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Regulation of aerobic fermentation in protozoans

VI. Comparative biochemistry of pathogenic and nonpathogenic protozoans[1]

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Summary

We have investigated the oxidation of carbohydrate by several protozoan organisms. The oxidation is incomplete and results in a mixture of organic acid and carbon dioxide. The phosphofructokinase and pyruvate kinase, enzymes normally under strict metabolic regulation, are not subject to the normal feedback inhibition or activation mechanisms found in other cells. Moreover, our investigations and those of others support the hypothesis that a primary pathway for glucose metabolism is to phosphoenolpyruvic acid and then to oxalacetic acid with subsequent reduction to succinic acid.

The oxidation of carbohydrate by protozoa is generally incomplete and results in a mixture of organic acids and carbon dioxide (von Brand, 1951); the kinds of acids and relative amounts thereof depend upon the particular organism or group of organisms studied (Ryley, 1956). In order to examine the regulatory processes involved in aerobic fermentation by eukaryotes, we have investigated a protozoan, *C. fasciculata*, which contains a mitochondrion but excretes organic acids during growth in air. It is grown readily in the laboratory to yield large quantities of cells and serves in many respects as a model for some of the pathogenic protozoans. Our studies of the regulation of aerobic fermentation in this organism (Marr, 1973, 1973a) have led to the postulate that there is very little allosteric regulation of the enzymes in the glycolytic pathway. Parallel investigations of the pathogenic protozoans, *Leishmania donovani* and *L. braziliensis* have demonstrated that two of the principle glycolytic enzymes, the phosphofructokinase (PFK) and the pyruvate kinase (PK) are very similar to

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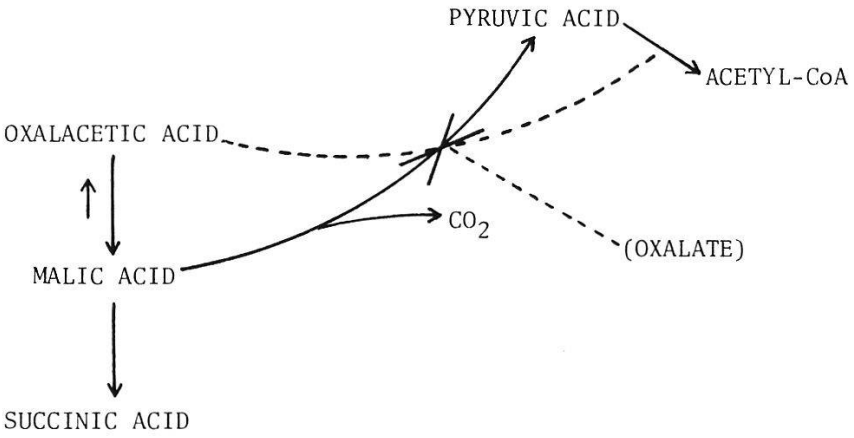

Fig. 1. Inhibition of the malic enzyme by intermediary metabolites.

those found in *Crithidia fasciculata.* Moreover, the growth characteristics and the utilization of glucose parallel the findings in the laboratory protozoan. Here we would like to present a comparative analysis of glycolysis in two pathogenic protozoans and a nonpathogenic laboratory protozoan and emphasize the usefulness of the latter in the study of the biochemistry of the pathogens.

Regulation of glycolysis in Crithidia fasciculata

The observations of Ryley (1956) and Cosgrove (1959) that protozoans form large amounts of succinic and pyruvic acids from glucose suggested that the reactions leading to and from oxalacetate (OAA) might be involved in any regulatory scheme. Consideration of the equilibria of these reactions indicates that control of the malic enzyme would be a reasonable means of regulating carbon flow toward gluconeogenesis, aerobic fermentation, or complete oxidation of dicarboxylic acids (Fig. 1). Although the physiological function of the malic enzyme varies with the organisms studied, or even the type of tissue within an organism, there is general agreement that, in both bacterial and mammalian systems, it is concerned with the generation of pyruvate and reducing equivalents for biosynthesis (Marr, 1973 and op. cit.). Moreover, it also has been shown that in some mammalian tissues a transhydrogenation reaction can take place between NADH and NADP involving the malic dehydrogenase and malic enzyme (Wise and Ball, 1964; Young et al., 1964; Pande et al., 1964). The phenomenon of aerobic fermentation implies that there must be direction of oxalacetate either toward oxidation and pyruvate formation or reduction and excretion as succinate. Our investigations showed that the malic enzyme in *C. fasciculata* was inhibited by OAA, oxalate, and acetyl-coenzyme A (CoA) (Marr, 1973). The inhibition was of a cumulative type and implied the existence of more than one site for the inhibitors. Both oxalacetate and acetyl-CoA were competitive inhibitors of the substrate, malate; oxalate was non-competitive with both substrate and the coenzyme, NADP (Fig. 1). The K_i for acetyl-CoA

