TRANSAMINASE AND GLUTAMATE DEHYDROGENASE ACTIVITY IN *RANGIA CUNEATA*

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INTRODUCTION

Energy metabolism in both aerobic and anaerobic organisms initially involves the reactions of glycolysis. However, many invertebrates appear to have developed an additional method of anaerobic ATP formation. Some examples included free living annelids (Schroff and Schottler, 1977), bivalves (Hochachka and Mustafa, 1972) as well as some bacteria (Dorn, et al., 1978). It appears that these organisms, which are capable of existing under sustained anoxic conditions, not only exhibited increased levels of enzymes associated with anaerobiosis but displayed a number of unique enzymatic pathways for anaerobic energy extraction (Hochachka and Somero, 1976). During anoxia these organisms relied upon the simultaneous mobilization of two energy sources, carbohydrates and amino acids. As a result, a multiplicity of end products were formed, such as (1) alanine, (2) succinate, (3) metabolic CO_2, (4) propionate, (5) acetate and alanopine (Hochachka, 1980).

Available evidence suggested that at least in some anaerobic invertebrates the metabolic fate of phosphoenolpyruvate (PEP) was quite different from that in other organisms. The metabolic pathway that appears to occur in these anaerobes is summarized below. As PEP is formed, it is channeled through a series of reactions resulting in the accumulation of two molecules of malate for every molecule of glucose that enters the pathway, instead of being converted to pyruvate by pyruvate kinase as this occurs in mammals. Malate may be converted to fumarate which, acting as a final electron acceptor, is reduced by NADH to succinate. Another malate molecule is a source of the NADH through the malic enzyme

reaction. The reduction of fumarate by the NADH (NADH fumarate reductase) appears to be mediated by the anaerobic electron transport system (NADH dehydrogenase, Coenzyme Q, etc.) associated with mitochondria, and to be coupled to the phosphorylation of ADP. The conversion of malate to pyruvate makes possible a glutamate-pyruvate transamination whereby α-ketoglutarate may be converted to succinyl CoA allowing for a potential substrate level phosphorylation (succinyl CoA synthetase) while alanine is a "switch" that controls the PEP branchpoint by inhibiting pyruvate kinase, thus making it possible to convert PEP into oxaloacetate by PEP carboxykinase. Therefore, the transaminase provides a means for the subsequent substrate level phosphorylation, another energy gain for the organism. In many organisms the α-ketoglutarate produced in the transamination is reconverted to glutamate by NADH linked glutamate dehydrogenase (GDH). Although the metabolic fate of PEP in anaerobic invertebrates just summarized was by no means established unequivocally, it is reasonable to hypothesize that the pathway also occurs in Rangia cuneata as well, unless proven otherwise.

The work was concerned with examination of transaminase and GDH in the mantle tissue of a bivalve, Rangia cuneata, which is readily available in this area of the country. It was undertaken in order to help elucidate roles that these enzymes may play in the invertebrate anaerobic metabolism.

REVIEW OF LITERATURE

Rangia cuneata is a predominant bivalve mollusc in oligohaline zones of estuaries from the Chesapeake Bay to the gulf coast where salinity levels are 5 pp/thousand and below. These zones are subjected to the greatest fluctuations in salinity because of irregular surges of river water during periods of high rain and surface runoff. From the time Rangia was first reported in North Carolina in the 1950's, it gradually increased in number. Tenore (1971) reported that the average density of the Pamlico River was 275 individuals/m^2.

Since many bivalves must accommodate temporary or indefinite periods of anoxia, created especially in the low tide when they close the shells completely in order to avoid dehydration, it is not surprising to find that these organisms utilize anaerobic metabolism to sustain life. Evidence supporting existence of various anaerobic metabolic pathways was based upon the accumulation of various end products of fermentation such as (1) alanine, (2) succinate, (3) metabolic CO_2, (4) propionate, (5) acetate and alanopine (Hochachka, 1980). Oxygen consumption was noted in facultative anaerobes but there was no evidence of complete oxidation of substrates to carbon dioxide and water. The quantity and types of the aforementioned metabolites differed somewhat among the organisms (Saz, 1971).

In the facultative anaerobes, glycogen (glucose) breakdown can be assumed to occur according to classical glycolysis (Figures 1, 2 and 3, Appendix) to phosphoenolpyruvate (PEP), as occurs in most living organisms. The metabolic production of succinate from PEP, however, was

believed to be one of the pathways in which an anaerobic energy yielding mechanism was associated, although the pathways from PEP to succinate have not been well established beyond hypothesis (Hochachka, 1976). Evidence indicated that PEP occupied the pivotal position for the aerobic-anaerobic transition (Mustafa and Hochachka, 1973a). PEP, the metabolic branchpoint, was thought to be converted to pyruvate via pyruvate kinase (PK) under aerobic conditions or oxaloacetate by PEP carboxykinase (PEPCK) under anaerobic conditions. A possibility of competition between the two enzymes for PEP was obvious when both enzymes occurred at significant levels in the same tissue. In fact, both enzymes occurred in the soluble fraction of the cell and consequently competed for the same PEP pool during the transition (Chen and Awapara, 1969a) in Rangia cuneata. A progressive inactivation of PK over a short period of time was suggested wherein PK and PEPCK, the latter of which is the enzyme that seems to direct PEPCK into the anaerobic pathway (Figure 4, Appendix), could not function simultaneously at a significant rate (Hochachka and Mustafa, 1973b). Inhibition of the rate of the PK reaction by H^+ and alanine, which were known to accumulate in anaerobiosis, appeared to account for the transition from aerobic to anaerobic metabolism. Molluscan bivalves were known to sustain substantial acidification of their tissues during anaerobiosis but to date, no definite acidifying agents have been postulated (Livingstone and Bayne, 1973).

The "all or nothing" operation of the aerobic-anaerobic pathways for glucose was postulated by Hochachka and Mustafa (1973a) stating that there had to be a decrease in the in vivo pH before PEPCK could function significantly. However, such a drop in pH was not caused by the accumu-

lation of acid end-products of the anaerobic pathway until PEPCK became functioning (Livingstone and Bayne, 1973). Mustafa and Hochachka (1973b) stated that because PEPCK and PK had essentially non-overlapping pH profiles, (PK pH optimum, 8.5, and PEPCK, 5.2), an automatic inhibition of PK occurred with a concomitant activation of PEPCK. Accompanied by this pH drop, L-alanine, known to accumulate along with succinate under anaerobic conditions, inhibited PK by increasing the $K_{m(PEP)}$ and decreasing the V_{max}. Inhibition by L-alanine appeared to be potentiated by decreasing pH. L-alanine reversed ITP inhibition of PEPCK and slightly activated the enzyme at low PEP concentration due to reduction in the apparent $K_{m(PEP)}$. These effects occurred at pH ranges (5-6), in which PK activity was very low and L-alanine inhibition of PK was high. Therefore, decreasing pH and increasing L-alanine concentration during the aerobic-anaerobic transition caused an exponential increase in PEPCK rate with an exponential decrease in PK rate. This simple "on/off" mechanism for PK and PEPCK was disputed by De Zwaan (1976). Although oyster mantle PEPCK showed a lower pH optimum than PK, the pH optima of the two enzymes overlapped in the region between pH 6.8 and 7.4. Livingstone and Bayne (1973) stated it was possible that a simultaneous flux of substrates through both pathways occurred and the extent as to which predominated, depended in part upon the degree of tissue hypoxia. However, they did agree with Hochachka and Mustafa (1972) that no matter what the control mechanism that operated at the PEP branchpoint, there did appear to be at least two linear paths of glucose fermentation, one leading to alanine accumulation and the other leading to succinate accumulation.

The oxaloacetate formed from PEP carboxylation was reduced to malate (Figure 4, Appendix) in anaerobic invertebrates. In the tissues of these organisms, cytoplasmic NAD^+-linked malate dehydrogenase (MDH) was very active (Hochachka, 1976). In addition to the formation of malate, MDH appeared to serve to maintain low oxaloacetate concentrations thereby preventing a significant reversal of PEPCK activity. Redox balance of the pathway up to this point can be achieved by the regeneration of NAD^+ by MDH required for the triose phosphate dehydrogenase (TDH) reaction, assuming of course that all of the carbons of glucose are channeled into this pathway during anaerobiosis (i.e., if PK is fully blocked).

Available evidence suggested that two alternate routes existed by which malate may be metabolized to form succinate and that both pathways competed equally for the cytoplasmic malate (Chen and Awapara, 1969a). One route involved malic enzyme that catalyzes conversion of malate to pyruvate accompanied by the reduction of $NADP^+$. Although malic enzyme was known to be thermodynamically readily reversible, strong kinetic barriers against carbon dioxide fixation tended to force the reaction in the decarboxylation direction (Hochachka and Mustafa, 1972). This reaction examined under varying pH conditions led to the finding that the decarboxylation reaction was greatly favored at alkaline pH values. At an acidic pH of approximately 5.2, the difference between the forward and the reverse reaction rates disappeared to the point where saturating concentrations of substrates provided a rate of carboxylation similar to that of decarboxylation. Since these acidic pH levels were encountered during anoxia, possible existence of other regulatory parameters, which strongly favor malic enzyme function in the direction of pyruvate formation in

vivo, were considered. For example, Hochachka and Mustafa (1973b) noted the malic enzyme showed a much greater affinity for malate than for pyruvate (K_m for malate was approximately .45 mM as compared to the K_m for pyruvate of 14 mM) and malate was also shown to have a product-inhibitory effect in the carboxylation direction. The ratio of $NADP^+$/NADPH regulated malic enzyme and therefore the reaction velocity was highly sensitive to the redox potential of the cell. During anoxia in these organisms, the maintenance of redox balance was perhaps as critical to the organism as the production of energy (Saz and Hubbard, 1957). The NADPH formed in the malic enzyme reaction was thought to reduce another molecule of malate to succinate (another route of malate metabolism to succinate) via fumarase and the fumarate reductase reaction (Saz and Lescure, 1969). The fumarate reductase reaction will be discussed in more depth later. As previously discussed, pyruvate kinase activity appeared to function at a progressively lower rate as the anoxic condition continued (Mustafa and Hochachka, 1971).

