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Histochemical Studies of Succinic Dehydrogenase in *Trypanosoma cruzi* and *Leishmania leproide*. (27567)

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Biochemical evidence accumulated in recent years shows that the Krebs cycle intermediates are utilized by the culture forms of *Trypanosoma cruzi*(1,2); moreover the presence of some of the enzymes involved, *viz.*, succinic dehydrogenase(3,4) and isocitric dehydrogenase(5), has been demonstrated by the manometric technic. Studies on the metabolism of leishmania are fragmentary; nevertheless, utilization of substrates of oxidative metabolism by the leptomonad form (in *L. tropica, donovani, brasiliensis*) has been reported(1,2,6).

Though the histochemical technic does not permit an accurate measure of enzyme activity, it offers the advantage of localizing the enzyme site in the cell and the possibility of applying it for *in vivo* and drug action studies. Therefore, we made an effort to develop a method which would permit the demonstration of succinic dehydrogenase in *T. cruzi* and leishmania. By this method we have demonstrated histochemically the presence of succinic dehydrogenase in *T. cruzi* obtained from cultures and infected mice, as well as in the leptomonad form of a variety of *L. brasiliensis*. We have also studied the effect of a drug (Spirotrypan) on the enzyme in *T. cruzi*.

Material and method. Two- to six-week-old cultures of a local strain of *T. cruzi*, grown at 26°C in the liquid medium described by Warren(7), were used. For *in vivo* experiments *T. cruzi* was collected, with a slight modification of the method described by Anderson *et al.*, for *T. equiperdum*(8), from the blood of mice at the peak of infection. For investigation of leishmania we utilized a strain employed previously for chemotherapeutic studies(9), a variety of *L. brasiliensis*, called by Pifano "*L. leproide*"(10). One- to 2-week-old cultures in Davis medium were used.

As incubation medium for staining, one of the more recent tetrazolium salts, 2,2'-di-p-nitrophenyl - 5,5' - diphenyl - 3,3'(3,3' - dimethoxy - 4,4' - biphenylene) ditetrazolium chloride (Nitro blue tetrazolium or "nitro-BT"), shown by Tsou *et al.*(11) to be a very effective histochemical reagent for succinic dehydrogenase, was selected. However, when this reagent was used as previously described (12-14) no reaction was obtained. Yet, we succeeded in staining the parasite by introducing an electron carrier in the staining medium, with a modification of the technic of Farber and Louviere for succinic dehydrogenase in mammalian tissue(15). The staining medium which finally led to the best results consisted of:

	ml
Sodium succinate, 0.2 M	.75
Nitro-BT, 3 mg/ml	.5
Brilliant cresyl blue, 0.5 mg/ml	.1
Phosphate buffer, 0.2 M, pH 7.4	.5
Sodium bicarbonate, 0.6 M	.15
Calcium chloride, 0.004 M	.3
Water	.7
	3.00

Demonstration of the enzyme. About 2 ml of *T. cruzi* culture was usually washed 2-3 times by centrifuging for 5 minutes with sub-

