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Comparative physiological studies on four species of hemoflagellates in culture. III. Effect of the Krebs' cycle intermediates on the respiration*

by

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The functioning of a Krebs' cycle in trypanosomes was investigated with negative results in *Trypanosoma evansi* by MARSHALL (14) and in *T. hippicum* by HARVEY (9). MOULDER (16) investigating the problem in the bloodstream form of *T. lewisi* concluded that, since addition of some of the intermediate compounds did not enhance the respiration rate of the flagellates, the tricarboxylic acid cycle is not active in these organisms. BAERNSTEIN (4) studied the malic dehydrogenase and found a fumarase and a fumaric hydrogenase in homogenates of the culture form of *Schizotrypanum cruzi*. SEAMAN (19) demonstrated succinic dehydrogenase activity in homogenates of *S. cruzi* and AGOSIN and VON BRAND (1) have studied this enzyme, which is linked to the mitochondrial fraction, in the same organism. The plant parasite, *Strigomonas oncopelti* (=*Phytomonas sp.* ?) seems to have several of the dehydrogenases of the cycle (18). VON BRAND and AGOSIN (5), in manometric experiments, demonstrated the oxidation of some of the intermediates of the cycle in the culture forms of *Leishmania tropica* and *S. cruzi* using whole organisms. Furthermore, these authors reported that the strong inhibition produced by malonate is reversed by succinate. Similar work was done by MEDINA *et al.* (15)

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using the culture form of *L. brasiliensis*. Finally AGOSIN and WEINBACH (2) have characterized a TPN-linked isocitric dehydrogenase in *S. cruzi*. The present report deals with experiments on the oxidation of Krebs' cycle intermediates by the culture forms of four species of hemoflagellates. Appreciation is expressed to Dr. Clark P. Read for his interest in this work and kind advice and criticism.

MATERIALS AND METHODS

ORGANISMS. *Endotrypanum schaudinni, Leishmania enriettii, Schizotrypanum cruzi* and *S. vespertilionis*. Details of the origin of the strains and of the culture medium used, have been given in a previous paper (22).

MANOMETRIC EXPERIMENTS. The organisms were harvested in the exponential phase of growth, washed by three repeated centrifugations in Krebs-Ringer phosphate (KRP), pH 7.2, and resuspended in an adequate volume of the same solution before use in manometric experiments or, in one series of experiments, in KRP at pH 4.5 (final pH 5.0). Oxygen consumption was determined by conventional Warburg respirometry using duplicate flasks for each substrate which, after addition, attained a final concentration of 0.01 M. Control vessels containing glucose were included in each experiment and endogenous respiration was recorded in two control flasks. The experiments were conducted under atmospheric air, at a temperature of 30° C, and oxygen uptake was followed for 2 hours. Details of the procedures have been already described (22). The intermediates, including pyruvate and acetate, were used as the sodium salt or the acid form neutralized with NaOH. Isocitric acid was prepared from the lactone form by hydrolysis in dilute NaOH according to the method of KREBS and EGGLESTON (12).

NITROGEN DETERMINATIONS. These were carried out either by the technique of KABAT and MAYER (11) or by the nesslerization procedure described by LANG (13). In both cases, the same standard solution of ammonium sulfate was used.

RESULTS

The results of the effect of acetate, pyruvate and the Krebs' cycle intermediates at two different pH's are presented in Tables 1 and 2. In none of the cases were the stimulations so clear cut at pH 7.2 as at the lower pH. At the higher pH, the stimulations varied, in general, between 10 and 40 per cent above the endogenous respiration and, in a few cases, some of the tricarboxylic acids depressed the respiration to values below those of endogenous controls. In one case (*L. enriettii*) acetate inhibited endogenous respiration and the phenomenon was true regardless of the pH. At pH 5.0 pronounced stimulation of respiration was observed upon addition of Krebs' cycle intermediates; in *E. schaudinni*, alpha-ketoglutarate, malate, and pyruvate yielded essentially the same respiratory rate as glucose. Succinate in the same species gave a stimulation

TABLE 1

Effect of Krebs' cycle intermediates on respiration at pH 7.2. Each value represents the mean of one duplicate determination, unless otherwise stated. Glucose and endogenous values are given for comparison