Once pyruvate was formed, the primary metabolic fate was its conversion to alanine by a transamination reaction in which α-ketoglutarate (α-KG) was produced from L-glutamate (Figure 7, Appendix). It was suggested that the transaminase was very important to the anaerobic pathway because it not only accounted for alanine formation but the formation of α-KG made possible an energy advantage, which will be discussed later. As previously mentioned, alanine was an important factor in regulation at the PEP branchpoint. In many organisms, α-KG produced in such a glutamate-pyruvate transamination was known to be reconverted to glutamate by glutamate dehydrogenase (GDH). In facultative anaerobes, however, the

specific activity of GDH was found to be very low and α-KG dehydrogenase (α-KG-DH) activity very high (Campbell and Bishop, 1970; Hammen, 1969). For this reason, it was believed that any GDH present would be outcompeted by α-KG-DH for the substrate, α-KG. In other words, the transaminase was thought to channel α-KG directly into the α-KG-DH reaction.

The potential metabolic sources of glutamate may be amino acids from decayed living materials in the environment. Bivalves were known to maintain very high concentrations of various amino acids in their tissues. For example, arginine and proline were found to occur in concentrations as high as 1 milligram per gram of wet tissue weight. Arginine and proline were known to be easily converted to glutamate and these reactions were demonstrated in molluscan tissues (Campbell and Bishop, 1970). It is possible that α-KG may also be a source for glutamate production by the direct action of GDH. However, as was stated previously, GDH activity was believed to be very low and therefore was not expected to contribute significantly to glutamate formation.

Suggestion that an energetic advantage was gained by the formation of α-KG through the transamination in facultative anaerobes was based on the high α-KG-DH in these organisms, which catalyzes the formation of succinyl-CoA (Figure 8, Appendix). The subsequent substrate phosphorylation catalyzed by succinyl-CoA synthetase was considered to be one of the important energy yielding reactions in facultative anaerobes (Figure 9, Appendix) (Mustafa and Hochachka, 1973a). The formation of propionate via propionyl-CoA synthetase presumably would be another source of a high energy phosphate (Figure 9, Appendix).

As previously mentioned, there were two pathways responsible for

the accumulation of succinate from malate in facultative anaerobes. Fumarate formed from malate by fumarase, was thought to enter the mitochondrion and to be reduced to succinate by NADH-fumarate reductase complex. It was believed that this complex was selected as a means of forming ATP in these facultative anaerobes as evidenced by a high affinity of this complex for fumarate and a relatively low affinity for succinate. The NADH produced in the α-KG-DH reaction was thought to supply the reductive power for the fumarate reductase reaction which was believed to be coupled to ATP formation. The NADPH formed during the cytosol malic enzyme reaction was thought to be another source of reducing power and enters the mitochondrion by a transhydrogenase. Saz (1971) introduced the possibility that fumarate was not anaerobically reduced to succinate by a single reductase but instead, there was a complex system of electron transfer components involved in the process (Figure 5, Appendix). Reduction was presumed, therefore, to occur in the direction from NADH to NADH dehydrogenase (f_{p_1}) to an ubiquinone (Coenzyme Q) to succinic dehydrogenase (f_{p_s}) and ultimately to fumarate. One ATP was formed between NADH and coenzyme Q for each molecule of fumarate reduced to succinate. In addition to the bivalves, this anaerobic pathway was believed to exist in parasites, free living helminths and some bacteria (Saz, 1971). Peck and Reddy (1978), while working with anaerobic fumarate reduction in bacteria, spectrophotometrically detected cytochrome b as an additional component of the fumarate reductase complex. There has been, however, little data to date that would indicate the nature of the electron transport components in Rangia cuneata except that Beasley (1979) showed evidence that the complex included rotenone-sensitive NADH dehydrogenase

and malonate-sensitive fumarate reductase or succinic dehydrogenase. It was assumed that the reductase consisted of complex I and complex II (Hatefi, et al., 1962) and that phosphorylation occurred in the Rangia system (Beasley, 1979).

Besides the previously discussed enzymatic reactions and their respective metabolites, Hochachka (1980), having investigated the anoxic oyster heart, suggested the possibility of a reductive condensation reaction involving pyruvate and alanine. The compound formed was identified as N-methylcarboxymethyl-alanine (alanopine) (Figure 7, Appendix). This reaction was catalyzed by the enzyme, alanopine dehydrogenase (ADH), which had a molecular weight of approximately 45,000 and displayed an almost absolute specificity for pyruvate as a keto acid substrate (Fields, et al., 1979). ADH displayed no regulatory properties such as activation or inhibition by glycolytic or tricarboxylic acid cycle intermediates as expected from the size of the enzyme molecule, and its role in this anaerobic pathway appeared to be speculative, if any.

The results of attempts to localize the enzymes involved in the anaerobic pathways of Rangia by Hochachka and Somero (1973), concurred with those of Chen and Awapara (1969a). All of the enzymes catalyzing the metabolism of glucose to succinate were found in the cytosol with the exception of fumarate reductase in the mitochondria. Although the localization and exact mechanisms of energy production varied among these organisms, by extracting energy via the fumarate reductase reaction along with substrate level phosphorylations, they appeared to be capable of tolerating indefinite periods of anoxia.

MATERIALS AND METHODS

ORGANISM

Rangia cuneata were collected in eastern North Carolina from Bath Creek and Roanoke Sound and maintained in an aerated tank containing the water the clams were in when gathered. The tank was refilled with distilled water as dessication occurred. The procedures that follow were conducted using mantle tissue.

TRANSAMINASE ACTIVITY

A. Qualitative Analysis

(1) Paper Chromatography - Two grams of mantle tissue were excised and homogenized in 20.0 ml of cold .05 M KPO_4 (potassium phosphate) buffer at pH 7.4 with a Brinkman Homogenizer, Model PT 10-35, for one minute at a medium speed setting of 8. Another two grams of mantle tissue were homogenized in 20.0 ml of cold detergent (1.1% cetyl trimethyl ammonia bromide and .85% KCl: to be called CTAB). Each homogenate was equally divided into two 50 ml centrifuge tubes. One tube from each homogenate was centrifuged (Sorvall Model RC2-B, head SS-34) in the cold for one minute at 8,000 x g. The supernatant was saved and the sediment was resuspended in 10.0 ml of its original homogenizing medium. The supernatants, the resuspended sediments and the homogenates were used for the enzyme detection. Two small centrifuge tubes for each of these enzyme preparations were then set up. To tube 1, 0.3 ml each of alanine

(0.2 M) and α-ketoglutarate (0.2 M) were added, and to tube 2, 0.3 ml each of sodium pyruvate (0.2 M) and L-glutamate (0.2 M). To prevent decarboxylation of α-keto acids by α-keto acid dehydrogenases, 0.4 ml of sodium arsenite (0.1 M) was also added in each reaction tube. The reaction was initiated with the addition of 1.0 ml of the appropriate enzyme preparation. These two tubes were then incubated for 45 minutes at 37°C in a constant temperature bath. At the end of the incubation, 6.0 ml of cold 95% ethanol was added to each tube and then allowed to stand in an ice bath until flocculent protein precipitate appeared. The tubes were then centrifuged for five minutes at 10,000 x g (Sorvall Model RC2-B, head SM-24). Each supernatant was chromatographed on Whatman number 1 filter paper for detection of amino acids and keto acids. The solvents employed were a n-propanol-water mixture (3:1) for amino acid detection and n-butanol saturated with 3% ammonia for keto acid detection. Every sample was spotted 8 times in the same location with each spot being allowed to dry prior to the next application. When spotting for keto acid detection, a spot of half-saturated 2,4-dinitrophenylhydrazine in 0.1 N HCl was also applied on top of the supernatant spots and the keto acid controls, in order to form yellow products, dinitrophenyl hydrazone derivatives of the α-keto acids at the spots. Both chromatograms were developed one to two hours, removed and placed in a chromatographic oven (100°C)

to dry. For detection of the amino acid spots, the appropriate paper was sprayed with ninhydrin reagent. The paper was then dried and heated in a chromatographic oven (100°C for 5-10 minutes).

(2) Amino Acid Analyzer - A Beckman Model 119B amino acid analyzer (9 x 510 mm column, resin type AA-20 and a resin bed height of 300 mm) was used to determine the amount of the amino acids produced by the transaminase reaction. The analysis involved a three buffer system of sodium citrate (Buffer A, pH 3.25; buffer B, pH 4.12 and buffer C, pH 6.40) and a buffer flow rate of 70.0 ml per hour and a ninhydrin flow rate of 35.0 ml per hour. The column temperature was 50°C and the recorder chart speed was set at 12 inches per hour. The transaminase reaction was carried out in the same way described above for paper chromatography. However, prior to the amino acid analysis, each of the supernatant samples obtained after the reaction was filtered through a 6-8 micron membrane filter. A 20 µl aliquot from each of the four samples was introduced into the column for analysis. (No sediment fraction from the CTAB-homogenate or from the phosphate-homogenate was used.) An additional amino acid analysis was conducted using rat liver as the enzyme source in order to compare its transaminase activity with the clam mantle.

