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Physiologic interactions between L-proline and D-glucose in *Leishmania tarentolae, L. donovani* and *Trypanosoma scelopori* culture forms[1]

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Summary

L-proline is more completely catabolized by *Leishmania tarentolae* and *Trypanosoma scelopori* than by *L. donovani.* In the two former organisms the activity of $^{14}CO_2$ derived from L-proline-^{14}C was as high or higher than that derived from D-glucose-^{14}C; in *L. donovani* more $^{14}CO_2$ is derived from D-glucose-^{14}C than from L-proline-^{14}C. Addition of unlabeled D-glucose to cells incubated with L-proline-^{14}C resulted in increased ^{14}C-activity of TCA-soluble fractions of *L. tarentolae* and *T. scelopori;* ^{14}C-activity of 5% TCA insoluble fractions of these cells remained unchanged. Some of the increased ^{14}C-label found in the soluble fraction was in glutamate and aspartate. This increased activity in glutamate and aspartate was accompanied by a decrease in alanine-^{14}C in the free amino acid pool. These results suggest that there are complex physiological interactions between glucose and proline in the metabolism of *L. tarentolae, L. donovani* and *T. scelopori.* In addition, proline appears to have a more central role in the amino acid metabolism of *L. tarentolae* and *T. scelopori* whereas glucose may be more significant in *L. donovani.*

Glucose has long been known as an important energy source for hemoflagellates. The importance of L-proline as an energy source for insect and/or culture stages of these organisms is also well established (von Brand, 1973). Most studies on glucose and proline (as well as other amino acids) metabolism utilize simplified in vitro systems in order to measure more readily substrate dissimilation and substrate effects upon endogenous respiration. We initiated a

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study of the relationships between proline and glucose utilization in culture forms of hemoflagellates in order to gain some understanding of the role of proline metabolism in general cell physiology. We measured the interplay between proline and glucose by following the distribution of ^{14}C from L-proline-^{14}C and D-glucose-^{14}C into various major cell fractions. The catabolism of each of the labeled substrates in the presence of the other unlabeled substrate was followed also. This was a comparative investigation and we were able to find differences in the response to these experimental conditions by three hemoflagellate species: *Leishmania tarentolae, L. donovani* and *Trypanosoma scelopori*. Some of the results have been previously described in abstract (Krassner and Flory, 1973).

Stock cultures of *Leishmania tarentolae*, strain TAR II, UCI variant were maintained at 26° C in 250 ml Erlenmeyer flasks containing 100 ml brain heart infusion (BHI) medium with 0.02 mg hemin. They were subcultured every 7 days. Cells used for experimental analysis were grown in 2500 ml wide bottomed Fernbach flasks containing 300 ml BHI and maintained at room temperature (~22° C) on a rotary shaker (60 rpm). Promastigotes were harvested during the exponential growth phase (day 4).

Stock cultures of *L. donovani* Malakal area Sudan strain (1S), obtained originally from Dr. L. A. Stauber, Rutgers University, were maintained in NNN medium with Locke's overlay (Taylor and Baker, 1968) at 26° C and were subcultured every 7 days. Cells used for cell fraction analysis were grown in 125 ml Erlenmeyer flasks with 30 ml used for the solid phase of the medium and 50 ml for the overlay. Cells used for thin layer chromatography studies were grown in NNN with 40 ml Locke's and 10 ml Cross's solutions in the overlay. The promastigotes were harvested during the exponential growth phase (day 7) as above.

Stock cultures of *Trypanosoma scelopori*, obtained originally from Dr. J. Chao, University of California, Los Angeles, were maintained in NNN medium with Locke's overlay as above. Cells used for experimental analysis were grown and harvested as above.

Parasites were counted in a Petroff-Hauser chamber.

$^{14}CO_2$ evolved from cells incubated in L-proline-^{14}C or in D-glucose-^{14}C was measured in respirometer flasks containing 0.5 ml hyamine hydroxide in the center well and the cell suspension in the main compartment. Immediately after addition of radioisotope to the main compartment three 100 μl samples were removed to determine initial radioactivity. The flask top and the side arm then were sealed. Flasks were incubated for 2 h at 27° C, the cells were killed with HCl, and the system allowed to sit 30 min for absorption of evolved CO_2. Hyamine hydroxide in the center well was transferred to scintillation fluid and counted in a Nuclear Chicago Unilux III scintillation counter (da Cruz and Krassner, 1971). The ^{14}C-label remaining in the main compartment (inside cells and in the medium) was also counted.

