition of anaerobic NADH oxidation was approximately 50%. This was in contrast with the rotenone inhibition which resulted in 82% inhibition of the anaerobic NADH oxidation. Applying the equation 4 (see Appendix, p. 46) again, one found the Km for fumarate was about one fifth of K_I for malonate in the NADH fumarate reductase reaction. This appeared to indicate that affinity of fumarate with the fumarate reductase was high, and to agree with the idea (Hochachka and Somero, 1973) that this enzyme complex was selected to function in anaerobic oxidative phosphorylation in facultative anaerobes, although the Km for fumarate in the NADH fumarate reductase of aerobes was unknown. In any case the malonate and the rotenone sensitivities demonstrated in the data certainly indicated that in all probability the fumarate reductase of Rangia, contained NADH dehydrogenase and succinic dehydrogenase similar to the ones commonly known in aerobic organisms.

A slight increase of the anaerobic NADH oxidation in the presence of oxaloacetate (see Table 3, Appendix) was observed. This result appeared to indicate that an active malate dehydrogenase was present in the Rangia mitochondrial fraction. Indeed the malate dehydrogenase activity (KCN + OAA) was higher than the NADH fumarate reductase activity (KCN + fumarate).

In the absence of KCN and in the presence of fumarate, malonate, and oxaloacetate an unexpectedly high rate of NADH oxidation was obtained (close to ten times higher than the anaerobic NADH fumarate reductase activity, (see Table 3, Appendix)). This result was hard to reconcile the previous finding (see Table 2, Appendix) in which NADH oxidation in the absence of KCN was only 20% higher than in the presence, for it appeared to indicate there was a highly active cytochrome system to oxidize NADH. However, if participation of the cytochromes in the NADH oxidation was assumed to be low in this instance, as was in many obligate and facultative anaerobes, postulation of a cyanide-sensitive malate dehydrogenase was necessary to explain the discrepancy, although such dehydrogenase has not been known.

The finding in this study, that the anaerobic NADH oxidation in the presence of fumarate was sensitive to both rotenone and malonate, indeed showed the presence of anaerobic NADH fumarate reductase in the Rangia cuneata mitochondrial fraction. However, all attempts to demonstrate coupled phosphorylation (^{32}Pi incorporation into organic ^{32}P) failed.

It was felt that the major reason for the lack of coupled phosphorylation stemmed from the method of isolation of the mitochondria.

The isolated mitochondria were probably in a highly uncoupled and disrupted state. In fact, the mantle mitochondrial fraction prepared without using a protease (Nagarse) or without being treated hypotonically did show the NADH fumarate reductase activity, indicating a disrupted mitochondrial membrane through which NADH freely entered the mitochondria. Demonstration of anaerobic oxidative phosphorylation associated with the NADH fumarate reductase in Rangia cuneata appeared to depend upon success in the future in isolating the mitochondria and maintaining their structural integrity.

SUMMARY

Light and electron micrographs of the mantle of Rangia cuneata, the common east and gulf coast clam, showed that distribution of mitochondria in this tissue appeared to be much lower than that in most aerobic organisms.

The NADH fumarate reductase, the electron transport components which catalyze anaerobic oxidation of NADH in the presence of the terminal electron acceptor fumarate, was present in the mantle mitochondria of this organism. The NADH fumarate reductase was sensitive to both rotenone and malonate, and thus considered to comprise at least NADH dehydrogenase and succinic dehydrogenase. All attempts to demonstrate coupled phosphorylation with the NADH fumarate reductase failed, which had been postulated to occur in considerable extent in many obligate and facultative anaerobes. The failure appeared to be due to the isolated mitochondria probably being in a highly uncoupled and disrupted state.

APPENDIX

FIGURE 1. LIGHT MICROGRAPH SHOWING OVERALL STRUCTURE AND DIFFERENTIATION OF CLAM MANTLE. THE OUTER AND INNER MANTLE CONTAIN RELATIVELY LARGE NUMBERS OF MITOCHONDRIA AND GLYCOGEN, WHILE THE MIDDLE MANTLE IS VIRTUALLY DEVOID OF MITOCHONDRIA AND CONTAINS NUMEROUS LIPID VESSICLES IN A COLLAGEN MATRIX. (O) OUTER MANTLE, (M) MIDDLE MANTLE, (I) INNER MANTLE, (MV) MICROVILLI. TOTAL MAGNIFICATION 680X.