B. Quantitative Analysis (spectrophotometry)

A stock solution of commercial glutamate dehydrogenase (GDH)

(Sigma Chemical Company, type II; protein content, 12 mg/ml; activity, 40 units/mg protein; one unit reduces 1.0 µmole of α-ketoglutarate to L-glutamate per minute at pH 7.3 at 25°C, in the presence of NH_4^+) was used to test the assay procedure that called for (Lowenstein, 1969) 2.0 ml of 0.1 M Tris buffer containing 0.4 M hydrazine sulfate, 10 mM $MgCl_2$, 5 mM EDTA, pH 8.5 (to be called Tris buffer mixture), .03 ml of NAD^+ (80 mg/ml) and .10 ml of 0.1 M L-glutamate (pH 8.5). The reaction was initiated with the addition of 1.0 ml of the GDH which had been diluted to a concentration of .6 units/ml with deionized water. When the GDH assay procedure was tested with CTAB (1.1% CTAB and .85% KCl) in the reaction medium, in the place of Tris buffer, the GDH was inhibited. Therefore, Triton-X 100 replaced the CTAB as a detergent medium for tissue homogenizing (0.1 M Tris-HCl buffer, pH 8.6, containing 1 mM mercaptoethanol and 2.5% Triton-X 100 mixture) (Tadakazu, 1979). Liver tissue was examined for transaminase activity for comparison, preliminary to the examination of the clam mantle tissue. One gram of tissue (liver or clam) was homogenized in 10.0 ml of Tris buffer mixture with a Brinkman Homogenizer for one minute at a speed setting of 8. Another one gram of tissue was also homogenized in 10.0 ml of Triton-X 100 mixture at the same speed setting (8) and duration with foaming kept at a minimum. Both samples were then centrifuged at 20,000 x g for 15 minutes (Sorvall Model RC2-B, head SM-24). In liver samples where turbidity was a problem, the extract was diluted 1:5 prior to the reaction. The reaction

medium for the transaminase in the direction of glutamate and pyruvate formation contained 1.0 ml of Tris buffer mixture, 0.5 ml of α-ketoglutarate (0.2 M), 0.5 ml of alanine (0.2 M), .06 ml of NAD^+ (0.1 M) and .02 ml (.6 units) of commercial GDH that had been diluted to 10 mg/ml with deionized water. The reaction was started with the addition of 1.0 ml of the appropriate enzyme preparation and the total reaction volume was 3.08 ml. The activity was estimated by following absorbance increase (NAD^+ reduction) at 340 nm with a Beckman DB spectrophotometer and a ten-inch linear potentiometric recorder (Model 1005). The transaminase was then assayed in the direction of α-ketoglutarate and alanine formation. Before the assay, the stock GDH was tested for the reverse direction and found to be fully active. The reaction medium consisted of 1.0 ml of α-ketoglutarate (0.2 M), 1.0 ml of Tris buffer mixture, .06 ml of NADH (80 μg/ml), and 1.0 ml of NH_4Cl (.01 M). The reaction was started with the addition of .02 ml (.6 units) of GDH that had been diluted to 10 mg/ml with deionized water. Total reaction volume was 3.08 ml. For the transaminase in this direction, the reaction medium contained 0.5 ml Tris buffer mixture, 0.5 ml of L-glutamate (0.2 M), 0.5 ml of pyruvate (0.2 M), .02 ml (.6 units) of stock GDH diluted to 10 mg/ml, .06 ml of NADH (80 μg/ml), and 0.5 ml of NH_4Cl (.01 M). The reaction was started with the addition of 1.0 ml of the enzyme extract (prepared in Triton-X 100 mixture of Tris buffer mixture). The activity was estimated by following the NADH oxidation at 340 nm.

Glutamate Dehydrogenase Activity

Glutamate dehydrogenase (GDH) was assayed in the direction of α-ketoglutarate formation. Liver tissue was examined before clam tissue for comparison. Tissue (liver or clam) homogenates were prepared in Tris buffer mixture and in Triton-X 100 mixture described previously in the section of the quantitative analysis for transaminase activity. The reaction medium contained 2.0 ml of Tris buffer mixture, .03 ml of NAD^+ (80 mg/ml) and .10 ml of L-glutamate. The reaction was started with the addition of 1.0 ml of the appropriate enzyme extract (Tris buffer mixture or Triton-X 100 mixture). The activity was estimated by following the NAD^+ reduction at 340 nm.

Determination of Oxygen and Carbon Dioxide by Warburg Respirometer

The Warburg constant volume respirometer was used to determine oxygen consumption and carbon dioxide evolution in rat liver and clam mantle tissue slices as well as homogenates from both of these sources (Umbreit and Burris, 1972). Two Warburg flasks were necessary to determine the volume of carbon dioxide evolved from the reaction mixture and one flask was used for the determination of O_2 consumption. An additional flask (thermobarometer) was used to correct for atmospheric pressure changes. The thermobarometer contained 3.4 ml of distilled water. The reaction mixture consisted of 0.5 ml of pyruvate (0.2 M), 0.5 ml of glutamate (0.2 M), 1.0 ml of KPO_4 buffer (.05 M, pH 7.4), .06 ml of NAD^+ (80 mg/ml), 0.33 gms tissue slice or 1.0 ml homogenate and deionized water to make the final volume 3.2 ml. To flask number one, used for O_2 determination, 0.20 ml of potassium hydroxide (6 N), was pipetted into the center well which contained a filter paper wick. Flasks numbered two and three, used

in measuring carbon dioxide evolution, contained 0.20 ml of 6 N sulfuric acid in the sidearms. The four flasks were attached with springs to the manometer and sealed. The gas vents were also sealed. The flasks were maintained in a constant temperature water bath at 37°C for 5-10 minutes. At the end of the equilibration period, all stopcocks were closed and the manometer fluid was adjusted for the first reading. After this initial reading, the number two flask was removed and the sulfuric acid in the sidearm was added to the solution in the main chamber. The manometers were read every 5 minutes after the initial readings for 60 minutes of incubation. At the end of the incubation period, flask number three was removed from the bath and the sulfuric acid was added to the reaction mixture. Readings were continued every 5 minutes for an additional 10 minutes on all four flasks. The above procedure was repeated with the reaction mixture in which either pyruvate or glutamate was omitted. Razor blades were used to prepare tissue slices on ice. The homogenates were prepared according to the procedure previously described for the quantitative analysis of the transaminase.

Protein Determination

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

RESULTS AND DISCUSSION

A transaminase was detected in the direction toward the formation of alanine and α-ketoglutarate as evidenced by the observed formation of these products (Table 1, Appendix). However, the reaction in the direction toward the formation of glutamate and pyruvate was not demonstrated. The formation of glutamate and the lack of formation of pyruvate in the enzyme extracts prepared in the phosphate buffer appeared to indicate glutamate was formed by a means other than the reversal of the transaminase detected. For example, relatively active glutamate dehydrogenase (GDH), as found in the mantle tissue (see later results), could have caused the rapid accumulation of glutamate. The lack of formation of the products (glutamate and pyruvate) by the extracts prepared in CTAB was probably a result not only of the slow transaminase reaction in the direction of glutamate and pyruvate formation but also the inactivation of glutamate dehydrogenase (as was indicated in Materials and Methods) by CTAB. Possible inhibition of the reversal of the transaminase by CTAB seemed unlikely since a transaminase is known to be readily reversible.

Glutamate and alanine where formed, were further analyzed quantitatively by an amino acid analyzer (Table 2, Appendix) to verify the results obtained by the paper chromatography experiments, although this analysis alone would not be sufficient to detect transaminases since keto acid detection was not possible. Concentrations of the products found were consistent with the findings of paper chromatography. For example, when alanine and α-ketoglutarate were used as initial substrates, glutamate

accumulation was observed only in the enzyme extracts prepared in the phosphate buffer and not in the ones in CTAB. In the samples where glutamate and pyruvate provided the initial substrates for the reaction, alanine found was comparable in all the enzyme extracts tested. In order to make comparisons to these findings, rat liver was analyzed. When glutamate and pyruvate were starting substrates with the enzyme extracts in the phosphate buffer, alanine concentrations found at the end of the reaction were approximately the same as those found in the mantle tissue experiments. Final glutamate concentrations, however, were higher than those found in the mantle tissue experiments when alanine and α-ketoglutarate were the initial substrates with the enzyme extracts in CTAB.

Data shown in Tables 1 and 2 appeared to suggest that transaminase in the Rangia mantle was functioning only in the direction of alanine and α-ketoglutarate production and that glutamate formation when occurred, was mainly accomplished by a process (such as GDH) other than transamination. The results of the liver analysis seemed to indicate that glutamate was very rapidly metabolized, for the transaminase in this tissue is known to be readily reversible.

Transaminase, as determined quantitatively by spectrophotometry (Table 3, Appendix) was found to be very active in rat liver (all extracts tested) in both directions. Mantle extract, however, showed no activity in the direction of pyruvate and glutamate formation under the same conditions. Activity in the direction of α-ketoglutarate and alanine formation for mantle tissue was as high as for liver tissue. These findings, coupled with the results of paper chromatography and amino acid analysis further strengthened the idea that this transaminase was

functioning only in one direction, namely α-ketoglutarate and alanine formation. Mustafa (1975) noted similar activity with oyster transaminase. He described the "unidirectional catalysis" by stating that there was an apparent product activation by alanine and product inhibition by glutamate (Hochachka and Somero, 1976). This certainly did not provide an adequate explanation for this process since it is extremely difficult to understand such activation and inhibition in transaminases. In any case the result (i.e., unidirectional transaminases) was not unique to this investigation here for Rangia.