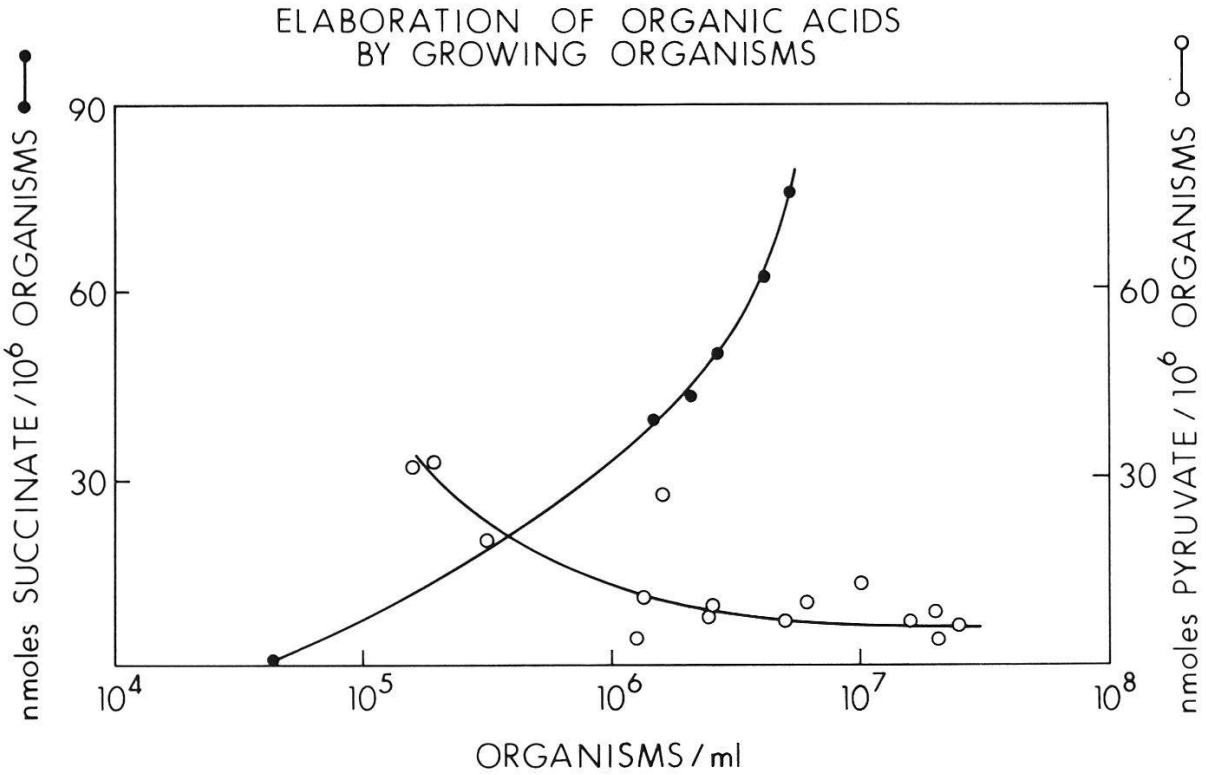

Fig. 2. Relationship of pyruvic and succinic acid excretion to organism growth.

was in agreement with that found in bacterial systems and suggested that the metabolite could be of physiological significance in the regulation of the malic enzyme in the cell. The K_i of oxalacetate was high, 0.7 mM, and its physiological meaning remains unclear. However, Bacchi et al. (1970) have indicated that OAA may be a major metabolic product in fermentation and subsequent investigations of our own (vide infra) have led to a similar conclusion. Since the apparent affinity constants of the malic enzyme and the fumarase were nearly equal (0.5 mM and 0.8 mM, respectively) an inhibition of the malic enzyme would give the fumarase a comparative advantage. The anticipated result of this type of modulation of the malic enzyme would be a reciprocal relationship between the amounts of pyruvic and succinic acids elaborated by this organism, since malic enzyme is at a branch point in the reductive metabolism of oxalacetate. This was demonstrated by measurement of these acids elaborated during growth (Fig. 2). Similar findings have been obtained in several *Leishmania* spp. (Ryley, 1956).

Because of the apparent importance of oxalacetate in the oxidative metabolism of this organism we investigated the source of this dicarboxylic acid. Bacchi et al. (1970) have shown, on kinetic grounds, that the phosphoenolpyruvate carboxykinase (PEPCK) is the most important enzyme in *C. fasciculata* with regard to the introduction of triose phosphate compounds into the tricarboxylic acid (TCA) cycle. This suggested that the pyruvate kinase (PK), which is of significance in many organisms as a point of metabolic regulation, might not

be subject to allosteric control in this protozoan. This could account, in part, for the failure of these organisms to oxidize carbohydrate completely. This study (Marr, 1974) showed that the PK was an allosteric protein located in the cytosol which could be activated by a decrease in pH. This decreased the substrate requirement from an apparent *n* value of 3 to 2. Activation by the nucleotide, ADP, was a unimolecular process. A wide variety of compounds was studied in an attempt to show activation or inhibition of the enzyme. Although it is well known that several amino acids, particularly alanine, inhibit the PK from other sources, no activation or inhibition could be demonstrated. Several hexoses and hexophosphates, dicarboxylic acids, and tricarboxylic acids were used, all without effect. These data suggest that the enzyme, although activated by hydrogen ion, is not subject to either feed-forward or feed-back regulation by heterotropic modifiers. This would support the contention of Bacchi et al. (1970) that the PEPCK acts as the major route for carbohydrate oxidation in this organism and bypasses the PK.