① 2.0μ

FIGURE 2. HIGHER MAGNIFICATION ELECTRON MICROGRAPH OF OUTER MANTLE SHOWING MITOCHONDRIA SURROUNDED BY NUMEROUS GLYCOGEN GRANULES. NOTE ALSO THE PROXIMITY OF THE MITOCHONDRIA TO THE CELL JUNCTION. (MI) MITOCHONDRIA, (G) GLYCOGEN GRANULES, (CJ) CELL JUNCTION, (MV) MICROVILLI. TOTAL MAGNIFICATION 45000x.

FIGURE 3. HIGHER MAGNIFICATION ELECTRON MICROGRAPH OF THE INNER MANTLE SHOWING THE MITOCHONDRIA, AS IN THE OUTER MANTLE, SURROUNDED BY NUMEROUS GLYCOGEN GRANULES. THE INNER MANTLE IS THAT PORTION OF THE MANTLE ADJACENT TO THE CLAM SHELL. NOTE THE LONGITUDINAL AND TRANSVERSE SECTIONS OF THE CILIA WHICH ARE INTERSPERSED AMONG MICROVILLI. (MI) MITOCHONDRIA, (G) GLYCOGEN GRANULES, (C) CILIA, (MV) MICROVILLI. TOTAL MAGNIFICATION 40000x.