SPECIES		Endogenous	Glucose	Citrate	cis-Aconitate	iso-Citrate	alpha-Ketoglutarate	Succinate	Fumarate	Malate	Oxaloacetate	Pyruvate	Acetate
E. schaudinni	S/E	**1.00**	13.80*	0.93	0.68	0.88	1.80	1.89	1.15	1.33	1.17	3.63	1.34
	S/G	**0.09***	1.00	0.07	0.13	0.07	0.12	0.35	0.21	0.09	0.22	0.28	0.19
L. enriettii	S/E	**1.00**	7.18*	1.09	1.29	0.66	1.22	1.57	1.14	1.22	1.29	1.00	0.62
	S/G	**0.14***	1.00	0.21	0.15	0.07	0.19	0.19	0.14	0.19	0.15	0.19	0.08
S. vespertilionis	S/E	**1.00**	3.04	1.15	1.00	1.00	1.27	1.12	1.00	1.44	1.02	1.00	0.99
	S/G	**0.36**	1.00	0.36	0.49	0.49	0.42	0.53	0.48	0.48	0.49	0.31	0.39
S. cruzi	S/E	**1.00**	2.78*	1.03	0.93	1.04	1.04	1.25	1.00	1.15	1.02	0.94	0.97
	S/G	**0.39***	1.00	0.46	0.43	0.51	0.48	0.58	0.46	0.53	0.47	0.42	0.45

S/E=ratio $\frac{N}{Q0_2}$ substrate/ $\frac{N}{Q0_2}$ endogenous; S/G=ratio $\frac{N}{Q0_2}$ substrate/ $\frac{N}{Q0_2}$ glucose $\frac{N}{Q0_2}$ = μl of 0_2/mg. N/hr.

* Mean of 15 duplicate determinations.

TABLE 2

Effect of Krebs' cycle intermediates on respiration at pH 5.0. Each value represents the mean of one duplicate determination, unless otherwise stated. Glucose and endogenous values are given for comparison .

SPECIES		Endogenous	Glucose	Citrate	cis-Aconitate	iso-Citrate	alpha-Ketoglutarate	Succinate	Fumarate	Malate	Oxaloacetate	Pyruvate	Acetate
E. schaudinni	S/E	1.00	9.65*	1.12	0.95	0.68	12.19	14.50	8.95	12.04	2.14	8.08	0.58
	S/G	0.10*	1.00	0.25	0.11	0.06	0.96	1.35	0.83	0.95	0.19	0.95	0.07
L. enrietii	S/E	1.00	4.93*	0.90	1.25	1.07	1.91	1.71	1.36	1.55	1.13	1.25	0.59
	S/G	0.20*	1.00	0.22	0.24	0.19	0.36	0.31	0.25	0.39	0.21	0.24	0.14
S. vespertilionis	S/E	1.00	2.70*	1.20	1.03	1.09	1.77	1.60	1.13	1.83	1.10	1.22	0.69
	S/G	0.37*	1.00	0.51	0.44	0.47	0.58	0.70	0.49	0.60	0.48	0.52	0.30
S. cruzi	S/E	1.00	2.56*	1.12	0.96	1.12	2.04	1.60	1.16	1.66	1.06	1.43	0.64
	S/G	0.39*	1.00	0.42	0.35	0.42	0.76	0.58	0.42	0.60	0.38	0.53	0.24

S/E and S/G ratios as in Table 1.
* Mean of 2 duplicate determinations.

35 per cent higher than that of the glucose control at the same low pH. In *L. enriettii* the values for respiratory stimulation are only slightly higher at the low pH, and for the two *Schizotrypanum* they were significantly higher in some instances. *L. enriettii, S. vespertilionis* and *S. cruzi* utilized pyruvate only at pH 5.0 at a very low rate. At the same low pH, acetate seemed to be toxic for all four species. The increase in respiration produced by the compounds at the low pH was quite stable. Examples are illustrated in figures 1, 2 and 3.