Since the pyruvate kinase was not under the usual type of heterotropic control we investigated the phosphofructokinase (PFK) in *C. fasciculata* to determine if this enzyme, usually a focal point of glycolytic modulation, was suject to feedback regulation (Ozanich and Marr, 1976). The enzyme was an allosteric protein, with respect to the substrate, and responded to AMP as a positive modifier. The addition of AMP decreased the apparent *n* value for fructose-6-phosphate from 3 to 1 (Fig. 3). The enzyme could be desensitized to the activator by freezing and thawing with a simultaneous conversion of the substrate activation curve from sigmoid to hyperbolic. It also was activated by the magnesium-ATP chelate and was inhibited by free ATP whenever the nucleotide was present in concentrations in excess of the divalent cation. This inhibition could be reversed either by the addition of magnesium to convert free ATP to the chelate or by the addition of AMP. Kinetic studies with AMP and ATP indicated that free ATP was a competitive inhibitor of the activator. Except for AMP, metabolic intermediates known to be positive or negative heterotropic modifiers of PFK in other systems were all without effect in this protozoan system. Concentrations of Mg-ATP and free ATP approximating those in the cell provide full activation of the enzyme.

Studies on the PFK of sheep brain (Passonneau and Lowry, 1962, 1963; Lowry and Passonneau, 1966), liver fluke (Mansour, 1962), guinea pig heart (Mansour, 1963), turtle heart (Lobes and Penny, 1974), skeletal muscle (Layzer et al., 1969), *Escherichia coli* (Atkinson and Walton, 1965; Reeves and Sols, 1973), rat and rabbit erythrocytes (Kuhn et al., 1974a, b), platelets (Akkerman et al., 1974), rat liver (Brand and Soling, 1974), and *Neurospora crassa* (Tsao and Madby, 1972), have indicated that the enzyme is the major regulator of the Pasteur effect. In these systems, where glucose plays an important role in energy metabolism and growth, the enzyme reacts with its substrate in a sigmoidal fashion and the second substrate, ATP, reacts in a hyperbolic manner. As ATP

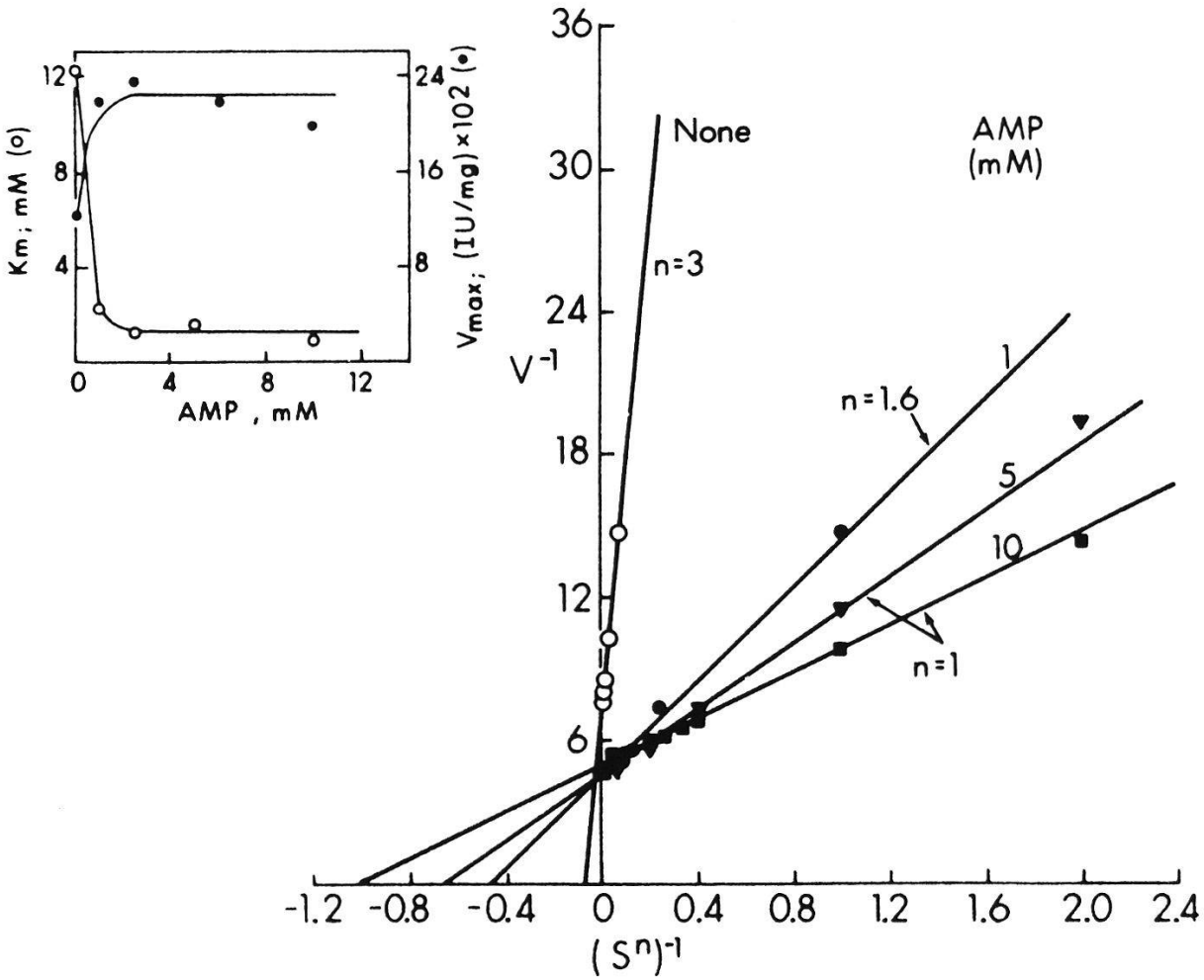

Fig. 3. Activation of the phosphofructokinase by AMP. Reproduced by permission of the International Journal of Biochemistry. The inset illustrates the relationship of the apparent K_m and apparent V_{max} to increasing concentrations of AMP.

