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Comparative physiological studies on four species of hemoflagellates in culture. IV. Effect of metabolic inhibitors on the respiration*

by

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Metabolic inhibitors in the study of hemoflagellates have been useful in demonstrating important physiological differences among the species. VON BRAND (3, 4), reviewed the literature up to 1951, and classified the blood forms of the trypanosomes parasitic in mammals by their reactions to certain metabolic inhibitors. Von Brand's groups corresponded approximately to the groups of the biological and morphological classification of HOARE (14). More recent contributions are those of MEDINA *et al.* (20) showing the sensitivity of *Leishmania brasiliensis* to iodoacetamide, the metabolic studies of RYLEY (25, 26) with *Strigomonas oncopelti* and ten species of trypanosomes, and the work of CHATTERJEE and GHOSH (9) on the action of several substances on the respiration of *L. donovani*. FULTON and SPOONER (13) demonstrated the parallelism between cyanide sensitivity or insensitivity and the presence or absence of cytochrome pigments in several species of trypanosomes representing all the groups of Hoare. In the present paper data are presented about the effect of five different metabolic inhibitors upon the respiration of four species of hemoflagellates. Appreciation is expressed to Dr. Clark P. Read for advice and criticism during this work.

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MATERIAL AND METHODS

ORGANISMS. Details of the origin of the strains of *Endotrypanum schaudinni, Leishmania enriettii, Schzotrypanum cruzi* and S. *vespertilionis* and the culture medium used, have been given in a previous paper (28).

MANOMETRIC EXPERIMENTS. The organisms were harvested in the exponential phase of growth, washed by three repeated centrifugations in Krebs-Ringer phosphate (pH 7.2) and resuspended in an adequate volume of the same solution before use in manometric techniques. Oxygen consumption, determined by conventional Warburg respirometry, was followed for 2 hours under air at a temperature of 31° C. Details of the procedure have been described previously (28). Three concentrations of each metabolic inhibitor, in duplicate flasks, were used, with glucose added in a final concentration of 0.01 M. Control flasks with the substrate but without the inhibitor were also set up and endogenous respiration was likewise determined in duplicate. Two flasks without the substrate and with the highest concentration of the inhibitor accompanied each experiment. The glucose and the inhibitor were placed from the beginning in the main compartment, readings being started after a 10 minute period of equilibration. Iodoacetic acid was dissolved in 0.1 N NaOH to give a 0.1 M solution. Sodium azide, sodium fluoroacetate and sodium arsenite were also prepared in 0.1 M solutions, the latter being neutralized with HCl. These four substances were used in final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. A 0.1 M solution of potassium cyanide was prepared and neutralized with HCL, and the inhibitor used in final concentrations of 0.46×10^{-3}, 10^{-4} and 10^{-5} M, as recommended by ROBBIE (22, 23). In those flasks without cyanide, 0.2 ml of a 0.5 M solution of KOH was used in the center well while in the others, 0.2 ml of a mixture of KCN-KOH was used, calculated from the data presented by ROBBIE (23). At the end of the 2 hour period one drop from each flask was examined under the microscope to determine the extent of motility, using the flasks containing glucose alone as the reference point.

NITROGEN DETERMINATIONS. These were carried out either by the technique of KABAT and MAYER (16) or by the nesslerization procedure described by LANG (18). In both cases, the same standard solution of ammonium sulfate was used.

RESULTS

In tables 1, 2 and 3 the degrees of inhibition produced by the inhibitors on each of the species are presented. Some of the patterns of these inhibitions are illustrated in figures 1 to 8. All the organisms were sensitive to the different substances but important quantitative differences can be pointed out. *E. schudinni* showed the highest sensitivity to the thiol inhibitors, iodoacetate being more effective than arsenite, as is frequently the case with other cells. The species of *Schizotrypanum* were not very sensitive to arsenite, a concentration of 10^{-4} M of the substance inhibiting the respiration of *S. cruzi* and *S. vespertilionis* about 6 per cent, in contrast to a respiratory in-