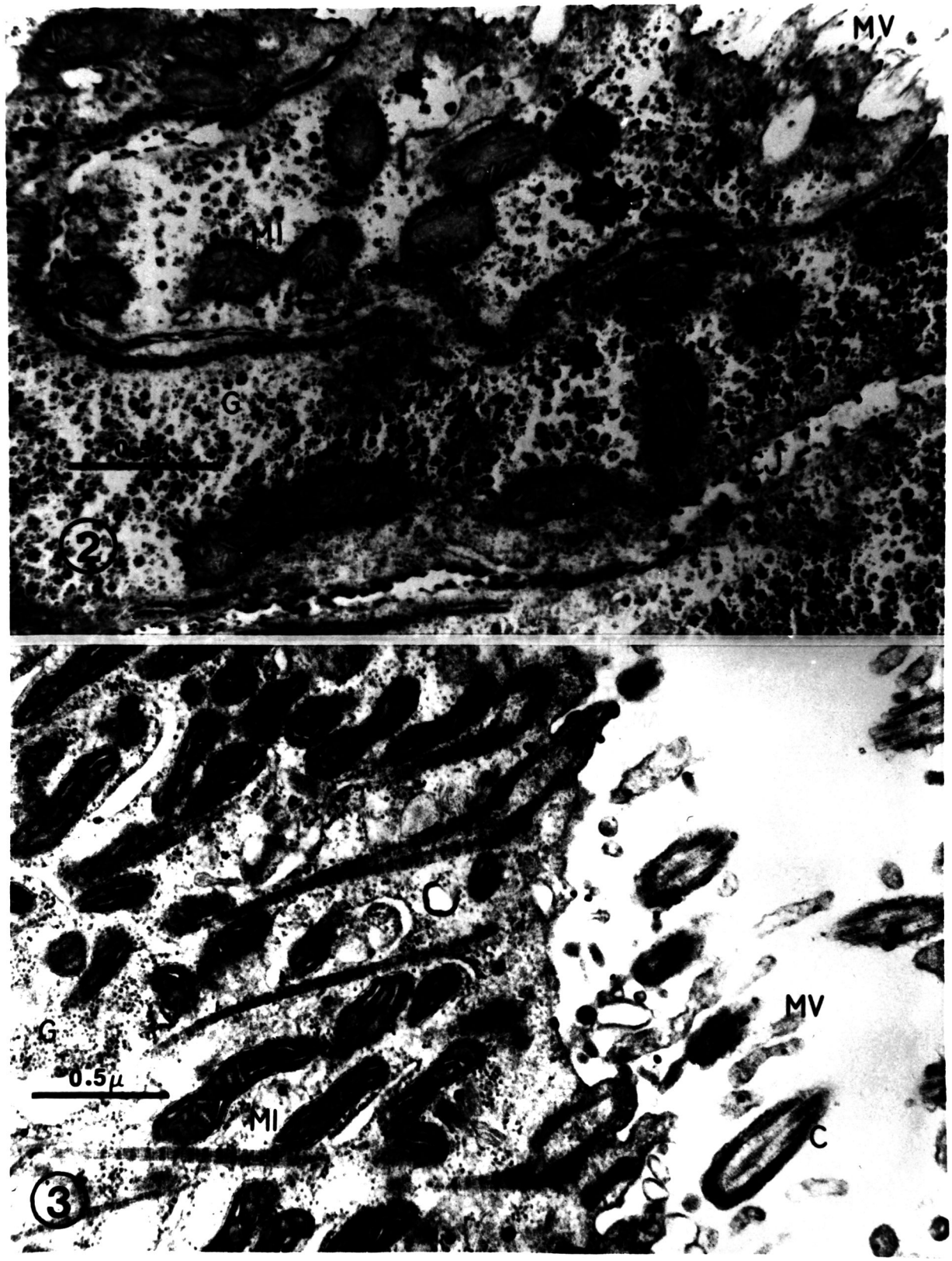
MV
MI
G
J
0.5μ
2
C
G
0.5μ
MI
MV
C
3

FIGURE 4. ANAEROBIC METABOLIC PATHWAY OF GLUCOSE DEGRADATION IN INVERTEBRATES. GLY-3-PH : GLYCERALDEHYDE-3-PHOSPHATE, 1,3 DPG : 1,3-DIPHOSPHOGLYCERATE, PEP : PHOSPHO-ENOLPYRUVATE, PEPCK : PEP CARBOXYKINASE, PK : PYRUVATE KINASE.

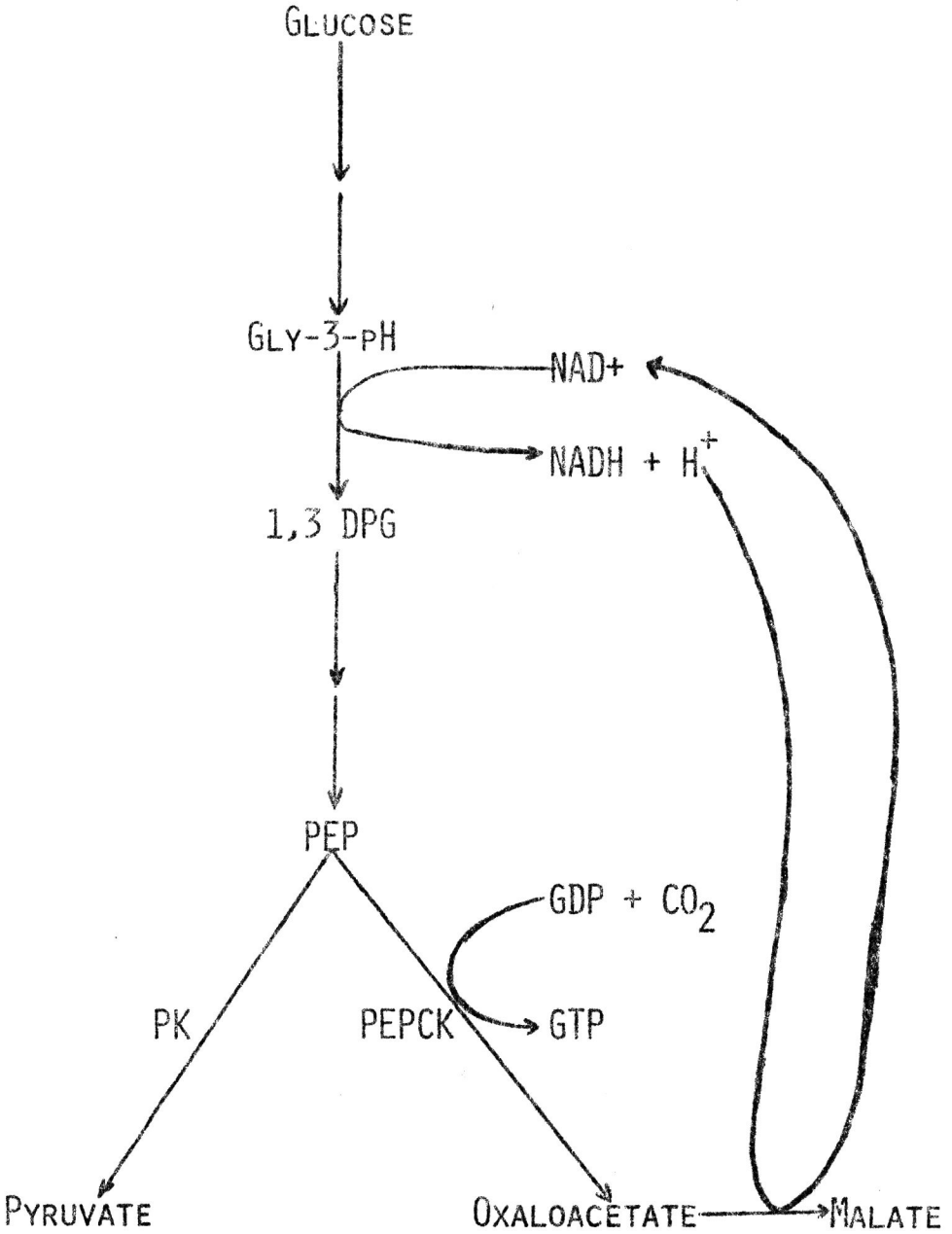
GLUCOSE
GLY-3-PH
NAD+
NADH + H+
1,3 DPG
PEP
GDP + CO2
PK
PEPCK
GTP
PYRUVATE
OXALOACETATE
MALATE