concentrations are increased beyond an optimum, there is a marked inhibition of the reaction. This inhibition is reversed by deinhibitors: 5′-AMP, ADP, and F6P (Passonneau and Lowry, 1962, 1963), FDP (Lowry and Passonneau, 1966), 3′, 5′-cyclic AMP (Mansour, 1963), NH_4^+, K^+, P_i (Layzer et al., 1969; et al., 1974b). In addition, PFK is susceptible to inhibition by citrate, PEP, 2-phosphoglycerate, 3-P-glycerate, $CaCl_2$, 2-3 diphosphoglycerate and pyruvic acid (Mansour and Setlow, 1972; Passonneau and Lowry, 1963; Lowry and Passonneau, 1966; Lobes et al., 1974; Kuhn et al., 1974b).

The PFK from organisms in which glucose is not utilized for growth, slime mold (Bauman and Wright, 1968), and *Arthrobacter crystallopoietes* (Ferninondus and Clark, 1969), is not a major regulatory enzyme. In the former, it displays hyperbolic kinetics with respect to F6P, and is not inhibited by high ATP concentrations. However, it is inhibited by ADP, FDP, and P_i and activated by NH_4^+. The PFK from *A. crystallopoietes* is not regulated by any of the positive or negative effectors.

The metabolism of *C. fasciculata* falls in between the examples discussed above. During its early logarithmic growth phase, *C. fasciculata* is dependent on amino acids for its metabolism (J. J. Marr, unpublished data). During the late logarithmic growth phase there is a change to glucose utilization and the organism begins aerobic fermentation. This uncontrolled utilization of glucose and production of acids is consistent with the hypothesis that PFK does not control glycolysis in this organism and with the in vitro data indicating that the known metabolic effectors have no influence on the enzyme. This system provides a model whereby AMP and ATP appear to play unimportant roles in the control of aerobic fermentation despite the demonstration of an in vitro response similar to that seen in systems in which the Pasteur effect occurs. To our knowledge this is the first demonstration of this phenomenon in a protozoan organism (see Ozanich and Marr [1976] for more detailed discussion).

As a result of these investigations on the enzymology of carbohydrate metabolism in protozoans, we postulated that carbohydrate was probably catabolized with very little regulation to succinic acid, pyruvic acid, and carbon dioxide. The pathway of degradation is probably through trioses to PEP and via the PEPCK to oxalacetate and thence to malate and succinate (Fig. 4). Moreover, since these organisms utilize glucose during the later stages of growth, we believe that amino acids may serve as a more significant source of carbon.

To test these hypotheses, we determined the sequence of appearance of metabolic products during aerobic fermentation of carbohydrates in vivo to verify the applicability of data derived in vitro (Marr et al., 1976). Organisms were harvested at various times during growth and methyl derivatives prepared from the acidified samples by modification of the method of Bricknell and Finegold (1973). These samples were assayed using gas chromatography (GLC). Compounds which could not be identified by gas chromatography were analyzed using a gas chromatograph-mass spectrophotometer (GLC-MS) interfaced with a PDP-12 computer. These methods have been described previously by Holmes et al. (1973). Analysis indicated that some lactic acid appears relatively early in the logarithmic phase. Since there is no measurable carbohydrate utilization during this time it may be a product of amino acid metabolism since the organisms have been shown to possess an alanine aminotransferase. Acetic and oxalacetic acids appear at mid-logarithmic phase as glucose is beginning to be utilized; pyruvic acid could not be demonstrated in any significant amounts at this time. From mid-logarithmic phase to stationary phase succinic acid appears in increasing amounts and the pH of the medium begins to decline in parallel with the glucose concentration, lending support to the concept that the source of the oxalacetic and succinic acids is carbohydrate and not amino acids. In late logarithmic and early stationary phase, pyruvic acid appears for the first time and becomes significant late in the stationary phase. These data are consistent with the hypothesis as proposed since oxalacetic acid appears