CO_2 evolution and fraction experiments were run in parallel for each substrate. Total counts in the main respirometer flask compartment (cells + media) for the two concurrent tests were about the same. Since initial concentrations of label added were identical in both procedures and recovery of ^{14}C-activity in CO_2 evolution experiments was close to 100%, the amount of $^{14}CO_2$ evolved from cells in parallel runs was assumed to be the same (i.e. the $^{14}CO_2$ activity measured in CO_2 evolution experiments should be the same as that produced by cells in the fraction experiments). This enabled us to calculate the per cent of total ^{14}C-activity present in $^{14}CO_2$, media, TCA soluble and TCA insoluble cell fractions.

Respirometer flasks were incubated as above for measurement of other ^{14}C fractions. At the end of the incubation period, cold PBS was added to the main compartment and the flasks set in an ice bath. All subsequent procedures were done at 4° C. The cell suspension was washed by centrifuging and the first supernatant and subsequent washings were counted to determine the amount of unincorporated label outside the cell as well as efflux (media fraction). A few drops of 4N HCl was added to this fraction before counting to liberate dissolved $^{14}CO_2$.

Cold trichloroacetic acid (TCA) was added to the pellet and the mixture chilled 10 min on ice. The mixture was centrifuged three times and the supernatant removed. The material was collected on filters and counted to determine acid insoluble radioactivity (assumed to be fraction containing ^{14}C incorporated into cell macromolecules). The initial supernatant and washings were combined and the activity also determined (unincorporated ^{14}C label in cell pools).

Thin layer chromatography studies were carried out by collecting radiolabeled flagellates on a Millipore filter which was placed immediately into 80% ethanol and sonified. After centrifugation, the supernatant fraction was transferred to a porcelain crucible and evaporated. The residue was redissolved in redistilled water. The evaporated material was placed onto thin-layer chromatography (TLC) plates and developed according to the method of Jones and Heathcote (1966).

Recovery of ^{14}C in evolved CO_2 and other fractions

We measured the oxidation of labeled substrates to CO_2 by culture forms of *L. tarentolae*, *L. donovani* and *T. scelopori* in the presence or absence of a second unlabeled substrate. ^{14}C in the acid soluble fraction, acid insoluble fraction, and that remaining in the incubation medium were also measured. The results are summarized in Table 1. Under the conditions employed ~80% of the initial ^{14}C added to the reaction mixture was taken up by *L. tarentolae* and *T. scelopori* when either proline-^{14}C or glucose-^{14}C was present. Relatively little proline-^{14}C was taken up by *L. donovani* and only 32% of the labeled glucose was used by this organism.

Total assimilation (uptake, incorporation and CO_2 evolution) of labeled

Table 1. ^{14}C-label recovered in evolved CO_2, acid (5% TCA) soluble and acid insoluble fractions of *Leishmania tarentolae, Leishmania donovani* and *Trypanosoma scelopori* culture stages incubated with ^{14}C-precursors