FIGURE 5. FORMATION OF SUCCINATE FROM MALATE IN INVERTEBRATES.

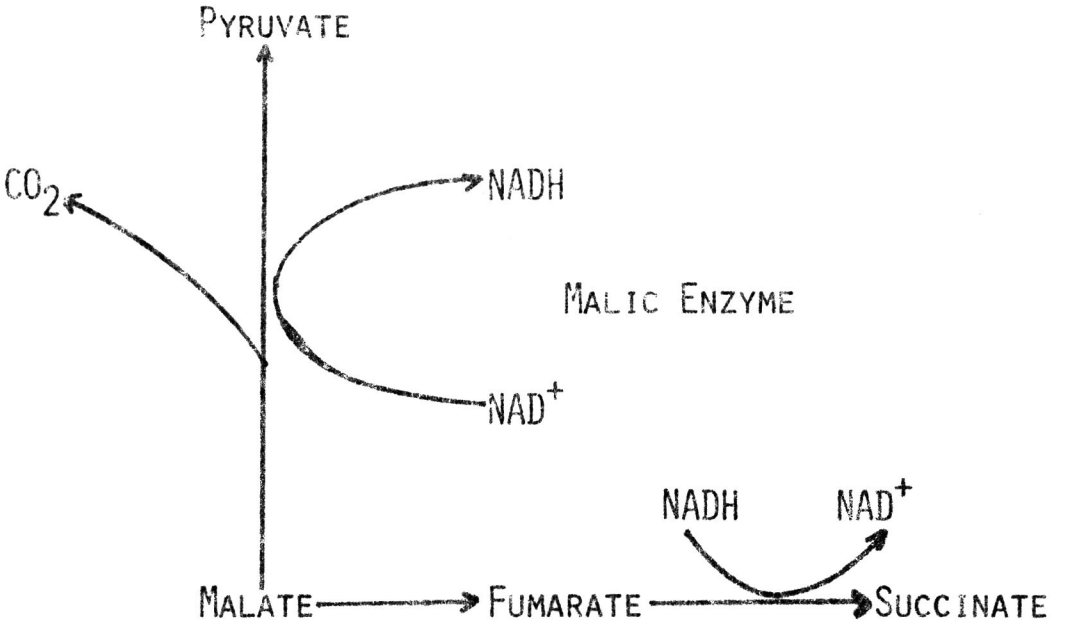
PYRUVATE
CO_2
NADH
MALIC ENZYME
NAD^+
NADH
NAD^+
MALATE
FUMARATE
SUCCINATE

FIGURE 6. ANAEROBIC METABOLIC PATHWAY OF GLUCOSE TO SUCCINATE IN INVERTEBRATES. PEP : PHOSPHOENOLPYRUVATE, OAA : OXALOACETATE, PYR : PYRUVATE, TH : TRANSHYDROGENASE.