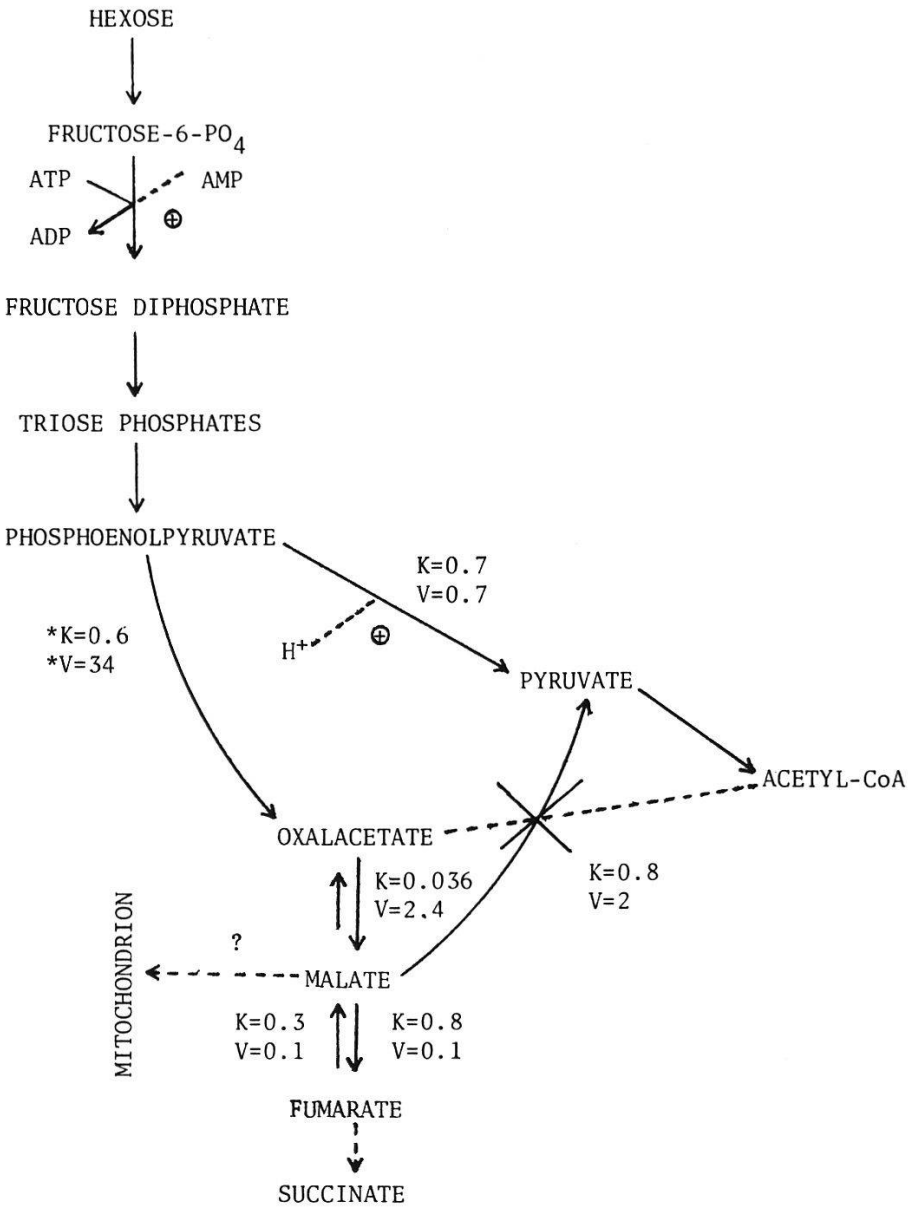

Fig. 4. Proposed scheme for aerobic fermentation in *C. fasciculata.* The symbol K refers to the apparent K_m and the symbol V to the apparent V_{max} for the indicated enzymes. The kinetic constants indicated with an asterisk are taken from Bacchi et al. (ref. 3). Metabolic products which affect the activity of an enzyme are indicated by dotted lines. If the compound activates the enzyme, it is indicated with a ⊕; if the product inhibits the enzyme, it is indicated by an X. The kinetic constants suggests that the principle flow of carbon is from phosphoenolpyruvate to oxalacetate and thence to succinate. This is indicated by the heavier arrow. The proportion of malate oxidized by the mitochondrion is unknown; this is indicated by a dotted line and question mark. Since the fumarate reductase has not yet been demonstrated in this organism, the conversion of fumarate to succinate is indicated by a →.

prior to the appearance of pyruvic and succinic acids (Fig. 4). When cells are grown in the absence of carbohydrate, none of these compounds can be identified by either gas chromatography or mass spectroscopy (Marr et al., 1976).

The proposed scheme of aerobic fermentation is lent some additional support by the inability to find citric, isocitric, and α-ketoglutaric acids in the cell

extracts by either GLC or GLC-MS. These methods should detect concentrations of about 0.1 μM. This does not indicate that the compounds do not exist, especially since the enzymes which utilize them are present; however, it does indicate that these compounds are present at concentrations much lower than those found for oxalacetic and succinic acids and supports the contention that the major pathway for fermentation is via the PEPCK to oxalacetic acid to succinic acid by a reversible TCA cycle.