TABLE 1

Effect of Sodium Iodoacetate and Sodium Arsenite on the respiration of the hemoflagellates

SPECIES		Na Iodoacetate			Na Arsenite		
		10^{-3} M	10^{-4} M	10^{-5} M	10^{-3} M	10^{-4} M	10^{-5} M
E. *schaudinni*	Respiration as % of control *	1.4	1.4	4.5	3.8	8.4	45.2
	Motility **	0	0	±	0	0	+
L. *enriettii*	Respiration as % of control	2.8	7.3	79.6	17.8	54.5	97.3
	Motility	0	±	++++	0	±	++++
S. *vespertilionis*	Respiration as % of control	13.6	24.3	74.8	33.1	93.8	93.8
	Motility	0	+	++++	+	++++	++++
S. ***cruzi***	Respiration as % of control	11.5	20.9	68.9	38.6	93.6	93.1
	Motility	±	+	++++	±	++++	++++

* Control = with glucose and without inhibitor.

** O = absence of movement; ± = only few organisms with movement + to +++ = intermediate degrees of motility; ++++ = normal motility of control.

TABLE 2

Effect of Potassium Cyanide and Sodium Azide on the respiration of the hemoflagellates

SPECIES		K Cyanide			Na Azide		
		0.46 x 10^{-3} M	10^{-5} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-5} M
E. schaudinni	Respiration as % of control	3.3*	12.6	43.8	19.6	75.7	108.2
	Motility	0	+	++	0	++	++++
L. enriettii	Respiration as % of control	9.0	20.1	45.4	37.5	62.5	97.0
	Motility	++++	++++	++++	0	++	+++
S. vespertilionis	Respiration as % of control	16.1	31.3	56.3	23.4	61.3	96.1
	Motility	++++	++++	++++	++	+++	++++
S. cruzi	Respiration as % of control	14.2	29.1	52.5	26.3	65.0	95.1
	Motility	++++	++++	++++	+	++	++++

* Figures and symbols as in Table 1.

hibition of almost 92 per cent for *E. schaudinni* at the same concentration of arsenite. *L. enriettii* in general exhibited an intermediate sensitivity to the thiol inhibitors and all the data seem consistent with the relative importance of carbohydrate metabolism in each of the four species (29). In the case of the thiol inhibitors, the degrees of inhibition corresponded fairly well to decreases in motility. Cyanide inhibited the respiration of the four species to approximately the same extent. In the presence of cyanide, only *Endotrypanum* exhibited a considerable depression of motility, which in the other three species was apparently normal, even with the highest concentration of inhibitor. Azide was a less effective inhibitor of respiration and the lowest concentration (10^{-5} M) was without effect on the respiration of any of the flagellates. The latter substance depressed the motility of all the species.

Fluoroacetate which is presumed to result in production of fluorocitrate which, in turn, has and inhibitory effect upon aconitase, produced similar degrees of respiratory inhibition with all the four species. *E. schaudinni* was slightly more sensitive to this compound than were the other species. This is correlated with the intensity of oxidation of the Kreb's cycle intermediates by the organisms (30). As in the case of cyanide, only in *E. schaudinni* was motility affected by fluoroacetate. In several instances the highest dilution of the inhibitor with some action upon the respiration began showing some inhithe inhibitor with some action upon the respiration began showing some inhibitory effect 45 or more minutes after the experiment was commenced. This might be due to a low rate of penetration and slow accumulation of an inhibitory amount inside the cell.

In Table 4 a comparison has been made of the effect of the highest concentration of each inhibitor upon the endogenous respiration of the parasites. Endogenous respiration was depressed in all cases to differing degrees, except that azide was without effect on *L. enriettii.* The endogenous respiration of the two species of *Schizotrypanum* was only slightly sensitive to the same inhibitor. It can be appreciated from Table 4 that, in the case of *Endotrypanum* and *Leishmania,* the remaining motility in the endogenous control is very low. The two species of *Schizotrypanum* showed high motility in the controls, after the same period.