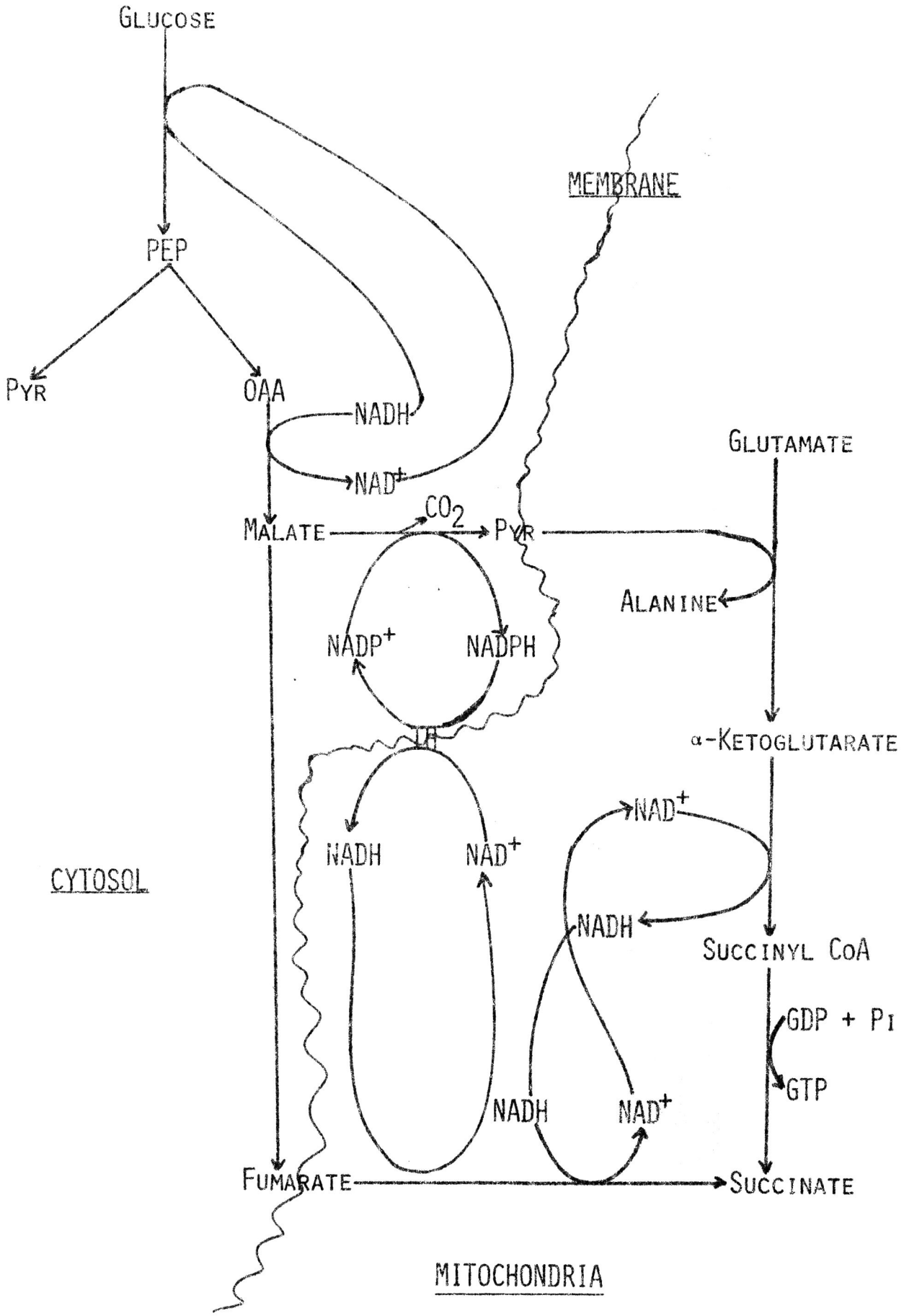
GLUCOSE
PEP
PYR
OAA
NADH
NAD+
MEMBRANE
GLUTAMATE
MALATE
CO2
PYR
ALANINE
NADP+
NADPH
α-KETOGLUTARATE
NAD+
NADH
NAD+
CYTOSOL
NADH
SUCCINYL CoA
GDP + Pi
GTP
NADH
NAD+
FUMARATE
SUCCINATE
MITOCHONDRIA