Enzymology of glycolysis in Leishmania species

General observations

Using the guidelines provided by the above studies in *C. fasciculata*, we examined the utilization of glucose by *Leishmania donovani* and *L. braziliensis* and showed that they are qualitatively and quantitatively similar to data derived from the laboratory protozoan model (Fig. 5). Since the organisms begin to metabolize glucose late in growth with a rather sharp decrease in pH, and, since some of the enzymes of the glycolytic scheme are activated by a decreased pH, the same experiments were done holding the pH constant. As shown in Fig. 6, the pattern of glucose utilization is not altered when the pH is

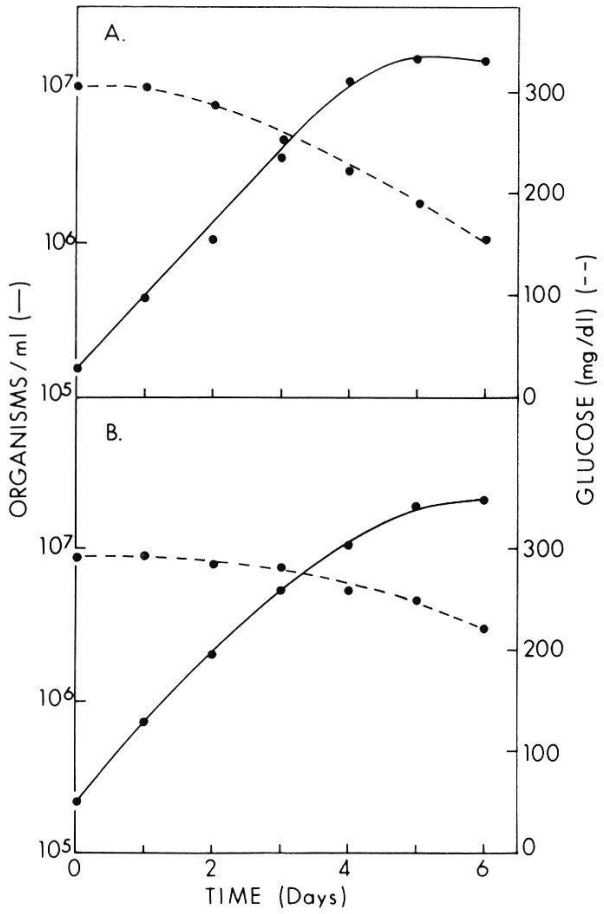

Fig. 5. Utilization of glucose by *L. donovani* and *L. braziliensis*. A. *L. donovani*; B. *L. braziliensis*.

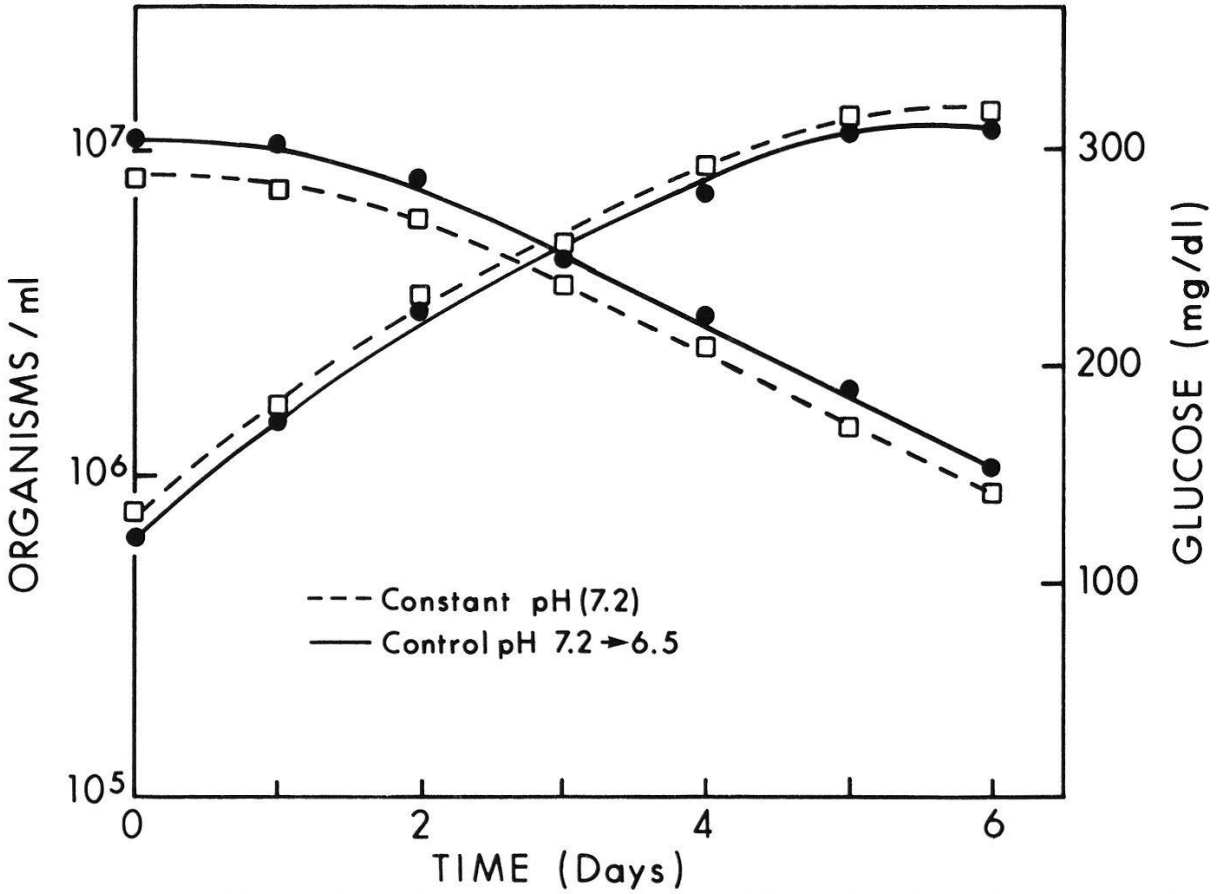

Fig. 6. Glucose utilization by *L. donovani* with constant pH. The dashed line indicates growth and glucose utilization when the pH was maintained at 7.2 Growth of organisms and their utilization of glucose when pH was not controlled are shown with the solid lines. The decrease in pH begins on the second day and declines in parallel with the glucose utilization to a low point of 6.5 on day 6.

