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Tricarboxylic acid and glyoxylate cycles in the *Leishmaniae*

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Summary

Cell-free extracts of four species of *Leishmania* (*L. brasiliensis, L. donovani, L. mexicana* and *L. tropica*) have been shown to contain all enzymes of the tricarboxylic acid cycle. However, the activity of citrate synthase is so low that it is doubtful whether this pathway is of significance in the metabolism of carbohydrate substrates. All four species show the presence of the two enzymes (isocitrate lyase and malate synthase) characteristic of the glyoxylate bypass and glyoxylate is present in cell-free preparations. The isocitrate lyase is relatively insensitive to phosphoenolpyruvate (PEP). The glyoxylate cycle in the *Leishmaniae*, thus, seems to be independent of control by C_3 compounds.

Key words: Leishmania, pyruvate dehydrogenase, tricarboxylic acid cycle, glyoxylate cycle.

A number of enzymes involved in carbohydrate metabolism have been previously demonstrated in various leishmanial species which suggest the operation of the Embden-Meyerhof pathway, the hexose monophosphate shunt and the tricarboxylic acid (TCA) cycle in these parasites (Bell, 1968; von Brand, 1973). However, these conclusions are generalizations pieced together from partial studies on different species. No single species has been thoroughly and systematically studied. In spite of the general validity of the concept of "unity in biochemistry" (Kluyver, 1931), there is the inherent danger that such generalizations could be misleading or even erroneous since examples are known where different species belonging to the same genus show differences in biochemical pathways. Differences between *Leishmania donovani* and *L. enrietti* in nitrogen metabolism especially in their transaminases have been documented (Chatterjee and Ghosh, 1957; Zeledón, 1960d). The two species differ substantially in the α-ketoglutarate-glutamate and the pyruvate-alanine transaminases.

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A comparative survey of enzymes of carbohydrate metabolism in four human species of *Leishmania* (*L. brasiliensis, L. donovani, L. mexicana* and *L. tropica*) which we completed recently, confirmed the occurrence of the central pathways in all four species (Martin et al., 1976). They all have the three irreversible enzymes, hexokinase, phosphofructokinase (PFK) and pyruvate kinase (PK), which are strategic points of allosteric regulation in other systems (Sanwal, 1970). However, Marr and Berens (vide supra) have investigated the PFK of *L. donovani* and *L. brasiliensis* and shown that they are not subject to the usual forms of allosteric control nor is the PK of *L. donovani.* However, we have reported previously that the activity of pyruvate kinase in *L. tropica* is regulated by adenosine monophosphate and fructose-1,6-diphosphate (Mukkada et al., 1974). Thus the fine control of glycolysis may be only variably present among the *Leishmaniae. L. tropica* was also shown to possess a carrier-mediated transport system for glucose (Schaefer et al., 1974; Schaefer and Mukkada, 1976). However, glucose is utilized by the organism only during late log to early stationary phases as is also true of *Trypanosoma brucei* (Evans and Brown, 1972), *Crithidia fasciculata* (Marr, 1974) and *Naegleria gruberi* (Weik and John, 1976). It appears that substrates other than glucose, possibly amino acids and their products are the primary growth substrates for the *Leishmaniae.* Definitive evidence for this has come from studies on *L. tarentolae* which is the only species that can be cultivated in a defined medium. This species grows normally in the complete absence of carbohydrates as long as proline and glutamate are available in the medium (Krassner, 1969; Krassner and Flory, 1971). A number of pathways have been established for amino acid catabolism among microorganisms (Barker, 1961; Greenberg, 1969), resulting directly or indirectly in the formation of acetyl-CoA or intermediates of the tricarboxylic acid cycle (Kornberg, 1965). The tricarboxylic acid cycle may assume major importance in the metabolism of these parasites.

Pyruvate dehydrogenase

All four species of *Leishmania* that we studied showed the presence of the pyruvate dehydrogenase complex which consists of pyruvate dehydrogenase (E. C. 1.2.4.1), dihydrolipoyl transacetylase (E. C. 2.3.1.12) and dihydrolipoyl dehydrogenase (E. C. 1.6.4.3). This multienzyme complex converts pyruvate to acetyl-CoA and provides the means for the entry of trioses into and their subsequent oxidation through the tricarboxylic acid cycle.