Liver and clam mantle exhibited similar (though clam showed slightly lower) levels of glutamate dehydrogenase activity (Table 3-III, Appendix). This raised the possibility that GDH was functioning in glutamate production (in extracts in the phosphate buffer; Tables 1 and 2, Appendix) when tested for transaminase in the direction of glutamate and pyruvate formation as mentioned previously. If the rate of glutamate production via GDH was considerably greater than the rate for the transaminase, then rapid glutamate accumulation could occur. It is possible that the incubation period was not sufficient for the transaminase to occur to a significant degree in the direction of pyruvate and glutamate formation and consequently only glutamate was detected as a result of GDH. The present finding that GDH in clam mantle was high was contrary to the belief that GDH in anaerobic invertebrates was low in general (Hochachka et al., 1973).

Oxygen consumption for tissue slices and tissue homogenates prepared in the buffer was approximately equal but values obtained for tissue homogenates prepared in the detergent were lower in both liver and clam

(Table 4, Appendix). Mantle preparations showed consistent oxygen uptake, though much lower than liver. All preparations appeared to contain sufficient endogenous substrates to cause equally high oxygen consumption regardless of the presence of two substrates or of one. The drop in the oxygen consumption in the tissue homogenates prepared in the detergent was probably caused by a disruption in membrane integrity by the detergent. Although Rangia was considered to be an essentially anaerobic organism (Chen and Awapara, 1969b), oxygen consumption was observed in Rangia mantle in this study here as well as in other bivalves. For example, Allen (1961) noted an increase in O_2 consumption when the mussel, M. edulis was placed in dilute sea water. He felt that this O_2 consumption, presumably associated with amino acid oxidases, would supply short chain keto acids which could be used as substrates in the transaminase reaction. It was, however, generally believed that although O_2 consumption occurred in anaerobic invertebrates, it did not contribute significantly to energy production (Allen, 1961; Chen and Awapara, 1969b; Stokes and Awapara, 1969; Mangum, 1973).

The amount of CO_2 evolution seemed to be independent of the methods of tissue preparations (Table 5, Appendix). The substrates in the reaction medium appeared to have an effect on the amount of CO_2 evolved, while that was apparently not the case on O_2 consumption. In each tissue preparation of liver and clam mantle, CO_2 evolution was highest with glutamate and pyruvate in the reaction medium. This was obviously expected since glutamate and pyruvate have their own metabolic sequence reactions that lead to CO_2 evolution. Some of such reactions are presumably common to both sequences. These common reactions probably resulted in the present

finding that the CO_2 evolution in the presence of both glutamate and pyruvate was less than the addition of the CO_2 evolution in the presence of glutamate alone and that in the presence of pyruvate alone in all liver enzyme preparations and in clam mantle slices (Table 5, Appendix). Although the CO_2 evolution in the presence of these two substrates in the clam mantle homogenates was higher than the addition of the CO_2 evolution under the other two conditions, it seemed unlikely that this result had any bearing on the metabolic uniqueness of clam mantle, since the clam mantle slices gave the different result.

The CO_2 evolution in clam mantle was considerably lower than in rat liver. This probably meant that clam was less metabolically active than rat.

Although it is possible to conclude that a "unidirectional" transaminase was present in Rangia mantle, as was mentioned previously it is difficult to conceive mechanistically how it works because of the readily reversible nature of transaminases. Alternate hypotheses involve reactions consuming one or more of the transaminase substrates. Recently, Hochachka (1980) reported a condensation reaction between pyruvate and alanine forming a product referred to as alanopine in the oyster. This reaction was catalyzed by alanopine dehydrogenase (ADH) (Figure 7, Appendix). A reaction such as this could account for the disappearance of pyruvate in the paper chromatography. It is entirely possible also that a similar dehydrogenase which catalyzes condensation of glutamate and pyruvate to form the product N-methylcarboxymethyl-glutamate (MCMG) (Figure 10, Appendix) might be present in the Rangia mantle. Presence of the enzyme MCMG dehydrogenase (glutamate forming), would account for the apparent

irreversibility of the transaminase reaction. Conversion of alanine and α-ketoglutarate in the presence of the mantle enzyme preparations to glutamate and pyruvate would lead to the formation of MCMG if a moderately active MCMG dehydrogenase is present in the tissue, thus preventing accumulation of glutamate and pyruvate. Such a speculation is not completely out of the question in view of the presence of alanopine dehydrogenase in the oyster and of saccharopine dehydrogenases (glutamate forming and lysine forming) in some organisms (Lehninger, 1975; Metzler, 1977).

SUMMARY

Active transaminase was present in Rangia cuneata mantle. It was detected only in the direction of α-ketoglutarate and alanine formation. Mechanisms responsible for the unidirectional nature of the enzyme remained unclear.

Active glutamate dehydrogenase (comparable or slightly lower level of activity than rat liver enzyme) was also found in the organism contrary to some reports which stated that anaerobic invertebrates contained low glutamate dehydrogenase.

The organism showed lower oxygen consumption and lower carbon dioxide evolution than rat liver, indicating lower metabolic activity.

APPENDIX

FIGURE 1. GLYCOLYSIS (GLUCOSE ⟶ FRUCTOSE - 1,6 - DIPHOSPHATE)

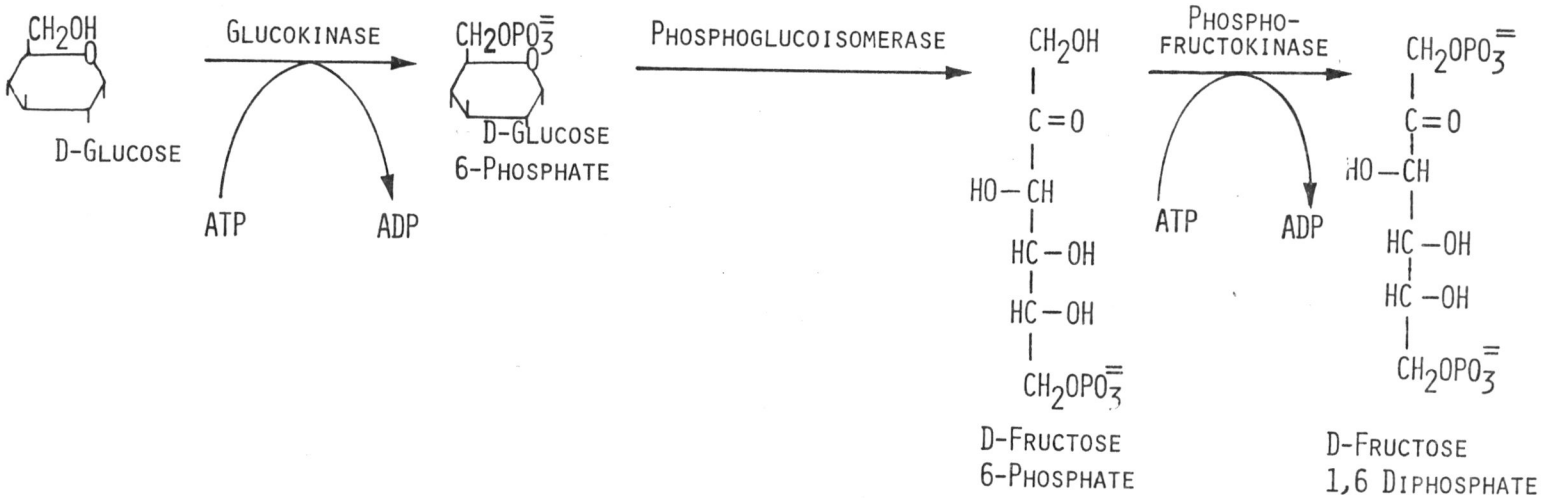

CH2OH
O
D-Glucose
Glucokinase
ATP
ADP
CH2OPO3=
O
D-Glucose
6-Phosphate
Phosphoglucoisomerase
CH2OH
C=O
HO—CH
HC—OH
HC—OH
CH2OPO3=
D-Fructose
6-Phosphate
Phospho-
fructokinase
ATP
ADP
CH2OPO3=
C=O
HO—CH
HC—OH
HC—OH
CH2OPO3=
D-Fructose
1,6 Diphosphate

FIGURE 2. GLYCOLYSIS (FRUCTOSE - 1,6 DIPHOSPHATE ⟶ 3 - PHOSPHOGLYCERATE)