DISCUSSION

Some evidence has been accumulated with trypanosomids showing that iodoacetate is a powerful inhibitor of triosephosphate dehydrogenase and that arsenite has a more specific action upon hexokinase (19, 27). *E. schaudinni* is the most sensitive to the thiol inhibitors and the two *Schizotrypanum,* very similar in all their reactions, were the least sensitive. *S. cruzi* and *S. vespertilionis* are only slightly sensitive to arsenite, indicating that either their -SH enzymes (e. g. hexokinase) have different properties or that an alternative pathway can be utilized in those cases once a block has been obtained. It is a well known fact that the same enzymes from different organisms may vary in reaction to a particular inhibitor (15). *L. enriettii* behaves in its response to the inhibitors,

TABLE 3

Effect of Sodium Fluoroacetate on the respiration of the hemoflagellates

SPECIES		Na Fluoroacetate 10^{-3}M	10^{-4} M	10^{-5}M
E. schaudinni	Respiration as % of control	41.2*	56.0	76.0
	Motility	+	++	+++
L. enriettii	Respiration as % of control	66.8	78.3	88.5
	Motility	+++	++++	++++
S. vespertilionis	Respiration as % of control	44.4	70.7	85.8
	Motility	++++	++++	++++
S. cruzi	Respiration as % of control	43.7	76.9	91.8
	Motility	++++	++++	++++

* Figures and symbols as in Table 1.

as already stated, as an intermediate of the other cases. CHATTERJEE *et al.* (10) have studied in detail the hexokinase of the kala-azar organism *L. donovani.* The enzyme was practically insensitive to thiol inhibitors such as p-chloromercuric benzoate and iodoacetate (10^{-4} M) giving the impression that the hexokinase of this hemoflagellate "is not markedly dependent upon functional -SH groups for its activity".

Since all organisms were sensitive to cyanide and azide, it is likely that heavy metal-containing enzymes play a role in their terminal respiration. Cytochrome b, at least, has been reported in *S. cruzi* by several authors (2, 13, 26). Sodium azide is regarded as a less potent inhibitor than cyanide at physiological pH, being more effective on the acid side (17), which may explain its lower action in the case of the hemoflagellates. *E. schaudinni* was the only species whose motility was affected by cyanide and azide. This indicates that some steps of energy metabolism are heavy-metal-dependent. These are not necessarily only terminal steps, since an aldolase activated by metals and inhibited by chelating agents has been reported in the flagellate protozoan *Trichomonas vaginalis* (1). RYLEY (24) has suggested that a metal ion is involved at some stage in the glucose fermentation process of *Trypanosoma lewisi.* Nevertheless, motility is also depressed in *E. schaudinni* by fluoroacetate, a substance that is not thought to affect energy metabolism at a level above that of pyruvate oxidation (12, 21). This seems to be another indication of the importance of the Kreb's cycle in the chemical economy of this organism. The effect of azide on the motility of the other three species may be related to the fact that this substance has been reported to inhibit phosphorylation and formation of energy-rich compounds during glycolysis (15). Cyanide may considerably decrease oxygen uptake by blocking the final oxidase system, with glycolysis continuing and producing the necessary energy for the normal movement of the parasites.

Fluoroacetate inhibitions are regarded as confirmation of the relative importance of the Kreb's cycle in the organisms (30). The species *T. pipistrelli,* reported by VON BRAND *et al.* (8) to be entirely insensitive to fluoroacetate, differs in this important aspect from our *S. vespertilionis.* According to the literature, these two trypanosomes from bats are neither biologically nor morphologically different and, for that reason, the two names are considered synonyms (11). The author favors the view that the *T. pipistrelli* culture of VON BRAND *et al.* (8) is not *S. vespertilionis* but is a different trypanosome from bats.

The present results on the degree of inhibition of *S. cruzi* by the different substances employed coincide with those reports in the literature as far as arsenite, cyanide, and fluoroacetate are concerned (5, 8, 26). Furthermore, VON BRAND and JOHNSON (5) have reported good motility in the flagellates, at the end of the experiment, in the presence of a concentration of 10^{-3} M cyanide. With the same species, *S. cruzi,* we obtained higher inhibition with iodoacetate and azide than reported by VON BRAND *et al.* (6), VON BRAND *et al.* (8), and RYLEY (26). *L. enriettii* seems to be more sensitive to cyanide than the three human species of the same genus (5).