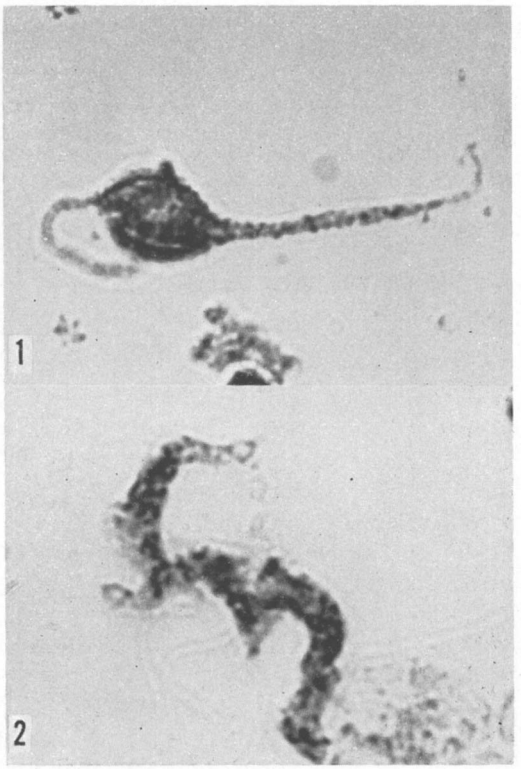

FIG. 1. Standard tetrazolium staining in *T. cruzi* culture (pH 7.6—40 min. incubation) with brilliant cresyl blue. (Lighter granules in center are the reddish ones.)

FIG. 2. *T. cruzi* from infected mice (same standard staining).

strate-free Krebs-Ringer solution(16) at pH 7.3-7.4. Prolonged washing could interfere with the staining. The deposit of the final washing, *ca.* 0.1-0.2 ml, was transferred to the incubation medium under nitrogen at 37°C. After 40 minutes incubation the reaction was terminated by adding 0.3 ml 40% formaldehyde to each tube and the parasites were collected by centrifugation. They were washed with 10 ml normal saline to which 1 ml 1% gelatin was added, centrifuged, spread over a slide, dried in incubator at 37°C and mounted in glycerine jelly. Photomicrograms were taken under oil immersion lens within 2-18 hours.

The same procedure could be used for demonstration of the enzyme in *T. cruzi* of infected mice, with the following details. A stock solution of 10 mg/ml heparin was diluted 1:10 with horse serum and 0.15 ml of this solution was added to the blood collected from each mouse. For the separation of parasites the narrow tubes used for hemoglobin assay were found convenient. Blood was centrifuged for 8 minutes at 2500 rpm, the parasites at the interface collected with a little plasma and added to the incubation medium.

L. leproide organisms contained in the condensation water of the Davis medium were prepared in the same way as *T. cruzi* cultures.

Results. T. cruzi culture. The presence of succinic dehydrogenase is indicated by the deep blue granules, with defined margin, throughout the cytoplasm and extending into the flagella (Fig. 1). There are faint reddish granules alongside the blue ones, which may be seen under certain conditions also in unstained specimens.

Since the enzymatic reaction is pH dependent and non-enzymatic reduction of the dye may occur at high pH(17), we examined the influence of pH on the reaction. Six solutions were prepared with phosphate buffer of pH varying from 5.9 to 8.2, giving a final pH for the incubation medium of 6.7-8.5. Practically no stained deposit was seen at pH 6.7. Some reaction occurred at pH 7 and this increased through 7.25 up to 7.6, the pH routinely employed. Further increase in pH caused no further increase in staining, indicating the enzymatic nature of the reaction. This is supported by the early appearance of the color of reduced tetrazolium. A definite reaction was observed in 10 minutes and the optimal time seems to be between 20-40 minutes, further increase in time of incubation being of no value.

It is well known that in mammalian tissue succinic dehydrogenase can be demonstrated by incubating with tetrazolium salt alone, although in case of low enzyme activity the reaction is enhanced by electron carriers such as methylene blue. On the other hand in case of *T. cruzi* no reaction was obtained with tetrazolium salt alone, and the presence of methylene blue, or better brilliant cresyl blue, was essential. A concentration of 17 μg/ml of brilliant cresyl blue gave the best results, although lower concentrations were also effective.

TABLE I

Exp.	Spirotrypan, mg/kg		Hr after treatment	Parasitemia	Mobility	Tetrazolium staining
1	100–150	i.v.	½–1	Unchanged (++)	Unchanged	Good
2	250	s.c.	2–3	" (++)	"	"
3	150	"	20	Markedly reduced	Markedly reduced	Staining in only 10% of parasites
	100	"	"	Slightly changed (+)	Slightly reduced	Weaker
4	150	"	"	" " (+)	Unchanged	"
	150	"	"	Markedly reduced	Markedly reduced	Staining in only 15% of parasites
	100	"	"	Unchanged (++)	Unchanged	Good

Succinic dehydrogenase requires a number of cofactors(18); in our work on *T. cruzi* we found it necessary to add calcium and bicarbonate for the tetrazolium reaction.