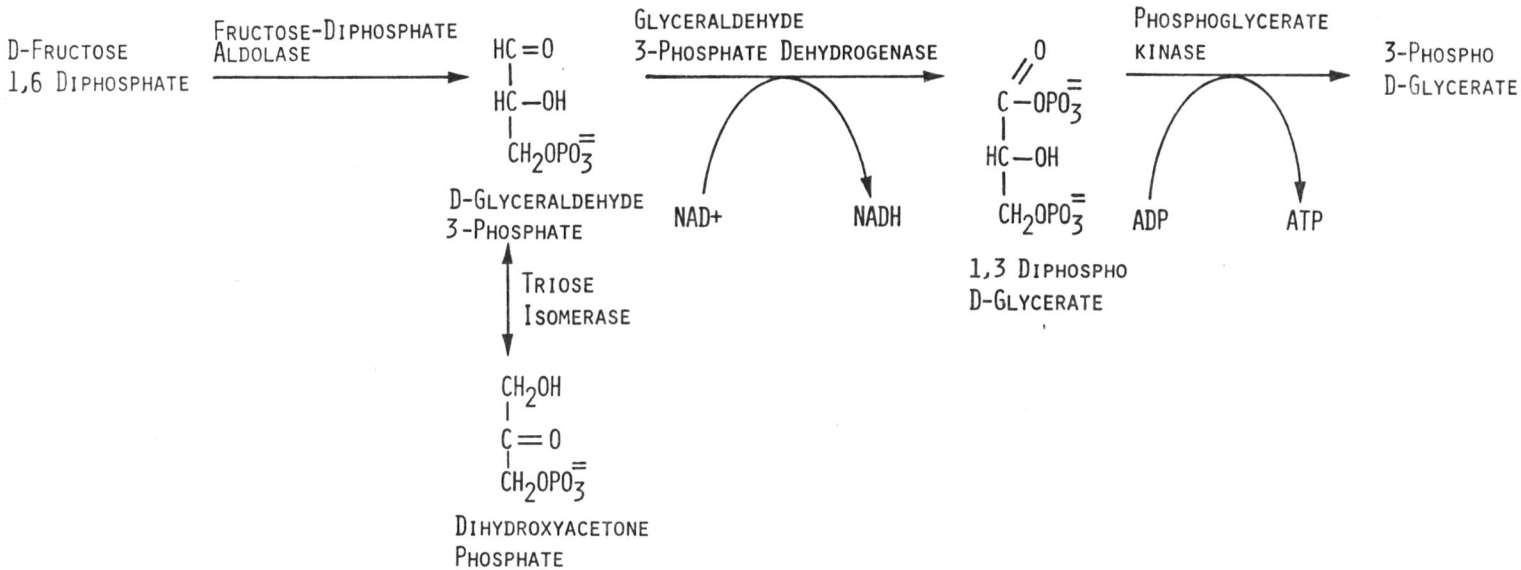

D-Fructose
1,6 Diphosphate
Fructose-Diphosphate
Aldolase
D-Glyceraldehyde
3-Phosphate
Triose
Isomerase
Dihydroxyacetone
Phosphate
Glyceraldehyde
3-Phosphate Dehydrogenase
NAD+
NADH
1,3 Diphospho
D-Glycerate
Phosphoglycerate
kinase
ADP
ATP
3-Phospho
D-Glycerate

FIGURE 3. GLYCOLYSIS (3 - PHOSPHOGLYCERATE ⟶ PHOSPHOENOLPYRUVATE ⟶ ANAEROBIC PATHWAY)

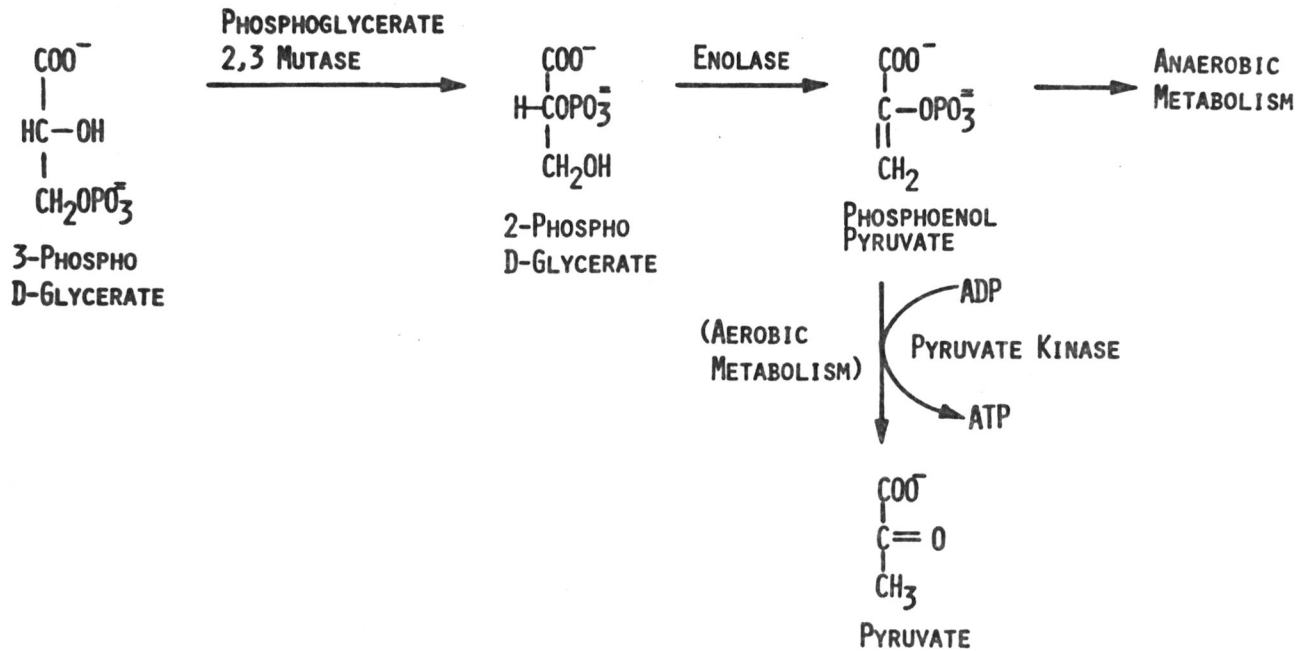

COO⁻
HC—OH
CH2OPO3=
3-Phospho D-Glycerate
Phosphoglycerate 2,3 Mutase
COO⁻
H—COPO3=
CH2OH
2-Phospho D-Glycerate
Enolase
COO⁻
C—OPO3=
CH2
Phosphoenol Pyruvate
Anaerobic Metabolism
(Aerobic Metabolism)
ADP
Pyruvate Kinase
ATP
COO⁻
C=O
CH3
Pyruvate

FIGURE 4. ANAEROBIC METABOLISM OF PHOSPHOENOLPYRUVATE (PEP), (PEP ⟶ MALATE) DURING ANAEROBIC METABOLISM, TWO MOLECULES OF MALATE ARE PRODUCED FOR EVERY MOLECULE OF GLUCOSE THAT ENTERS THE PATHWAY

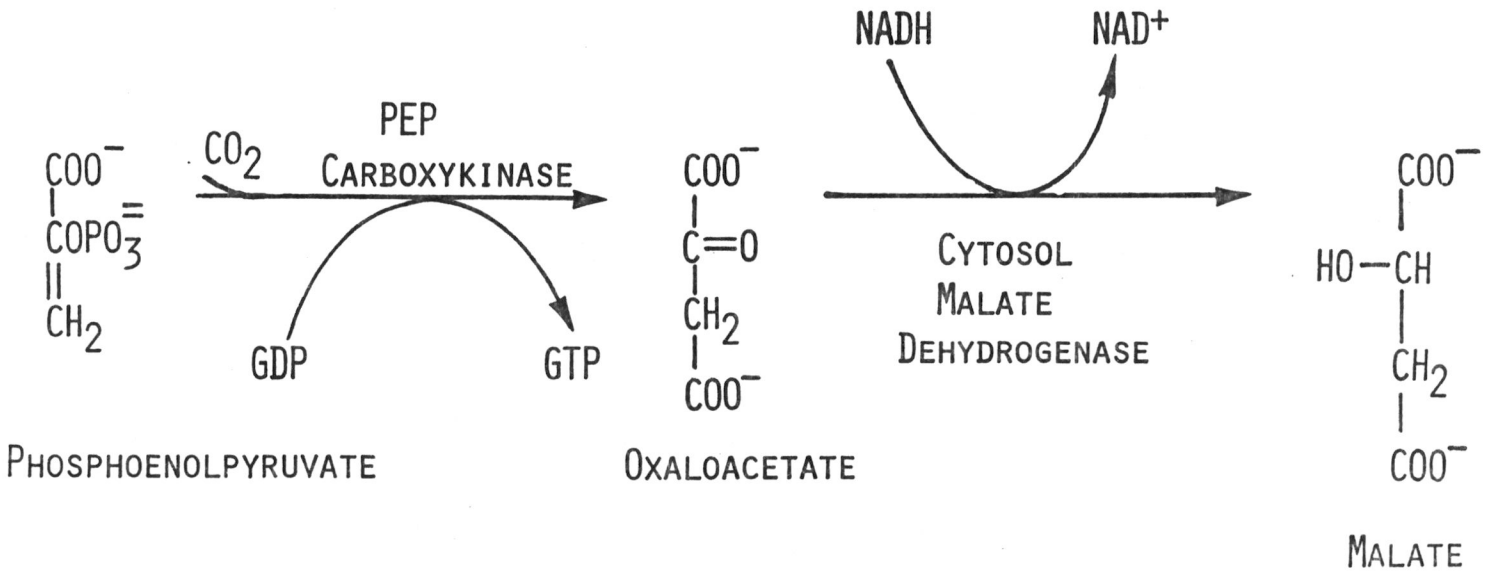

NADH
NAD+
PEP
CARBOXYKINASE
CO_2
GDP
GTP
CYTOSOL
MALATE
DEHYDROGENASE
PHOSPHOENOLPYRUVATE
OXALOACETATE
MALATE

FIGURE 5. ANAEROBIC METABOLISM OF MALATE
(MALATE ⟶ SUCCINATE)