FIGURE 7. ELECTRON TRANSPORT SEQUENCE OF FUMARATE REDUCTASE AND SITES OF INHIBITION BY ROTENONE AND MALONATE, FP_1 : NADH DEHYDROGENASE, FP_S : SUCCINIC DEHYDROGENASE.

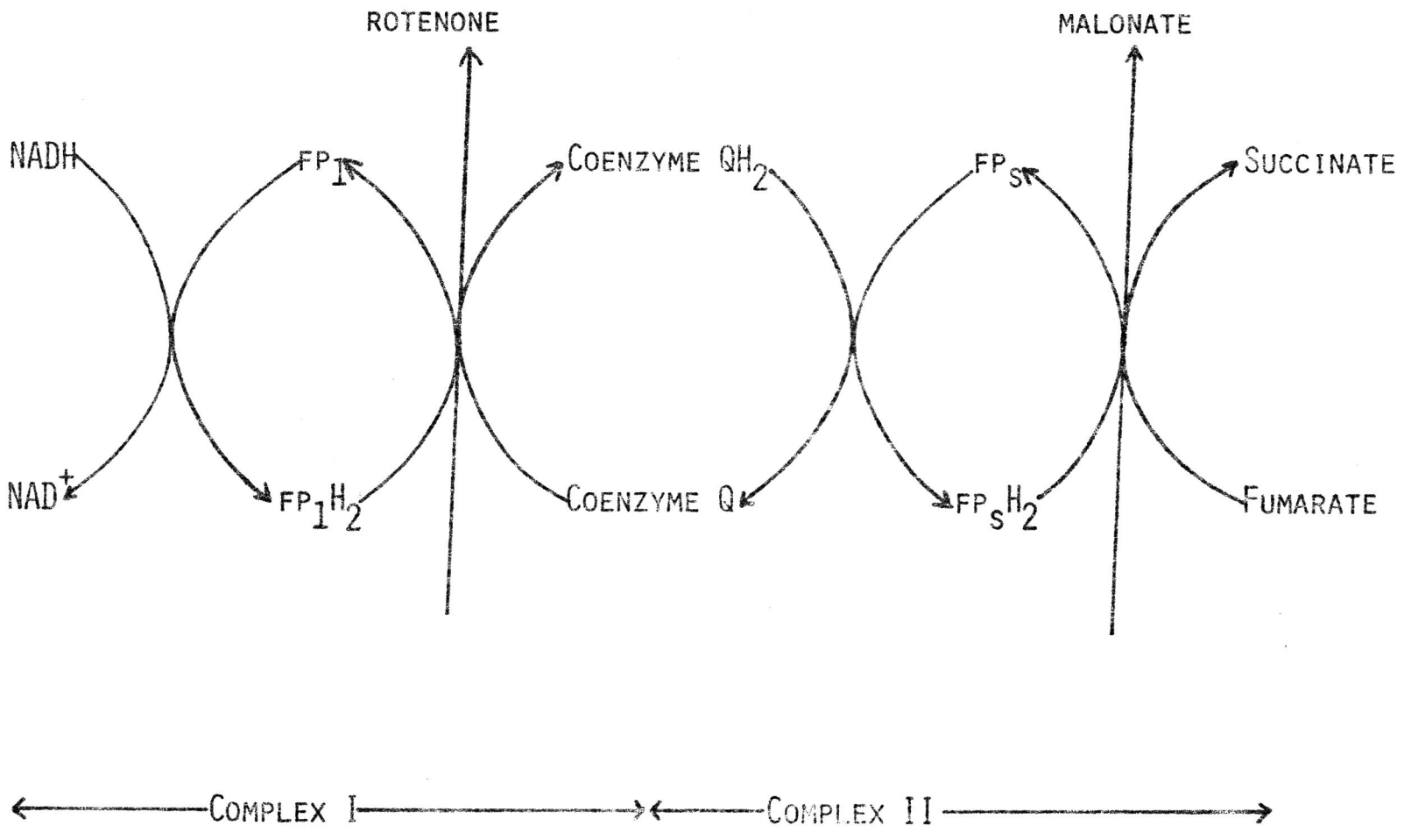
ROTENONE
MALONATE
NADH
FP I
COENZYME QH2
FP S
SUCCINATE
NAD+
FP1H2
COENZYME Q
FPSH2
FUMARATE
COMPLEX I
COMPLEX II