Unlike preparations of mammalian tissue which require addition of succinate for histochemical demonstration of the enzyme, in *T. cruzi* tetrazolium reaction could be obtained in the absence of added substrate, the endogenous substrate being sufficient. This raised a doubt about the specificity of the dehydrogenase reaction. But the participation of succinic dehydrogenase is demonstrated by the finding that parasites, washed several times in substrate-free Krebs-Ringer solution show very weak staining. Furthermore, in parasites washed with 0.05 M malonate and incubated in the medium with 0.1 M malonate the tetrazolium reaction was blocked.

T. cruzi in infected mice. In trypanosomes isolated from peripheral blood the tetrazolium staining was very similar to that described for the culture form (Fig. 2). However, the stained granules were not visible in the flagella and the reddish granules were less conspicuous.

To show the specificity of the reaction for succinic dehydrogenase, we incubated a suspension of *T. cruzi* in plasma with 0.1 M and 0.2 M malonate for 40 minutes prior to incubation in succinate-free medium containing malonate in the respective concentrations. Malonate caused a marked decrease in the staining and a large number of parasites were unstained.

Effect of spirotrypan on the enzyme. Experiments were performed both *in vitro* and *in vivo* to see whether 2-di-(β,γ-dihydroxypropyl)- aminophenol-<4-arseno-5>-β-(benzoxazolyl-(2)-mercapto)- propionic acid, sodium salt (spirotrypan), a drug which clears *T. cruzi* infection in mice, has any effect on succinic dehydrogenase. The trypanosomes, pooled with the plasma from 4-6 infected mice, were incubated with different concentrations of the drug for 40 minutes, centrifuged and placed in the tetrazolium solution. Minimal spirotrypan concentrations of 0.3-0.5 mg/ml were found to reduce the tetrazolium reaction: the staining was less granular, weak or absent, though a few well-stained parasites were also present. With increasing concentration of spirotrypan the effect was more pronounced. The minimum concentration of spirotrypan which inhibited the tetrazolium reaction also affected the mobility of the parasites.

The results of the experiments performed *in vivo* seem to indicate that the tetrazolium reaction, considering both intensity of staining and number of unstained parasites, is reduced in relation to the drug action on parasitemia (Table I).

L. leproide *culture.* Using the same technic as for *T. cruzi* culture, succinic dehydrogenase could be demonstrated in the flagellate form of *L. leproide.* The stain distribution is about the same as in *T. cruzi, i.e.,* dispersed in granular form throughout the cytoplasm; and a few red granules are also visible. The reaction is inhibited by repeated washing and incubation with malonate instead of succinate. Practically no reaction occurred in the absence of brilliant cresyl blue.

Discussion. The histochemical method developed for succinic dehydrogenase in *T. cruzi* and *L. leproide* was shown to be specific for the enzyme, in spite of the presence of

endogenous substrate, by inhibition of the reaction after repeated washing and/or incubation with malonate. The speed of the reaction (within 10 minutes) and the pH dependence are also consistent with the enzymatic nature of the reaction. The optimal pH fits in with the pH curve shown for *T. cruzi* homogenate by the manometric method. The need for calcium is also in conformity with the activating effect of calcium on succinic dehydrogenase(18).

In our experiments under anaerobic conditions better results were obtained, although formazans are not readily reoxidized in air (11). The most likely explanation is that oxygen would reoxidize brilliant cresyl blue, and thus compete with tetrazolium for electrons from the dye (brilliant cresyl blue), as suggested before for methylene blue(15).