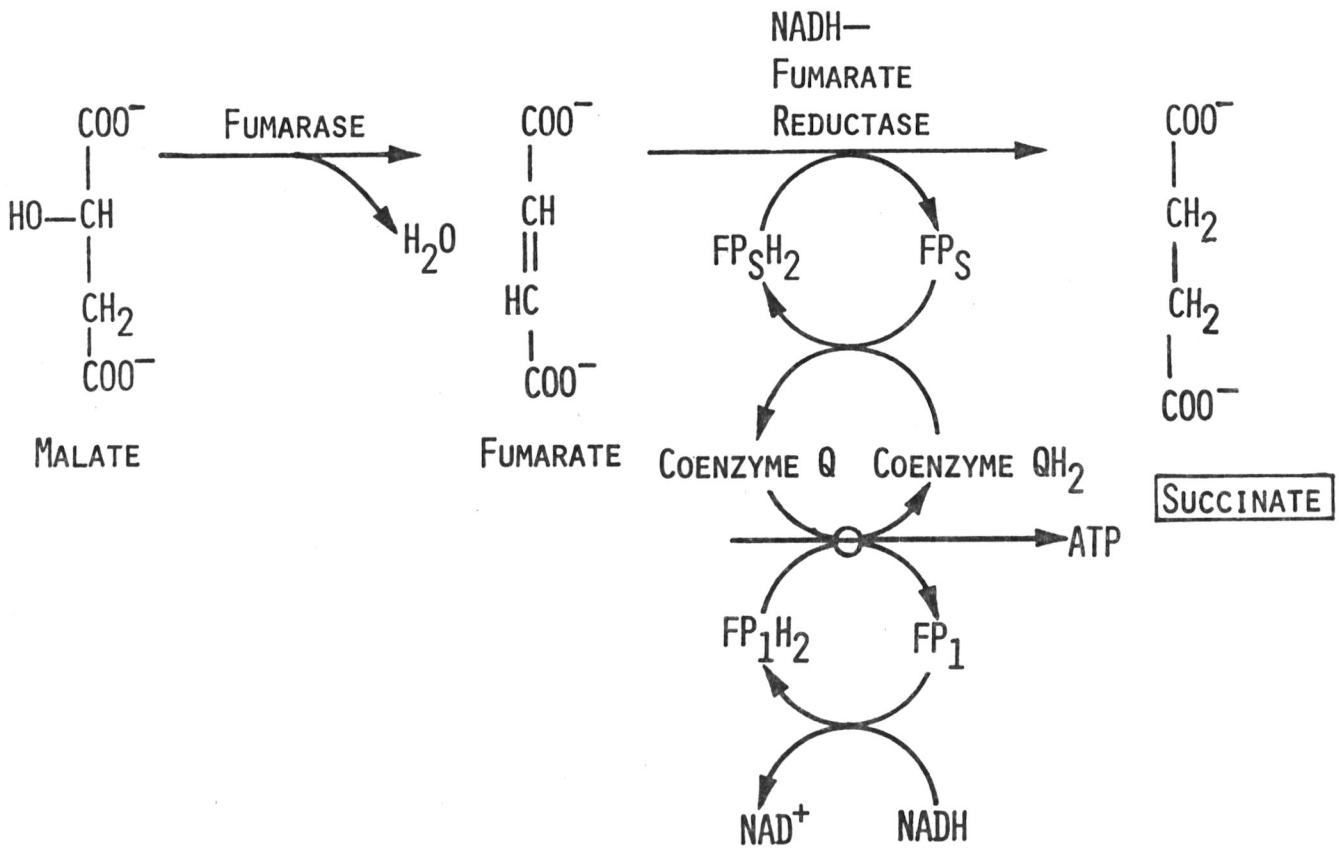

COO⁻
HO—CH
CH2
COO⁻
MALATE
FUMARASE
H2O
COO⁻
CH
HC
COO⁻
FUMARATE
NADH—
FUMARATE
REDUCTASE
FPSH2
FPS
COENZYME Q
COENZYME QH2
ATP
FP1H2
FP1
NAD+
NADH
COO⁻
CH2
CH2
COO⁻
SUCCINATE

FIGURE 6. ANAEROBIC METABOLISM OF MALATE
(MALATE ⟶ ACETATE, ETC.)

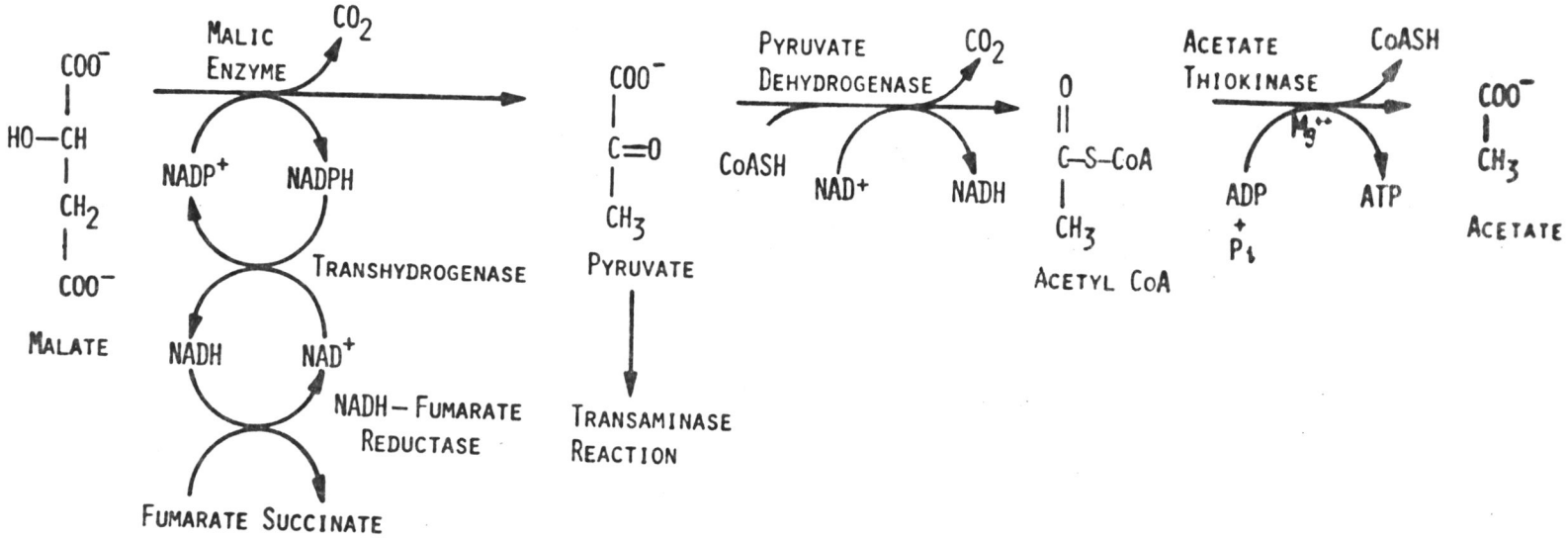

COO⁻
HO—CH
CH2
COO⁻
MALATE
MALIC ENZYME
CO2
NADP+
NADPH
TRANSHYDROGENASE
NADH
NAD+
NADH – FUMARATE REDUCTASE
FUMARATE SUCCINATE
COO⁻
C=O
CH3
PYRUVATE
TRANSAMINASE REACTION
PYRUVATE DEHYDROGENASE
CO2
CoASH
NAD+
NADH
O
C–S–CoA
CH3
ACETYL CoA
ACETATE THIOKINASE
CoASH
Mg++
ADP
+
Pi
ATP
COO⁻
CH3
ACETATE