Tricarboxylic acid cycle

Several species of *Leishmania* have been shown to utilize substrates such as citrate, α-ketoglutarate and succinate (Bell, 1968; Ghosh and Datta, 1969). Oxygen uptake in *Leishmania* spp. is reported to be stimulated by pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate and oxalacetate (Zeledón, 1960a, c). Enzymes of the tricarboxylic acid cycle reported in *Leishmania* spp.

include NADP-linked isocitrate dehydrogenase (Janovy, 1972), succinate dehydrogenase (Guha et al., 1956; Krassner, 1966). NAD-linked malate dehydrogenase (Krassner, 1968) and fumarase (Chatterjee and Datta, 1974). Voller et al. (1963) demonstrated that in *L. tropica,* label from ^{14}C-glucose was concentrated in succinate and to a lesser extent in fumarate, malate, aspartate, glutamate and alanine. All these led to the overwhelming acceptance of a functional tricarboxylic acid cycle in these organisms. However, positive proof for a complete cycle was lacking since α-ketoglutarate dehydrogenase, a crucial enzyme in the cycle was not demonstrated in any species. The ability to utilize intermediates of the tricarboxylic acid cycle does not necessarily constitute evidence for the occurrence of a complete cycle. Pyruvate entering the cycle as acetyl-CoA could reach α-ketoglutarate and then be converted to glutamate by reductive amination. An active glutamate dehydrogenase (E. C. 1.4.1.3) is present in the four species that we examined (Martin et al., 1976). Similarly, the accumulation of succinate previously reported in *Leishmania* spp. (Zeledón, 1960b) could be accounted for by the carboxylation of pyruvate to oxalacetate by pyruvate carboxylase and subsequent reduction to succinate. Such a pathway exists in a number of invertebrates including protozoa (Hochachka and Mustafa, 1972; Marr, 1973a, b, 1974). Carboxylation of pyruvate is the principal method of heterotrophic CO_2 fixation for the replenishment of C_4 acids in some *Leishmaniae* (Martin et al., 1976). Thus the demonstration of α-ketoglutarate dehydrogenase was of central importance in establishing the existence of a complete tricarboxylic acid cycle. Attempts to assay the enzyme in cell-free extracts by various procedures gave consistently negative results until it was recognized that the leishmanial enzyme has an absolute requirement for thiamine pyrophosphate (TPP). TPP is an activator of the enzyme and optimal results were obtained in presence of 0.3 mM TPP.

Table 1 shows that all enzymes of the tricarboxylic acid cycle are present in the species of *Leishmania* that we examined. Citrate synthase, succinate dehydrogenase and α-ketoglutarate dehydrogenase activities are extremely low. In contrast, isocitrate dehydrogenase and malate dehydrogenase are quite active. The isocitrate dehydrogenase is NADP-linked. No NAD-linked activity was detected as is the case in *Trypanosoma cruzi* (Agosin and Weinbach, 1956) and *Fasciola hepatica* (Prichard and Schofield, 1968). Malate dehydrogenase shows both NAD- and NADP-linked activities and activities are comparable with either coenzyme. Since the specific activities of citrate synthase and α-ketoglutarate dehydrogenase, the two critical enzymes of the TCA cycle, are very low, the functional efficiency of this pathway remains open to question.

As indicated earlier, all species we studied show an active glutamate dehydrogenase. This would imply a competition between α-ketoglutarate dehydrogenase and glutamate dehydrogenase for the common substrate α-ketoglutarate, which might favor the latter enzyme. The control of these two enzymes will largely determine whether α-ketoglutarate is further oxidized through the tri-

Table 1. Enzymes of the tricarboxylic acid cycle (from Martin, Simon, Schaefer and Mukkada, 1976)