FIGURE 8. ELECTRON TRANSPORT SEQUENCE OF AEROBIC SYSTEM AND OF NADH FUMARATE REDUCTASE AND SITES OF INHIBITIONS BY INHIBITORS. FP_1 : NADH DEHYDROGENASE, FP_S : SUCCINIC DEHYDROGENASE, CYTO : CYTOCHROME.

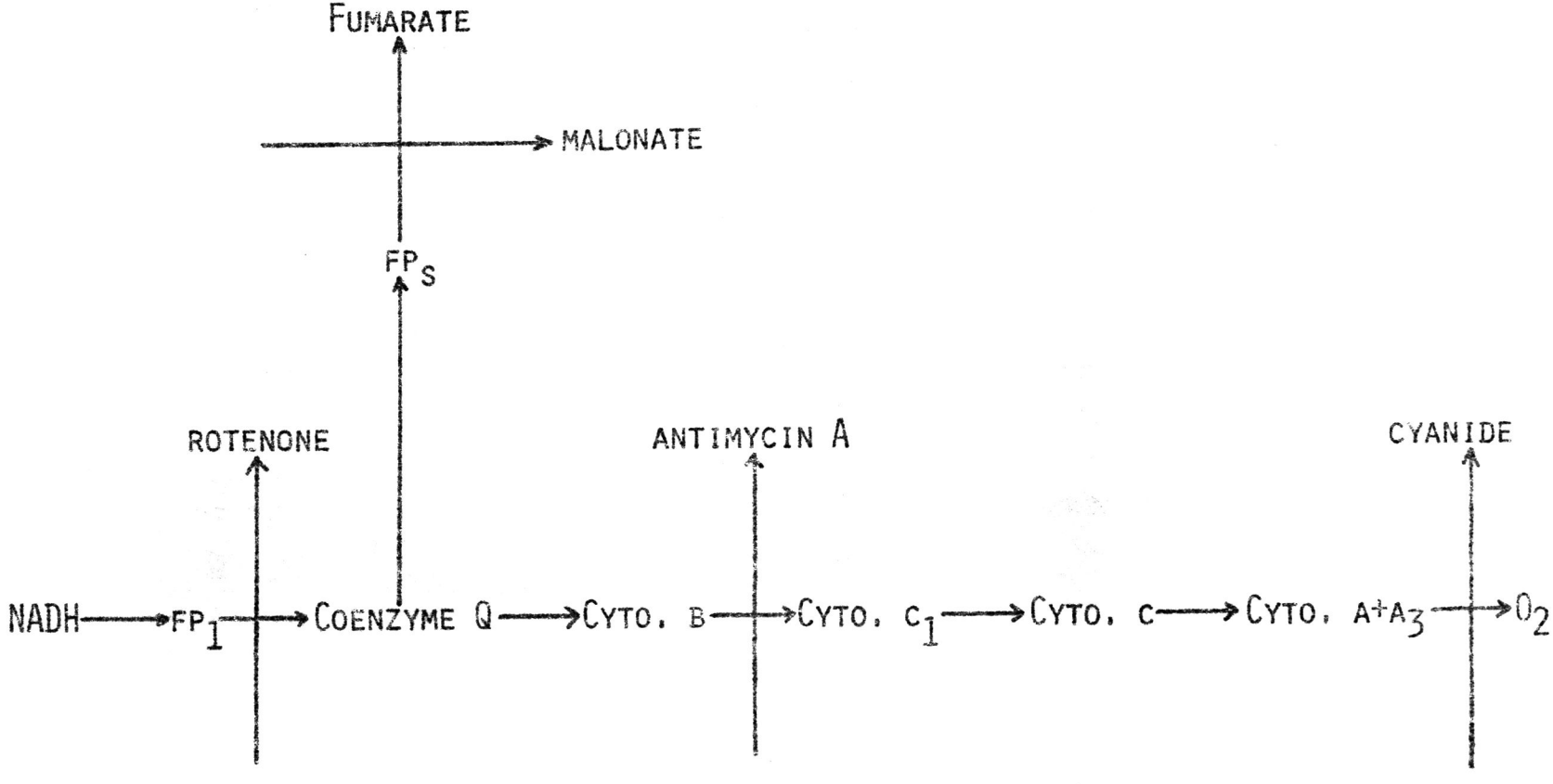
FUMARATE
MALONATE
FP S
ROTENONE
ANTIMYCIN A
CYANIDE
NADH
FP 1
COENZYME Q
CYTO. B
CYTO. C 1
CYTO. C
CYTO. A+A3
O2