TABLE 4

Effect of the highest concentration of inhibitor on the endogenous respiration of the hemoflagellates

SPECIES		Iodoacetate 10^{-3}M E	Iodoacetate 10^{-3}M E+I	Arsenite 10^{-3}M E	Arsenite 10^{-3}M E+I	Cyanide 0.46×10^{-3}M E	Cyanide 0.46×10^{-3}M E+I	Azide 10^{-3}M E	Azide 10^{-3}M E+I	Fluoroacetate 10^{-3}M E	Fluoroacetate 10^{-3}M E+I
E. schaudinni	% of respiration	100.0	47.0*	100.0	10.0	100.0	27.3	100.0	28.3	100.0	0.0
	Motility**	±	0	0	0	±	0	+	0	0	0
L. enriettii	% of respiration	100.0	0.0	100.0	50.0	100.0	0.0	100.0	100.0	100.0	8.6
	Motility	+	0	+	0	+	0	±	±	+	+
S. vespertilionis	% of respiration	100.0	39.7	100.0	54.2	100.0	6.4	100.0	83.5	100.0	51.4
	Motility	++	0	++++	±	+++	+	++++	++	++++	++
S. cruzi	% of respiration	100.0	30.3	100.0	50.9	100.0	0.0	100.0	82.5	100.0	42.2
	Motility	++	0	+++	0	++	+	+++	++	+++	++

E = Endogenous; E + I = Endogenous + Inhibitor

* Respiration of Endogenous plus inhibitor expressed as relative percentage of Endogenous.

** Motility as compared with the control with glucose and without inhibitor.

The effect of the inhibitors upon the endogenous respiration of the flagellates may be interpreted in several ways. The low endogenous respiration of *E. schaudinni,* inhibited by all the substances tested, was more affected by arsenite than by iodoacetate, perhaps because the former compound interferes with the oxidative decarboxylation of pyruvate. Fluoroacetate was the only one that reduced the remaining respiration of this species to zero, further suggesting that the short survival of flagellate in the absence of glucose is due to the oxidation of small amounts of the products of carbohydrate metabolism. Endogenous respiration of *L. enriettii* was more sensitive to iodoacetate, cyanide and also to fluoroacetate but, curiously enough, not at all to azide. This could be either a failure of the compound to penetrate the cells in the absence of glucose, or complete insensitivity of the oxidative processes in the cell. Endogenous respiration of the two *Schizotrypanum* is not greatly depressed by the inhibitors except for cyanide. Amino acid oxidation may account for a major part of endogenous respiration in *Schizotrypanum.* The findings by RYLEY (26) that the culture-form of *S. cruzi* produces ammonia when respiring in the absence of any substrate lends further support to this idea. On the other hand, the oxidation of lipids, poorly investigated as energy sources in this organism, may account for another part of the respiration. In connection with this, VON BRAND *et al.* (7) have pointed out that "the culture-form of *T. cruzi* contains considerably more lipids than carbohydrates."

SUMMARY

Two thiol inhibitors, two inhibitors of heavy metal catalysis and an inhibitor of the Kreb's cycle, were tested against the respiration of *Endotrypanum schaudinni, Leishmania enriettii, Schizotrypanum cruzi* and *S. vespertilionis,* both in the presence and absence of glucose.

All the species were more or less sensitive to iodoacetate, *E. schaudinni* being the most sensitive one. This species and *L. enriettii* were inhibited to a great extent by arsenite, whereas the respiration of the two *Schizotrypanum* was considerably more resistant to this substance. This is explained in terms of the more important role that -SH enzymes play in the case of the first two species. Furthermore, a good correlation between motility and degree of inhibition by the thiol inhibitors was observed.