Enzyme	Specific activity (nmoles/min/mg protein)				Assay procedure
	L. brasiliensis	*L. donovani*	*L. mexicana*	*L. tropica*	
Citrate synthase (E. C. 4.1.3.7)	0.29	0.05	0.11	0.04	Srere (1969)
Aconitase (E. C. 4.2.1.3)	41.5	38.8	23.3	55.9	Racker (1950)
NAD-linked isocitrate dehydrogenase (E. C. 1.1.1.41)	n.d.*	n.d.	n.d.	n.d.	Siebert (1963)
NADP-linked isocitrate dehydrogenase (E. C. 1.1.1.42)	28.5	115.2	80.1	35.3	Siebert (1963)
α-ketoglutarate dehydrogenase (E. C. 1.2.4.2)	0.8	1.5	1.6	1.5	Reed and Mukherjee (1969)
Succinate dehydrogenase (E. C. 1.3.99.1)	2.8	1.6	0.7	1.2	Bonner (1955)
Fumarase (E. C. 4.2.1.2)	44.4	37.3	44.9	32.3	Racker (1950)
NAD-linked malate dehydrogenase (E. C. 1.1.1.37)	132.0	238.0	61.8	372.3	Ochoa (1955a)
NADP-linked malate dehydrogenase (E. C. 1.1.1.40)	202.6	44.5	130.6	28.4	Ochoa (1955b)

* n.d. = not detected

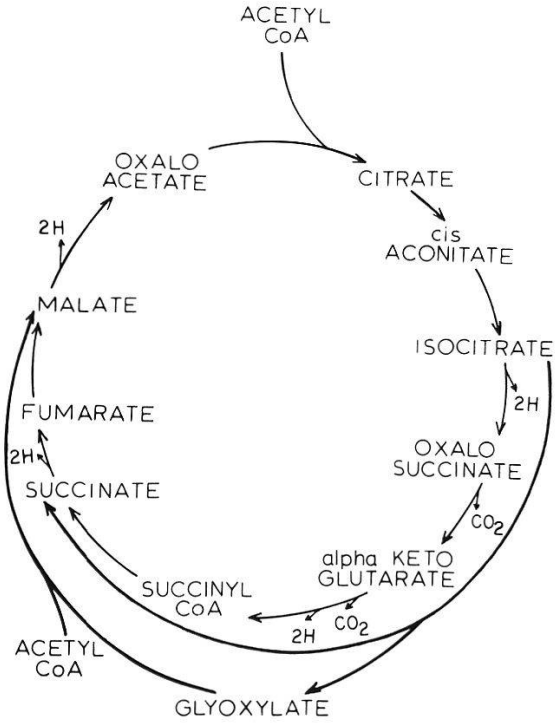

Fig. 1. Tricarboxylic acid and glyoxylate cycles in *Leishmania tropica.*

carboxylic acid cycle or be reductively aminated to glutamate. Glutamate dehydrogenase also may oxidize glutamate to α-ketoglutarate and provide tricarboxylic acid cycle intermediates. This is quite relevant for the *Leishmaniae* grown in vitro in complex media rich in amino acids. The substitution of carbohydrates with glutamate and proline in the medium for *L. tarentolae* without adverse effects tends to support this (Krassner, 1969; Krassner and Flory, 1971).

Glyoxylate bypass

Since the medium in which we grow the organisms is rich in gluconeogenic substrates, we investigated the function of the glyoxylate bypass. The glyoxylate cycle is an anaplerotic pathway which replenishes dicarboxylic acid intermediates especially when 2-carbon substrates such as acetate or other products of fatty acid metabolism are used as principal carbon sources. This cycle deviates from the tricarboxylic acid cycle principally in the aldol cleavage of isocitrate into succinate and glyoxylate by isocitrate lyase. This bypasses the two oxidative decarboxylation steps at isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Fig. 1). Glyoxylate then reacts with acetyl-CoA to form malate, a reaction catalyzed by malate synthase.

As shown in Table 2, cell-free extracts of all the four species of *Leishmania* contain isocitrate lyase and malate synthase, the two enzymes characteristic of the pathway (Simon, Martin and Mukkada, unpublished data). Since isocitrate lyase from a number of organisms has been demonstrated to play a pivotal role in regulating the glyoxylate cycle, this enzyme was chosen for further study. It

Table 2. Enzymes of the glyoxylate bypass (from Simon, Martin and Mukkada, unpublished)

Organism	Specific activity (nmoles/min/mg protein)*	
	Isocitrate lyase	Malate synthase
L. brasiliensis	0.9	2.7
L. donovani	4.4	9.6
L. mexicana	2.5	0.8
L. tropica	1.5	1.8

* Both enzymes were assayed according to the procedure of Dixon and Kornberg (1959).