held constant. This suggests that the initiation of glucose metabolism is not dependent upon an alteration in pH nor does the decrease in pH associated with this metabolism activate the pyruvate kinase and phosphofructokinase in an autocatalytic manner. It is not possible to exclude the possibility of local concentrations of acid in the cell which could bring about autoactivation. However, there is no evidence to indicate that the cytoplasmic enzymes of glycolysis are spatially arranged within the cytosol. The reason for the induction of glycolysis at the late stage in growth remains unclear although there is a temporal correlation between a decrease in certain amino acids in the medium and the initiation of glycolysis (Berens and Marr, unpublished observations).

Regulation of glycolysis in Leishmania sp.

Our investigations of the phosphofructokinase (PFK) of *L. donovani* and *L. braziliensis* have shown that both enzymes are similar to that of *C. fasciculata.* As shown in Fig. 7, the responses of the enzymes to the substrate, fructose-6-phosphate, are sigmoidal with an apparent *n* value for the substrate of 2 in the PFK from *L. donovani* and an *n* value of 3 for the enzyme from *L. braziliensis.*

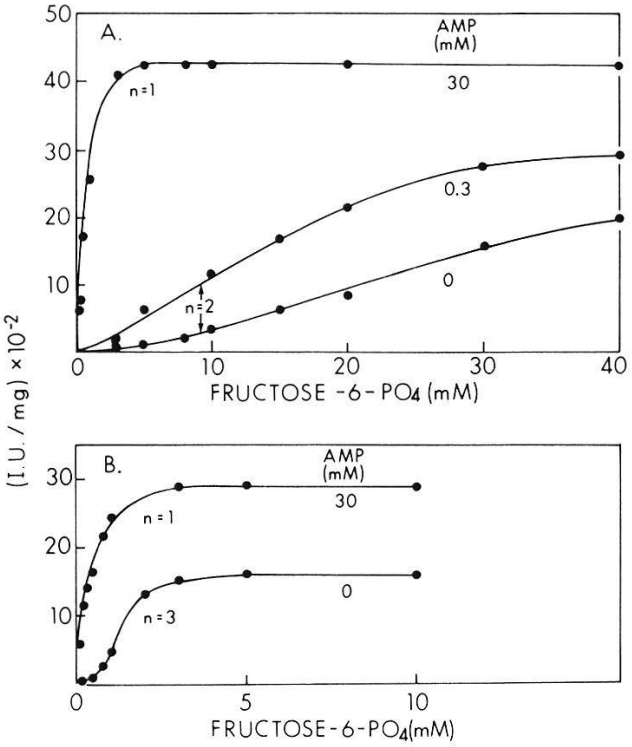

Fig. 7. Activation of the phosphofructokinase from *L. donovani* and *L. braziliensis* by AMP. A. The enzyme obtained from *L. donovani* and B. the enzyme from *L. braziliensis.* In each case, the enzyme is maximally activated by AMP at concentrations of 30 mM; however, the apparent K_a for AMP in each case is much lower and lies in the physiological concentration range for this compound (Berens and Marr, manuscript in preparation).

In each case the addition of saturating concentrations of AMP decreased the apparent *n* value to 1. In each instance there is a corresponding decrease in the apparent K_m for the substrate and an increase in the V_{max}. Both enzymes respond to AMP in a hyperbolic manner although the apparent K_m for the nucleotide is much lower for the enzyme from *L. braziliensis.* The requirements for the nucleotide can be eliminated by decreasing the pH.

As has been shown with the PFK from *C. fasciculata* (Ozanich and Marr, 1976), the enzyme from *L. donovani* responds to increasing ATP concentrations in a first-order manner until the concentration of ATP exceeds that of the metal ion. Increasing the concentration of the nucleotide cause it to behave as an inhibitor, suggesting that the free or non-chelated form of the nucleoside triphosphate acts as an inhibitor of the enzyme. This has already been shown for the enzyme from *C. fasciculata.* The concentrations of the chelate and free ATP for various ratios of total ATP to Mg^{++} were determined and it was clear that free ATP acted as an inhibitor of the enzyme. The details of this work will be published at a later date (Berens and Marr, J. Protozool., in press).