The flagellates were more sensitive to cyanide than to azide. No arrest in motility was observed with *L. enriettii, S. vespertilionis* and *S. cruzi* in the case of cyanide. Some suggestions are made to explain the response of the flagellates to these two inhibitors. It seems likely that cytochrome pigments of some kind are important in the final respiration of the organisms.

The inhibition produced by fluoroacetate is regarded as further indication of the presence of a complete tricarboxylic acid cycle in the flagellates.

The effect of the inhibitors upon the endogenous respiration of the parasites provided some clues as to the nature of this process, which showed some differences among the three genera studied.

RESUMEN

Se prueba el efecto de cinco inhibidores metabólicos (arsenito, iodoacetato, cianuro, azida de sodio y fluoroacetato) sobre la respiración de *Endotrypanum schaudinni, Leishmania enriettii, Schizotrypanum cruzi* y *S. vespertilionis,* en presencia y ausencia de glucosa.

E. schaudinni fue el más sensible a iodoacetato y esta especie y *L. enriettii* fueron bastante sensibles a arsenito mientras que los dos *Schizotrypanum* se mostraron más resistentes a esta sustancia. Tal comportamiento parece encontrar explicación en la mayor importancia que tienen las enzimas con grupos -SH en el caso de las dos primeras especies. Además se encontró una buena correlación entre la disminución de motilidad y el grado de inhibición producida por los dos inhibidores de grupos tiólicos en todas las especies.

Los flagelados se mostraron más sensibles al cianuro que a la azida de sodio. No se observó efecto en la motilidad por parte del cianuro en el caso de *L. enriettii, S. vespertilionis* y *S. cruzi.* Se hacen algunos comentarios tratando de explicar las respuestas de los flagelados a estos dos inhibidores. Parece posible que pigmentos de tipo citocromo son importantes en la respiración final de los mismos.

La inhibición producida por el fluoroacetato se considera como una prueba más de la existencia de un ciclo de Krebs completo en los flagelados.

El efecto de las sustancias sobre la respiración endógena muestra ciertas diferencias entre los géneros estudiados, y da alguna indicación sobre el tipo de proceso metabólico en los mismos.

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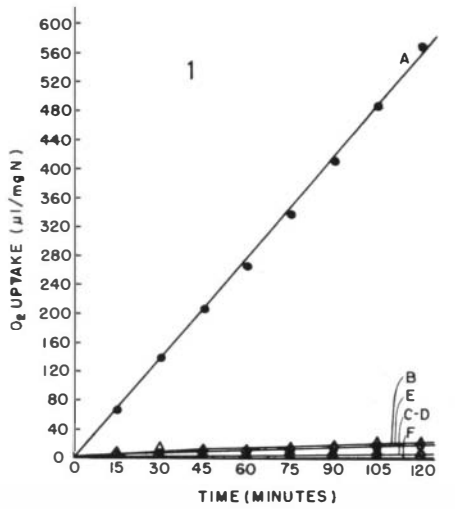
Fig. 1: Effect of Sodium Iodoacetate on the respiration of *E. schaudinni* A = glucose, B = glucose + 10^{-5} M. Io., C = glucose + 10^{-4} M. Io., D = glucose + 10^{-3} M. Io., E = endogenous, F = endogenous + 10^{-3} M. Io.

Fig. 2: Effect of Sodium Iodoacetate on the respiration of *S. cruzi.* A = glucose, B = glucose + 10^{-5} M. Io., C = glucose + 10^{-4} M. Io., D = glucose + 10^{-3} M. Io., E = endogenous, F = endogenous + 10^{-3} M. Io.

Fig. 3: Effect of Sodium Arsenite on the respiration of *E. schaudinni.* A = glucose, B = glucose + 10^{-5}M. ars., C = glucose + 10^{-4}M. ars., D = glucose + 10^{-3}M. ars., E = endogenous, F = endogenous + 10^{-3}M. ars.