DISCUSSION

The present observations on the utilization of Krebs' cycle intermediates by four species of hemoflagellates indicate that the external pH is an important factor in the detection of their oxidation. The influence of low pH can be understood in terms of the ionization of the intermediates. It is known that the unionized molecules of weak electrolytes penetrate cells by diffusion more readily than the ions (10). This account for the differences observed at the two different pH's. It may also be postulated that the lower pH will modify the permeability properties of the cell membrane making the substrate available to the enzymes. Furthermore, the data suggest that the tricarboxylic acids penetrate the cell with great difficulty; this is in agreement with the results obtained by VON BRAND and AGOSIN (5) in the case of *S. cruzi* and *L. tropica.* The latter authors observed somewhat more utilization of the tricarboxylic acids by *S. cruzi* in isotonic KCl at pH 5.6.

The vigorous utilization of pyruvate by *E. schaudinni,* with evidence of a very active Krebs' cycle in this species, suggests that glucose might be oxidized completely, or nearly so, by this parasite. The low rate of oxidation of pyruvate by *L. enriettii* and the two *Schizotrypanum* is consistent with the observation that the compound is only partially oxidized by the flagellates. Changes in utilization rate of the compound in cultures, accompanied by physiological and morphological changes in the case of *S. cruzi* (7), await further investigation. A diphasic growth curve has been reported in cultures of the latter species (6, 8). We agree with VON BRAND and AGOSIN (5) that a fundamental change in metabolic pattern of the organisms is not necessarily responsible for the second peak shown by *S. cruzi.* As the pH drops as a consequence of carbohydrate fermentation, the organisms are able to metabolize pyruvic and succinic acids (two important products first accumulated in the medium), but it is not known whether the organisms of the second growth curve utilize these products at a higher rate than those belonging to the first growth curve.

The data presented here suggest that the complete tricarboxylic acid cycle is present in the four organisms studied, although it might have a somewhat different physiological function in each particular case. The possibility that only a part of the cycle may operate is apparent. BAERNSTEIN (3) could not demonstrate aconitase in the culture form of *S. cruzi* and has presented (4), for the same organism, the scheme of a partial cycle with a very active malic dehydrogenase coupled to a fumarase and a fumaric hydrogenase, giving rise

to succinate. The system, at the same time, would be connected in some way to the cytochromes, presumably through flavoproteins. On the other hand, the recent demonstration of isocitric dehydrogenase in *S. cruzi* by AGOSIN and WEINBACH (2) and the sensitivity of the flagellate to fluoroacetate (21), seem to support the occurrence of the complete cycle.

It may be that in *Schizotrypanum* and *Leishmania* the tricarboxylic acid cycle plays a minor role in energy production but supplies the precursors of a series of amino acids, as seems to be the case in *Escherichia coli* (17) and in *Mycobacterium butyricum* (20). We believe that, at least for *Schizotrypanum,* transminating enzymes may play a very important role in connection with this phase of energy metabolism. As far as *E. schaudinni* is concerned, the Krebs' cycle is probably quite important as a pathway for pyruvate oxidation and consequent energy production; the inhibition by fluorecetate with a corresponding decrease in motility seems to support this idea (21). Likewise, the high rate of succinate oxidation by this organism suggests the importance of the succinoxidase system.

SUMMARY

The effect of the Krebs' cycle intermediates upon respiration of the culture forms of *Endotrypanum schaudinni, Leishmania enriettii, Schizotrypanum vespertilionis* and *S. cruzi* was examined at two different hydrogen ion concentrations. At pH 7.2, low stimulations with some of the intermediates were observed. At pH 5.0 the stimulations in general were more evident, and alpha-ketoglutarate, malate, and pyruvate were oxidized by *E. schaudinni* at the same rate as glucose while succinate produced a respiratory stimulation 35 per cent higher than that of glucose. At the same low pH, the other three species were able to utilize some pyruvate, whereas acetate inhibited all of them. The increase in respiration produced by the tricarboxylic acids was almost negligible, regardless of the pH, and in some instances the substances rather had a depressive action on the respiration. It is believed that the tricarboxylic acid cycle is present in the four species and the possibility of its importance in roles other than energy production, in *Leishmania* and *Schizotrypanum,* is discussed.