In addition to AMP, several other compounds, known to be positive or negative effectors of the enzyme obtained from other sources, were tested on the PFK of these two *Leishmaniae.* None of these had any effect: K^+, NH_4^+, 3–5′ cyclic AMP, P_i, Ca^{++}, ADP, citrate, PEP, GTP, pyruvate, F-1-6-diP, 2,3-diPG,

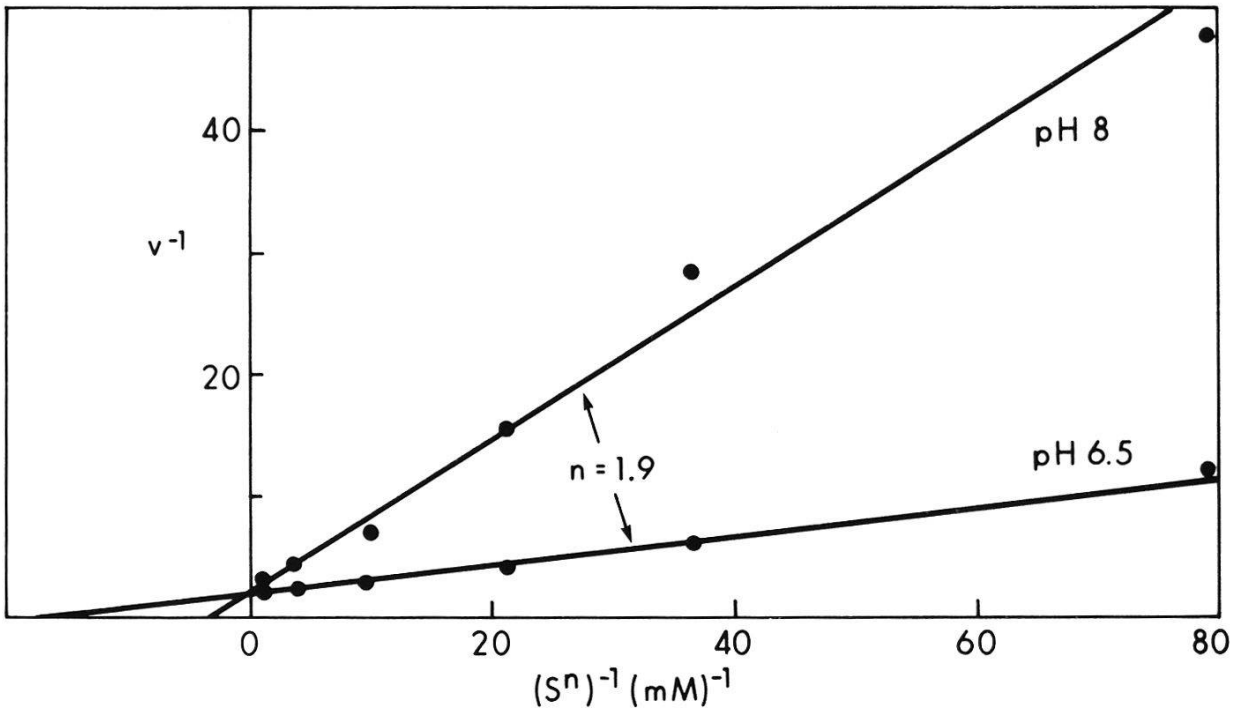

Fig. 8. Activation of the pyruvic kinase from *L. donovani* by hydrogen ion. Reducing the pH decreases the apparent K_m by half while leaving the apparent V_{max} unchanged. The apparent *n* value does not decrease during activation.

2-PG, and 3-PG. In addition, none of twenty amino acids served as effectors of the enzymes.

In addition to the PFK, the pyruvate kinase (PK) of *L. donovani* was investigated. The enzyme from this protozoan appears to behave in a manner similar to that of *C. fasciculata* (Marr, 1974). It is an allosteric protein with respect to its substrate, PEP, it responds in a sigmoidal manner to increasing concentrations of PEP and has an apparent *n* value of 2 for its substrate. Decreasing the pH from 8 to 6.5 (Fig. 8) activates the enzyme by decreasing the apparent K_m from 0.52 mM to 0.23 mM. The apparent V_{max} was unchanged and the apparent *n* value remains at 2. This is somewhat different from the corresponding enzyme studied in *C. fasciculata.* The PK from that organism showed a decrease in apparent *n* value for the substrate at pH 6.3. Nevertheless, both enzymes are activated by an acid pH although the mechanism may differ. The following substances were tested for their ability to activate or inhibit the PK from *L. donovani*: glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1-6 diphosphate, α-glycerophosphate, lactate, malate, succinate, fumarate, galactose, galactose-1-phosphate, mannose, mannose-6-phosphate, trehalose, ATP, and twenty amino acids. All of these were without effect. Thus, as in *C. fasciculata,* this enzyme does not appear to be suited for metabolic regulation. Investigation of the PK from *L. braziliensis* is in progress.

In summary, the enzymatic data derived from the two pathogenic *Leishmaniae* parallel very closely the evidence accumulated during our investigations

of *C. fasciculata,* a nonpathogenic model protozoan. Glucose catabolism is relatively unresponsive to metabolic regulation and the accumulated data lend support to the concept that aerobic fermentation occurs via a reversal of the TCA cycle from oxalacetate to succinate. Similar conclusions were reached by Chatterjee and Datta (1973) using anaerobic cells of *L. donovani,* and Klein et al. (1975) using the trypanosomatids, *T. mega, T. brucei,* and *C. fasciculata.* In addition, the observations that glucose is not utilized until late in the growth cycle and our electrode data indicating that deamination occurs very early in growth and continues through log phase to the point where glucose is catabolized, suggests that amino acids may be the primary substrate for growth in these organisms and that glucose is a secondary substrate.

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