Fig. 4: Effect of Sodium Arsenite on the respiration of *S. cruzi.* A = glucose, B = glucose + 10^{-5}M. ars., C = glucose + 10^{-4}M. ars., D = glucose + 10^{-3}M. ars., E = endogenous, F = endogenous + 10^{-3}M. ars.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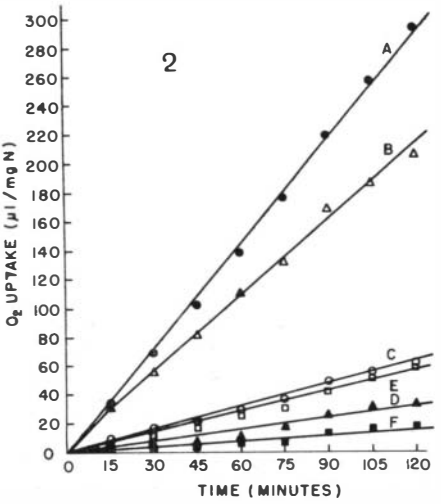
A
B
E
C-D
F
O_2 UPTAKE (µl/mgN)
TIME (MINUTES)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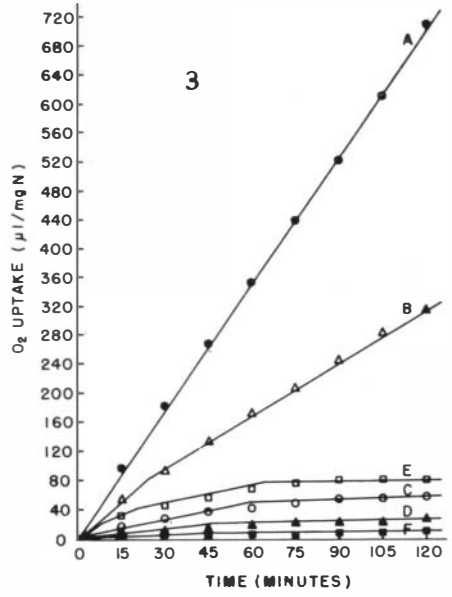
O_2 UPTAKE (µl/mgN)
TIME (MINUTES)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O_2 UPTAKE (µl/mgN)
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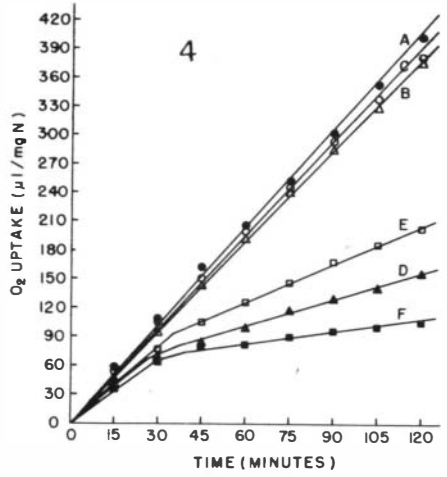
4
A
C
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E
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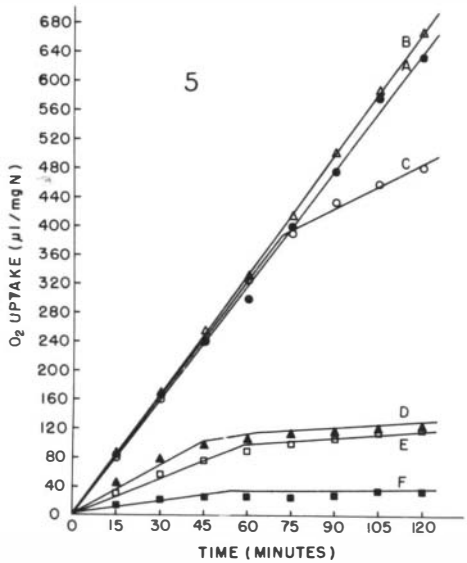
F
O_2 UPTAKE (µl/mgN)
TIME (MINUTES)