TABLE 1. SUCCINIC DEHYDROGENASE ACTIVITY IN ISOLATED MITOCHONDRIAL FRACTIONS OF *RANGIA* MANTLE AND RAT LIVER. SEE THE TEXT FOR THE DETAILS OF THE CONDITIONS.

NMOLES OF FERRIEYANIDE REDUCED/MIN/MG PROTEIN

CONDITIONS	*RANGIA* MITOCHONDRIAL FRACTION	RAT LIVER MITOCHONDRIAL FRACTION
WITHOUT MALONATE		
(KCN + ROTENONE)	54.4	316
(KCN)	54.4	
WITH MALONATE		
(KCN)	26.1	52.5
(KCN)	28.8	

TABLE 2. NADH FUMARATE REDUCTASE ACTIVITY BY MITOCHONDRIAL FRACTIONS ISOLATED FROM DIFFERENT SOURCES. SEE THE TEXT FOR THE DETAILS OF THE CONDITIONS. FUM : FUMARATE, ROT : ROTENONE, W/O : WITHOUT.

NMOLES OF NADH OXIDIZED/MIN/MG PROTEIN

Oxidation Conditions and/or Inhibitors	Clams Living in Anoxia (5 days) in Brackish Water with 1 mM Fumarate	Clams Living in Anoxia (5 days) in Brackish Water	Rat Liver	Clams Living in O_2
KCN + FUM	3.41	2.77	15	5.58
KCN + FUM + ROT	0.74	0.82	15	0.49
KCN (W/O FUM) (ENDOGENOUS)	1.09	1.06	10	1.43
FUM (W/O INHIBITORS)				6.69

TABLE 3. NADH FUMARATE REDUCTASE ACTIVITY IN THE PRESENCE OF MALONATE AND OXALOACETATE. SEE THE TEXT FOR THE DETAILS OF THE CONDITIONS. OAA : OXALOACETATE.

NMOLES OF NADH OXIDIZED/MIN/MG PROTEIN

Oxidation Conditions	Activity
KCN + fumarate	4.23
KCN + fumarate + malonate	2.08
KCN + fumarate + malonate + rotenone	0.75
KCN + fumarate + OAA	6.71
KCN + OAA	8.80
fum + OAA + malonate	43.3

Derivation of the ratio of the competitively inhibited rate to the uninhibited rate of an enzyme reaction

From the Michaelis-Menton equations for the competitively inhibited and the uninhibited reactions (Lehninger, 1975):

$$\frac{1}{v} = \frac{Km}{Vm}\frac{1}{[S]} + \frac{1}{Vm} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\frac{1}{v_I} = \frac{Km}{Vm}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{Vm} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

one can have

$$\frac{v_I}{v} = \frac{Km\,K_I + K_I\,[S]}{Km\,K_I + Km\,[I] + K_I\,[S]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Since $Km\,K_I$ is small, compare with other terms,

$$\frac{v_I}{v} = \frac{[S]}{\frac{Km}{K_I}[I] + [S]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

where

v_I : the rate of the inhibited reaction

v : the rate of the uninhibited reaction

$[S]$: the concentration of the substrate

$[I]$: the concentration of the competitive inhibitor

Km : Michaelis constant for the substrate

K_I : Inhibitor constant

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