RESUMEN

Se estudia el efecto de las sustancias intermediarias del ciclo de Krebs en la respiración de las formas de cultivo de *Endotrypanum schaudinni, Leishmania enriettii, Schizotrypanum vespertilionis* y *S. cruzi,* a dos diferentes concentraciones de iones de hidrógeno. A pH 7.2 se observaron estímulos pequeños; a pH 5.0 los estímulos respiratorios fueron más evidentes y en el caso de *E. schaudinni,* alfa-cetoglutarato, malato y piruvato fueron oxidados con la misma intensidad que glucosa y el succinato produjo un estímulo respiratorio 35 por ciento más elevado que el producido por el carbohidrato. Al mismo pH

ácido, las otras tres especies fueron capaces de utilizar piruvato, mientras que el acetato fue inhibitorio para todas. Los ácidos tricarboxílicos produjeron un aumento insignificante a cualquiera de los dos pH y en algunos casos hubo un efecto depresivo sobre la respiración. Se concluye que el ciclo de Krebs existe en las cuatro especies y se discute la posibilidad de que el mismo tenga importancia en otros aspectos metabólicos, que no sean la producción de energía, en el caso de *Leishmania* y *Schizotrypanum*.

REFERENCES

1. AGOSÍN, M., & T. VON BRAND
   1955. Characterization and intracellular distribution of the succinic dehydrogenase of *Trypanosoma cruzi*. *Exp. Parasitol.*, 4: 548-563.

2. AGOSÍN, M., & E. C. WEINBACH
   1956. Partial purification and characterization of the isocitric dehydrogenase from *Trypanosoma cruzi*. *Biochim. et Biophys. Acta*, 21: 117-126.

3. BAERNSTEIN, H. D.
   1953. The enzyme systems of the culture form of *Trypanosoma cruzi*. *Ann. N. Y. Acad. Sci.*, 56: 982-994.

4. BAERNSTEIN, H. D.
   1953. Malic dehydrogenase and related enzymes in the culture form of *Trypanosoma cruzi*. *Exp. Parasitol.*, 2: 380-396.

5. BRAND, T. VON & M. AGOSÍN
   1955. The utilization of Krebs cycle intermediates by the culture forms of *Trypanosoma cruzi* and *Leishmania tropica*. *J. Infect. Dis.*, 97: 274-279.

6. CHANG, S. L.
   1947. Studies on hemoflagellates. I. A semisolid medium and a fluid medium with a solid base for growing various species of *Leishmania* and *Trypanosoma cruzi*. *J. Infect. Dis.*, 80: 164-171.

7. CHANG, S. L.
   1948. Studies on hemoflagellates IV. Observations concerning some biochemical activities in culture, and respiration of three species of *Leishmania* and *Trypanosoma cruzi*. *J. Infect. Dis.*, 82: 109-118.

8. CHANG, S. L. & W. O. NEGHERBON
   1947. Studies on hemoflagellates II. A study of the growth rates of *Leishmania donovani*, *L. brasiliensis*, *L. tropica* and *Trypanosoma cruzi* in culture. *J. Infect. Dis.*, 80: 172-184.

9. HARVEY, S. C.
   1949. The carbohydrate metabolism of *Trypanosoma hippicum*. *J. Biol. Chem.*, 179: 435-453.

10. JAMES, W. O.
    1953. The use of respiratory inhibitors. *Ann. Rev. Plant Physiol.*, 4: 59-90.

11. KABAT, E. A., & M. M. MAYER
1948. *Experimental Immunochemistry*, 567 pp. Charles C. Thomas, Illinois.

12. KREBS, H. A., & L. V. EGGLESTON
1944. Micro-determination of iso-citric and cis-aconitic acids in biological material. *Biochem. J.*, 38: 426-437.

13. LANG, C. A.
1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. *Anal. Chem.*, 30: 1692-1694.

14. MARSHALL, P. B.
1948. The glucose metabolism of *Trypanosoma evansi* and the action of trypanocides. *Brit. J. Pharmacol.*, 3: 8-14.

15. MEDINA, H., D. AMARAL, & M. BACILA
1955. Estudos sobre o metabolismo de protozoarios do gênero *Leishmania*. I. Vias de oxidação da glicose e do acetato pela *Leishmania brasiliensis* Vianna, 1911. *Arq. Biol. Tecnol.*, 10: 97-102.