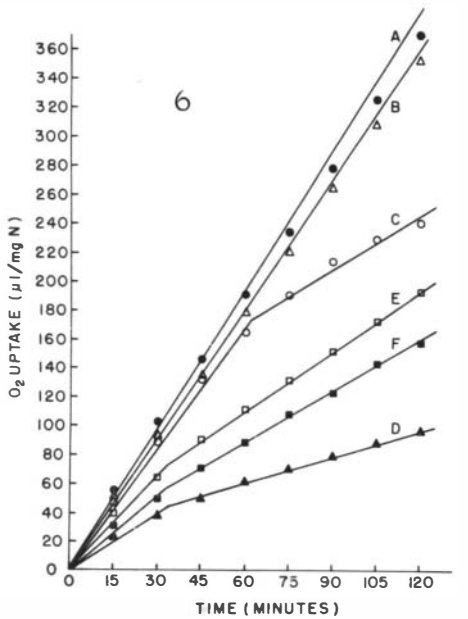
Fig. 5: Effect of Sodium Azide on the respiration of *E. schaudinni*. A = glucose, B = glucose + 10^{-5}M. azi., C = glucose + 10^{-4} M. azi., D = glucose + 10^{-3} M. azi., E = endogenous, F = endogenous + 10^{-3} M. azi.

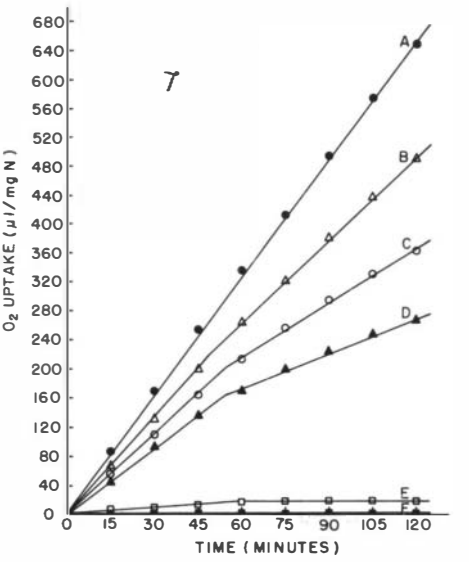
Fig. 6: Effect of Sodium Azide on the respiration of *S. cruzi* A=glucose, B=glucose+10^{-5}M. azi., C=glucose+10^{-4} M. azi., D = glucose + 10^{-3} M. azi., E = endogenous F = endogenous + 10^{-3} M. azi.

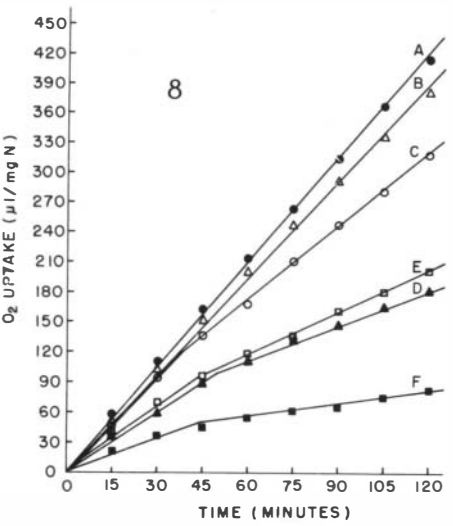
Fig. 7: Effect of Sodium Fluoroacetate on the respiration of *E. schaudinni* A = glucose, B = glucose + 10^{-5}M. fluo., C = glucose + 10^{-4}M. fluo., D = glucose + 10^{-3}M. fluo., E = endogenous, F = endogenous + 10^{-3} M. fluo.

F. 8: Effect of Sodium Fluoroacetate on the respiration of *S. cruzi*. A = glucose, B = glucose + 10^{-5} M. fluo., C = glucose + 10^{-4} M. fluo., D = glucose + 10^{-3}M. fluo., E = endogenous. F = endogenous + 10^{-3} M. fluo.


5
O_2 UPTAKE (µl/mg N)
TIME (MINUTES)
A
B
C
D
E
F


6
O_2 UPTAKE (µl/mg N)
TIME (MINUTES)
A
B
C
D
E
F


7
O_2 UPTAKE (µl/mg N)
TIME (MINUTES)
A
B
C
D
E
F


8
O_2 UPTAKE (µl/mg N)
TIME (MINUTES)
A
B
C
D
E
F