shows peak levels of activity in cells during late log phase. In many organisms, the specific activity of isocitrate lyase can be enhanced by growing the cells on acetate. Efforts to increase the enzyme in *L. tropica* by using 20 mM acetate in place of glucose in the medium were unsuccessful. However, we have been able to purify the enzyme 68–70 fold from cell-free extracts prepared by sonication of washed cells (unpublished data). A typical purification protocol included ultracentrifugation of a crude extract at 100,000 x *g* for 1 h followed by ammonium sulfate fractionation of the supernate and chromatography of a 40% ammonium sulfate precipitate through a Sephadex G-200 column. Active fractions from the G-200 column were pooled and applied to a DEAE cellulose column and eluted with a linear gradient of 0.1–0.8 M NaCl. The enzyme appeared in a sharp peak (unpublished data) and had a pH optimum around 6.8 and showed typical hyperbolic kinetics with respect to substrate. The partially purified enzyme has an apparent K_m of 0.34 mM and a V_{max} of 0.93 nmoles/mg/min for isocitrate.

Isocitrate lyase from *Escherichia coli* and a number of other organisms is subject to regulation by C_3 compounds especially phosphoenolpyruvate (Ashworth and Kornberg, 1963; Kornberg, 1966) which is an allosteric inhibitor and a repressor of the enzyme. Thus phosphoenolpyruvate controls the synthesis of the enzyme ("coarse" control) and its activity ("fine" control). The leishmanial enzyme, however, is relatively insensitive to phosphoenolpyruvate and does not appear to be under control by C_3 compounds (unpublished data). It is uncertain whether this is related to our observation that peak levels of isocitrate lyase are observed in cells in late log phase, which corresponds to the period of maximal glucose utilization and presumably also the formation of C_3 precursors (Mukkada et al., 1974).

The *Leishmaniae* seem to be capable of oxidizing glucose to acetyl-CoA but it is not certain that it is oxidized beyond this point although all enzymes of the tricarboxylic acid cycle are present. The activity of citrate synthase is extremely low (almost negligible) which would restrict the formation of citrate from acetyl-CoA and oxalacetate. It is thus unlikely that these organisms can grow on

glucose as the sole source of carbon and energy. However, this block in citrate formation is offset by their ability to utilize glutamate and its precursor, proline. It appears that not only do these organisms utilize glucose very late in the growth cycle (Mukkada et al., 1974) but that when they do, they utilize the reactions of the Embden-Meyerhof pathway principally in a biosynthetic capacity. This is comparable to bacterial mutants deficient in citrate synthase (Kornberg, 1963). The relatively low levels of activity of α-ketoglutarate dehydrogenase as compared to the very active glutamate dehydrogenase and the equilibrium position of the latter enzyme which generally favors glutamate formation (Sanwal, 1970) would indicate another rate-limiting step in the tricarboxylic acid cycle at the level of α-ketoglutarate dehydrogenase in the *Leishmaniae.*

The functional significance of the glyoxylate cycle in *Leishmania* spp. is not clearly understood. This pathway is widespread among prokaryotes and fungi, as well as fatty seeds during germination, and in some algae but is rare in animal cells. It is present, however, in *Tetrahymena* (Hogg and Kornberg, 1963) especially during glyconeogenesis. While the presence of isocitrate lyase and malate synthase does not necessarily indicate a functional glyoxylate cycle in the *Leishmaniae,* its significance cannot be ignored since these organisms grow principally on glyconeogenic substrates. Deproteinized samples of cell-free extracts of *L. tropica* were found to contain glyoxylate (6–7 nmoles/mg protein) as determined by the method of McFadden and Howes (1960). The glyoxylate content of the cell-free extracts was enhanced by incubating such extracts with isocitrate under appropriate conditions for the isocitrate lyase reaction. Thus, it is clear that glyoxylate is formed in vivo and it appears that the glyoxylate cycle contributes, at least in part to its formation.

It is possible that in *Leishmania* as in *Tetrahymena,* the glyoxylate cycle plays a role in glyconeogenesis when there is a mobilization of fats and their conversion to glycogen. The tricarboxylic acid and glyoxylate cycles could operate independently since the glyoxylate enzymes could be spatially separated from the tricarboxylic acid cycle enzymes although this has not been shown.

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