FIGURE 7. TRANSAMINASE, GLUTAMATE DEHYDROGENASE, ALANOPINE DEHYDROGENASE

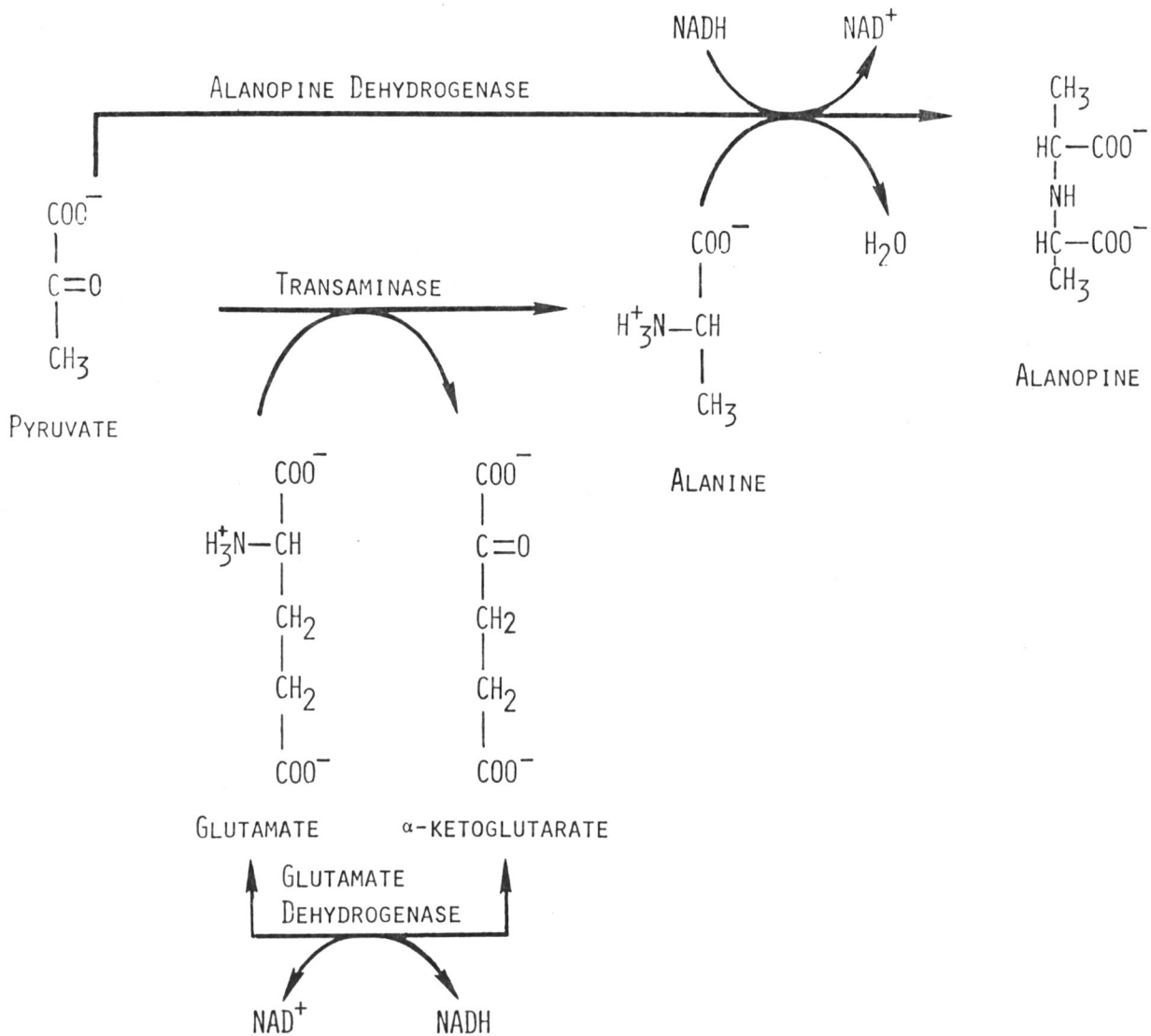

NADH
NAD+
ALANOPINE DEHYDROGENASE
H2O
TRANSAMINASE
PYRUVATE
ALANINE
ALANOPINE
GLUTAMATE
α-KETOGLUTARATE
GLUTAMATE DEHYDROGENASE
NAD+
NADH

FIGURE 8. METABOLISM OF α-KETOGLUTARATE

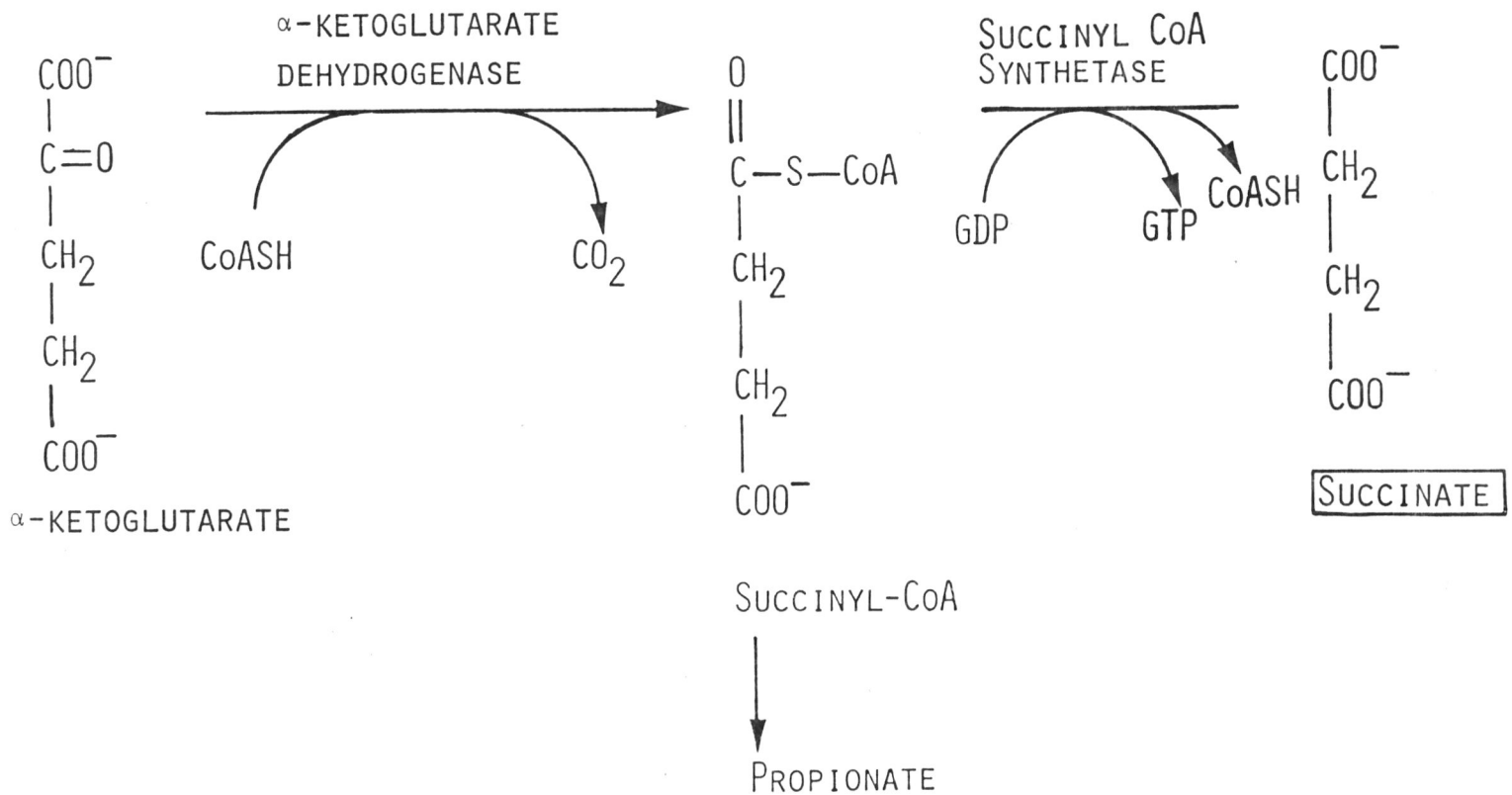
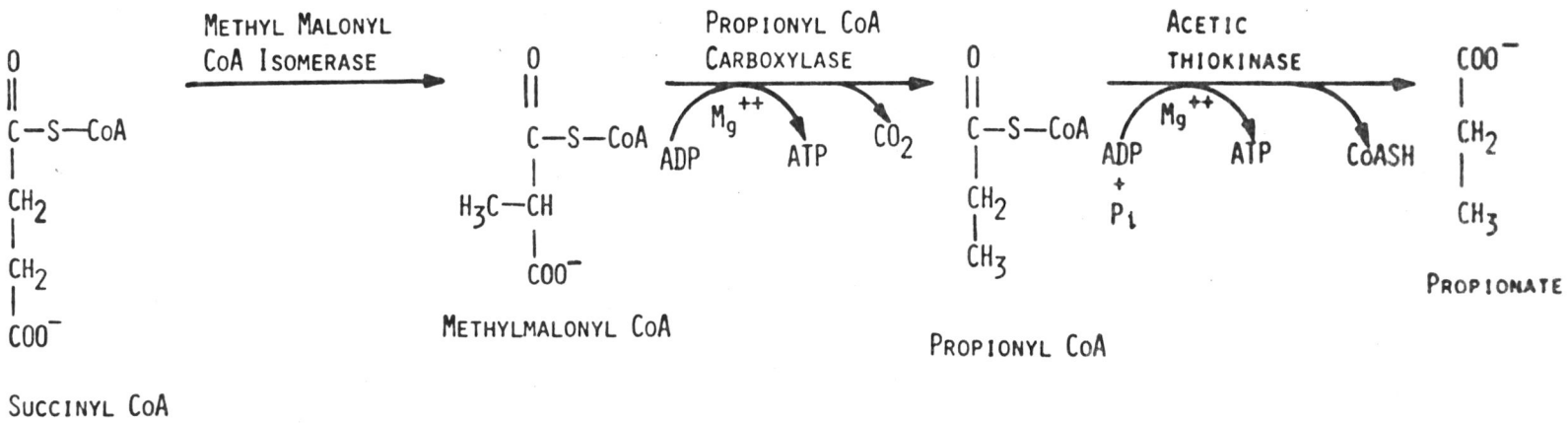

α-KETOGLUTARATE
DEHYDROGENASE
CoASH
CO2
α-KETOGLUTARATE
SUCCINYL CoA
SYNTHETASE
GDP
GTP
CoASH
SUCCINATE
SUCCINYL-CoA
PROPIONATE

FIGURE 9. METABOLISM OF SUCCINYL CoA



SUCCINYL CoA

METHYLMALONYL CoA

PROPIONYL CoA

FIGURE 10. GLUTAMATE AND PYRUVATE CONDENSATION FORMING THE HYPOTHETICAL PRODUCT N-METHYL CARBOXYMETHYL-GLUTAMATE (MCMG).