16. MOULDER, J. W.
1948. The oxidative metabolism of *Trypanosoma lewisi* in a phosphate saline medium. *J. Infect. Dis.*, 83: 33-41.

17. ROBERTS, R. B., D. B. COWIE, R. BRITTEN, E. BOLTON, & P. H. ABELSON
1953. The role of the tricarboxylic acid cycle in amino acid synthesis in *Escherichia coli*. *Proc. Natl. Acad. Sci.*, 39: 1013-1019.

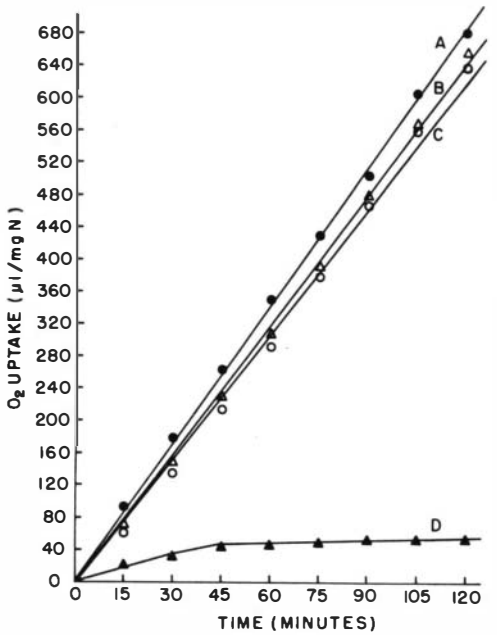
18. RYLEY, J. F.
1955. Studies on the metabolism of the Protozoa. 4. Metabolism of the parasitic flagellate *Strigomonas oncopelti*. *Biochem. J.*, 59: 353-361.

19. SEAMAN, G. R.
1953. The succinic dehydrogenase of *Trypanosoma cruzi*. *Exp. Parasitol.*, 2: 236-241.

20. WRIGHT, D. E.
1959. The formation of keto and amino acids by *Mycobacterium butyricum* growing in a chemically defined medium. *J. Gen. Microbiol.*, 20: 554-565.

21. ZELEDÓN, R.
1959. Unpublished data.

22. ZELEDÓN, R.
1960. Comparative physiological studies on four species of hemoflagellates in culture. I. Endogenous respiration and respiration in the presence of glucose. *J. Protozool.* 7: 146-150.



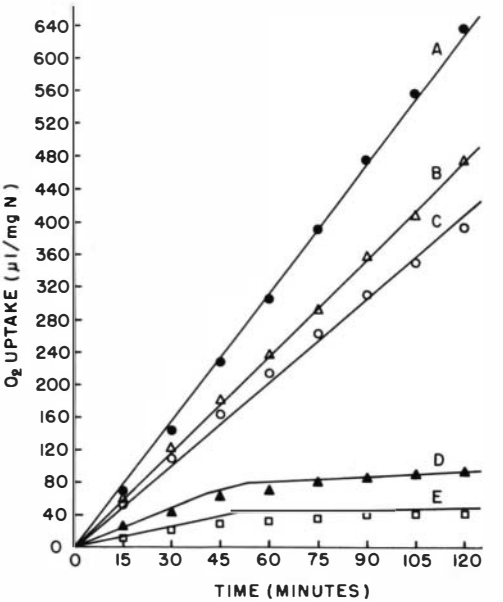


Fig. 1: Effect of Krebs' cycle intermediates on respiration of *E. schaudinni* at pH 5.0. Glucose and endogenous patterns are given for comparison. A=glucose, B= alpha-ketogluturate, C=malate, D= endogenous.

Fig. 2: Effect of Krebs' cycle intermediates on respiration of *E. schaudinni* at pH 5.0. Glucose and endogenous patterns are given for comparison. A=succinate, B= glucose, C = fumarate, D=oxaloacetate, E=endogenous.

Fig. 3: Effect of pyruvate on the respiration of *E. schaudinni* at pH 5.0. Glucose and endogenous patterns are given for comparison. A=glucose, B=pyruvate, C= endogenous.