T. cruzi gave no reaction with the tetrazolium dye alone even after 90 minutes incubation, while the reaction occurred rapidly in presence of methylene blue or brilliant cresyl blue. This would suggest that tetrazolium is unable to accept electrons by the usual direct sequence from succinic acid in *T. cruzi*. The additional dye seems to transfer the electrons to tetrazolium acting as an intermediary electron carrier. The physiological sequence of electron transfer would remain of course to cytochromes and finally to oxygen. In this connection, Baernstein and Tobie(19) identified cytochrome b in *T. cruzi*, but could not find cytochrome c. We selected brilliant cresyl blue because in the manometric method, of all the artificial electron carriers, the highest activity was obtained with this dye, nearly twice as active as methylene blue (Agosin and von Brand, 4).

It has been shown that the tetrazolium salt is bound non-specifically to the cytoplasm (20,21) prior to its reduction. Due to this initial binding the position of the reduced dye gives a fairly accurate picture of the site of enzyme activity. The low lipid solubility of nitro-BT reduces the chance of nonspecific staining and false localization. The appearance of the diformazan deposits in the form of relatively large granules in the cytoplasm is consistent with its location in mitochondria. Agosin and von Brand(4) in their studies on succinic dehydrogenase of *T. cruzi* by the manometric method reported most of the enzyme activity in the "mitochondria" and "microsome" fractions. Guha *et al.*(22) using various histochemical stains reported that both succinic dehydrogenase and cytochrome oxidase are present in the mitochondria of *L. donovani*. We feel that the localization of the enzyme activity for succinic dehydrogenase could be convincingly demonstrated by extending the tetrazolium reaction for the parasites to electron microscopy. Formazan is electron dense and the tetrazolium technic has been used to demonstrate the enzyme by electron microscopy in mammalian tissue(23-25).

Apart from the deep blue diformazan deposits separate faint red granules were also observed in the cytoplasm. The latter might be due to low enzyme activity reducing the dye to red monoformazan(13). This possibility, however, can be excluded, because the reddish granules could be observed in some unstained preparations although they were less marked. An additional indication which may point in the same direction is that after usual tetrazolium staining and formol-fixation of the trypanosomes, treatment with a reducing agent, 0.1 M sodium dithionite ($Na_2S_2O_4$), did not turn the red color into blue as one could expect if they were monoformazans.

The possibility that these might be volutin granules was also investigated. RNA has been demonstrated in the volutin granules of protozoa(26). Since ribonuclease specifically removes RNA from a preparation(27) we incubated formol-fixed and frozen-thawed *T. cruzi* cultures with 1 mg/ml crystalline protease-free ribonuclease for one hour(13); in both cases the red granules were unaffected by ribonuclease and showed no difference from control samples incubated without the enzyme. It was therefore concluded that these were neither monoformazan deposits nor volutin granules. They seem to be normally present in the cytoplasm.

The effect of spirotrypan, an arsenobenzol derivative with clearing action on *T. cruzi in vivo*, on the parasitic enzyme must be interpreted with caution. The inhibitory effect *in*

vitro with a concentration of 0.3-0.5 mg/ml and *in vivo* 20 hours after 150 mg/kg leads to the suggestion that the drug could act by blocking the enzyme. However, to reveal this effect *in vivo* the parasites had to be greatly reduced in number by the treatment. We feel therefore that a quantitative histochemical method would be needed for evaluation of enzyme activity after treatment, with a sufficient number of parasites still remaining in the peripheral blood.

Summary. Succinic dehydrogenase has been demonstrated histochemically in *T. cruzi* and *L. leproide* – leptomonad form. The protozoa were stained for the enzyme with nitro blue tetrazolium in presence of brilliant cresyl blue. Optimal requirements for the reaction have been described. Enzyme activity is indicated by blue deposits of diformazan in granular form in the cytoplasm. The reaction is weak in absence of calcium and bicarbonate and is inhibited by malonate. Spirotrypan had some inhibitory effect on the tetrazolium reaction in *T. cruzi* both *in vitro* and *in vivo*.

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