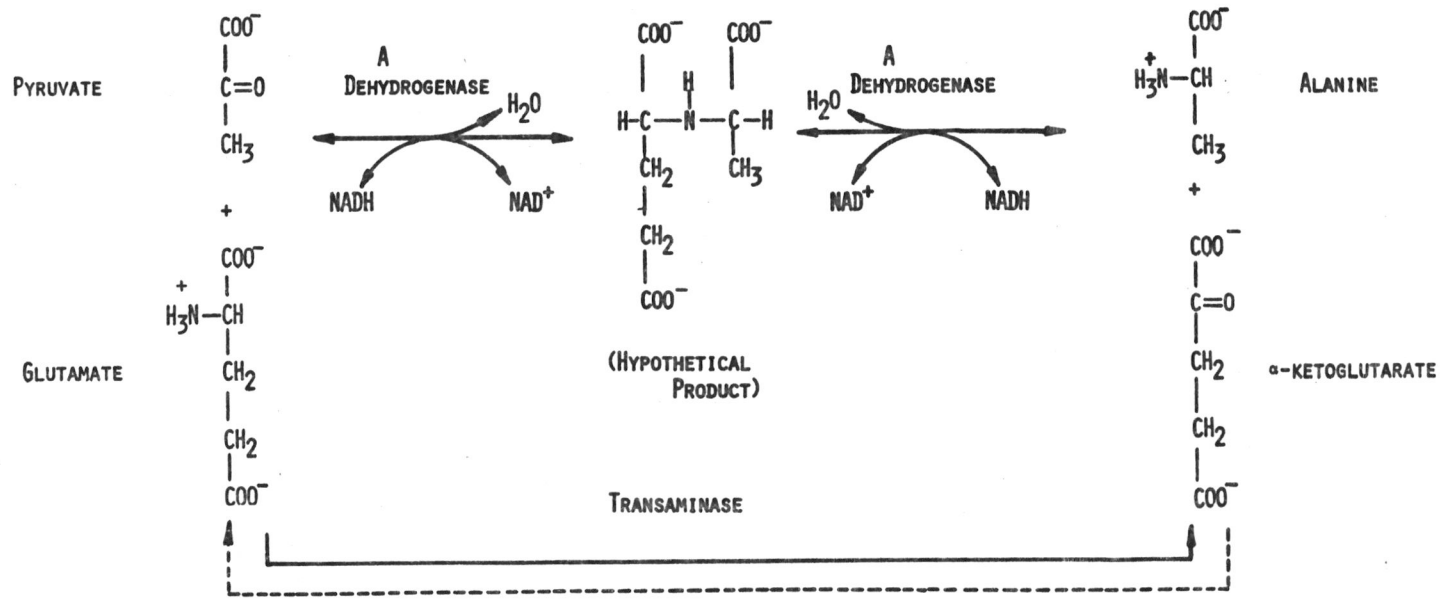

PYRUVATE
A
DEHYDROGENASE
H_2O
NADH
NAD^+
(HYPOTHETICAL
PRODUCT)
A
DEHYDROGENASE
H_2O
NAD^+
NADH
ALANINE
GLUTAMATE
α-KETOGLUTARATE
TRANSAMINASE

TABLE 1. DETECTION OF TRANSAMINASE PRODUCTS WITH DIFFERENT INITIAL SUBSTRATES AND ENZYME PREPARATIONS. SEE TEXT FOR DETAILS. ALA:ALANINE, α-KG:α-KETOGLUTARATE, GLU:GLUTAMATE, PYR:PYRUVATE, CTAB:CETYL TRIMETHYL AMMONIA BROMIDE (DETERGENT)

PAPER CHROMATOGRAPHY

STARTING SUBSTRATES:	(ALA - α-KG)		(GLU - PYR)	
DETECTED PRODUCTS:	GLUTAMATE	PYRUVATE	ALANINE	α-KG
HOMOGENATE - CTAB	–	–	+	+
SUPERNATANT - CTAB	–	–	+	+
SEDIMENT - CTAB	–	–	+	+
HOMOGENATE - BUFFER	+	–	+	+
SUPERNATANT - BUFFER	+	–	+	+
SEDIMENT - BUFFER	+	–	+	+

(–) NO PRODUCT OBSERVED

(+) PRODUCT OBSERVED

TABLE 2. I AND II REPRESENT GLUTAMATE AND ALANINE CONCENTRATIONS WHEN STARTING SUBSTRATES AND ENZYME PREPARATIONS WERE VARIED IN MANTLE TISSUE. THE DATA IN III WAS OBTAINED FROM RAT LIVER. SEE TEXT FOR DETAILS. GLU:GLUTAMATE, ALA:ALANINE, α-KG:α-KETOGLUTARATE, PYR:PYRUVATE, CTAB:CETYL TRIMETHYL AMMONIA BROMIDE

AMINO ACID ANALYSIS

(μMOLES/ML)

I.

STARTING SUB.:	ALA-α-KG	ALA-α-KG	ALA-α-KG	ALA-α-KG
ENZYME PREP.:	HOMOGENATE-CTAB	HOMOGENATE-BUFFER	SUPERNATANT-CTAB	SUPERNATANT-BUFFER
INITIAL ALA	30.0	30.0	30.0	30.0
FINAL ALA	26.0	48.0	32.0	26.0
FINAL GLU	0.96	18.0	0.93	17.0

II.

STARTING SUB.:	GLU-PYR	GLU-PYR	GLU-PYR	GLU-PYR
INITAL GLU	30.0	30.0	30.0	30.0
FINAL GLU	23.0	26.0	22.0	27.0
FINAL ALA	14.0	19.0	12.5	17.0

III.

STARTING SUB.:	ALA-α-KG	ALA-α-KG		GLU-PYR	GLU-PYR
ENZYME PREP.:	HOMOGENATE-CTAB	SUPERNATANT-CTAB		HOMOGENATE-BUFFER	SUPERNATANT-BUFFER
INITIAL ALA	30.0	30.0	INITIAL GLU	30.0	30.0
FINAL ALA	16.83	38.0	FINAL GLU	13.0	23.0
FINAL GLU	2.78	4.5	FINAL ALA	15.0	27.0

TABLE 3. TRANSAMINASE AND GDH ACTIVITIES

I REPRESENTS TRANSAMINASE ACTIVITY IN THE DIRECTION OF GLUTAMATE AND PYRUVATE FORMATION. II SHOWS TRANSAMINASE ACTIVITY IN THE DIRECTION OF α-KETOGLUTARATE AND ALANINE FORMATION. III REPRESENTS GLUTAMATE DEHYDROGENASE (GDH) ACTIVITY IN THE DIRECTION OF α-KETOGLUTARATE FORMATION. SEE TEXT FOR DETAILS.

TRANSAMINASE ACTIVITY & GDH ACTIVITY

(μMOLES/MIN/GM WET TISSUE WT.)

	LIVER EXTRACT	MANTLE EXTRACT
I.		
BUFFER SUPERNATANT	5.26 ±.21	UNDETECTABLE
DETERGENT SUPERNATANT	7.26 ±.48	UNDETECTABLE
II.		
BUFFER SUPERNATANT	3.21 ±.24	3.41 ±.19
DETERGENT SUPERNATANT	4.06 ±.25	3.68 ±.26
III.		
BUFFER SUPERNATANT	4.45 ±.25	2.95 ±.41
DETERGENT SUPERNATANT	4.76 ±.16	3.16 ±.30

(MEAN ± STANDARD ERROR)

TABLE 4. O_2 CONSUMPTION IN RAT LIVER AND MANTLE TISSUE WITH DIFFERENT SUBSTRATES. SEE TEXT FOR DETAILS. GLU:GLUTAMATE, PYR:PYRUVATE

MANOMETRY

(μMOLES/MIN/GM WET TISSUE WT.)

O_2 CONSUMPTION

	GLU & PYR	GLU	PYR
LIVER SLICES	.384	.342	.308
LIVER - BUFFER HOMOGENATE	.377	.462	.490
LIVER - DETERGENT HOMOGENATE	.253	.226	.243
MANTLE SLICES	.197	.191	.188
MANTLE - BUFFER HOMOGENATE	.180	.183	.130
MANTLE - DETERGENT HOMOGENATE	.100	.132	.110

TABLE 5. CO_2 EVOLUTION IN RAT LIVER AND MANTLE TISSUE WITH DIFFERENT SUBSTRATES. SEE TEXT FOR DETAILS. GLU:GLUTAMATE, PYR:PYRUVATE

MANOMETRY

CO_2 Evolution

(μMOLES/MIN/GM WET TISSUE WT.)

	GLU & PYR	GLU	PYR
Liver slices	.737	.424	.476
Liver - buffer Homogenate	.818	.620	.595
Liver - detergent Homogenate	.872	.661	.565
Mantle slices	.417	.230	.257
Mantle - buffer Homogenate	.420	.115	.131
Mantle - detergent Homogenate	.443	.169	.186

LIST OF ABBREVIATIONS

ADH: alanopine dehydrogenase

ADP: adenosine 5'-diphosphate

ATP: adenosine 5'-triphosphate

CoA: coenzyme A

CTAB: cetyl trimethyl ammonia bromide

EDTA: ethylenediamine tetraacetate

GDH: glutamate dehydrogenase

GTP: guanosine 5'-diphosphate

ITP: inosine 5'-triphosphate

KCl: potassium chloride

α-KG: α-ketoglutarate

α-KG-DH: α-ketoglutarate dehydrogenase

KOH: potassium hydroxide

KPO_4: potassium phosphate

MCMG: N-methylcarboxymethyl-glutamate

MDH: malate dehydrogenase

$MgCl_2$: magnesium chloride

NAD^+: nicotinamide adenine dinucleotide, oxidized form

NADH: nicotinamide adenine dinucleotide, reduced form

$NADP^+$: nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH: nicotinamide adenine dinucleotide phosphate, reduced form

NH_4Cl: ammonium chloride

PEP: phosphoenolpyruvate

PEPCK: phosphoenolpyruvate carboxykinase

PK: pyruvate kinase

TDH: triose phosphate dehydrogenase

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ABSTRACT

Earl Hampton Crumpler, Jr. TRANSAMINASE AND GLUTAMATE DEHYDROGENASE ACTIVITY IN RANGIA CUNEATA. (Under the direction of Takeru Ito) Department of Biology, June 1980.

Active transaminase was present in Rangia cuneata mantle. It was detected only in the direction of α-ketoglutarate and alanine formation. Mechanisms responsible for the unidirectional nature of the enzyme remained unclear.

Active glutamate dehydrogenase (comparable or slightly lower level of activity than rat liver enzyme) was also found in the organism contrary to some reports which stated that anaerobic invertebrates contained low glutamate dehydrogenase.

The organism showed lower oxygen consumption and lower carbon dioxide evolution than rat liver, indicating lower metabolic activity.