Substrates tested	Percent of total ^{14}C-recovered□ in:											
	Leishmania tarentolae				*Leishmania donovani*				*Trypanosoma scelopori*			
	CO_2	*media*	*acid soluble*	*acid insoluble*	*CO_2*	*media*	*acid soluble*	*acid insoluble*	*CO_2*	*media*	*acid soluble*	*acid insoluble*
L-proline-^{14}C*	*67.0 ± 0.0(4)*○	*17.8* ± 2.8(4)	*6.8* ± 1.5(4)	*8.8* ± 1.5(4)	*4.4* ± 2.8(6)	*96.6* ± 4.8(6)	*2.3* ± 1.6(6)	*0.7* ± 0.7(6)	*53.3* ± 3.0(6)	*28.3* ± 5.5(6)	*13.5* ± 7.2(6)	*4.7* ± 1.0(6)
D-glucose-^{14}C**	57.0 ± 9.2(4)	19.8 ± 2.4(4)	17.3 ± 9.8(4)	6.3 ± 0.5(4)	22.2 ± 3.6(6)	67.7 ± 2.9(6)	10.3 ± 2.8(6)	0.7 ± 0.4(6)	48.3 ± 7.7(6)	18.7 ± 1.0(6)	23.8 ± 7.9(6)	9.2 ± 2.3(6)
L-proline-^{14}C + L-proline-^{12}C△	3.0 ± 0.0(2)	94.0 ± 0.0(2)	3.0 ± 0.0(2)	0.4 ± 0.0(2)	0.3 ± 0.1(4)	96.5 ± 1.2(4)	1.6 ± 0.7(4)	0.1 ± 0.0(4)	1.0 ± 0.0(2)	96.0 ± 1.4(2)	2.9 ± 1.3(2)	0.1 ± 0.0(2)
D-glucose-^{14}C + D-glucose-^{12}C△	2.0 ± 0.0(2)	96.0 ± 0.0(2)	1.5 ± 0.1(2)	0.3 ± 0.0(2)	0.9 ± 0.2(4)	98.1 ± 2.2(4)	1.1 ± 0.2(4)	0.1 ± 0.1(4)	1.2 ± 0.0(2)	97.6 ± 0.0(2)	1.0 ± 0.0(2)	0.2 ± 0.0(2)
L-proline-^{14}C + D-glucose-^{12}C△	24.0 ± 0.0(2)	58.0 ± 1.4(2)	15.5 ± 0.7(2)	8.5 ± 0.7(2)	1.8 ± 0.3(4)	95.8 ± 1.5(4)	1.9 ± 0.9(4)	0.3 ± 0.3(4)	27.3 ± 5.7(4)	32.3 ± 2.8(4)	32.0 ± 8.8(4)	5.5 ± 1.7(4)
D-glucose-^{14}C + L-proline-^{12}C△	36.0 ± 0.0(2)	31.0 ± 4.2(2)	35.5 ± 6.4(2)	8.0 ± 0.0(3)	8.0 ± 0.0(3)	75.0 ± 10.2(3)	9.1 ± 4.3(3)	0.3 ± 0.3(3)	32.0 ± 8.1(4)	24.0 ± 1.8(4)	32.5 ± 9.3(4)	10.5 ± 3.0(4)

* UL 0.1 μCi (spec. activ. 180 mCi/mM)
** UL 0.1 μCi (spec. activ. 116 mCi/mM)
△ 10 mM
□ Average total CPM recovered 150,000
○ ( ) = number of determinations

Incubation time and cell number are given in text.

proline by *L. tarentolae* and *T. scelopori* was reduced by ~94% when 10 mM proline-^{12}C was included in the incubation media. A similar drop in assimilation was found when unlabeled glucose was added to reaction mixtures containing glucose-^{14}C. Addition of the ^{12}C co-substrate diluted ^{14}C-activity more than a million-fold so that the lower assimilation of ^{14}C-label in the presence of ^{12}C-substrate is not surprising. This effect was not noted in the case of *L. donovani* because the amount of proline-^{14}C taken up was small even without the co-substrate. Addition of glucose-^{12}C to *L. donovani* did result in a dilution effect (drop of ~94%). The dilution effect in all cases resulted in marked drops in ^{14}C-activity of the various cell fractions.

Changes were seen in the level of ^{14}C recovered from the different cell fractions when unlabeled glucose was added to proline-^{14}C and when unlabeled proline was added to glucose-^{14}C in the reaction mixture. These changes were not completely due to a dilution effect although modest decreases in total assimilation of ^{14}C were found. Addition of glucose-^{12}C to proline-^{14}C resulted in a 49% drop of assimilated label by *L. tarentolae;* the drop was only 6.0% in *T. scelopori.* When proline-^{12}C was added to labeled glucose in the incubation medium, the drop in ^{14}C-assimilation was 14% in *L. tarentolae,* 22% in *L. donovani* and only 6.0% in *T. scelopori.*

These results suggest that addition of the opposite ^{12}C-substrate to the reaction mixture potentiated uptake of the ^{14}C-substrate and worked against the dilution effect. This view is supported by the results found with ^{14}C-activity of TCA insoluble cell fractions; addition of the other ^{12}C-substrate caused no change in ^{14}C-labeled TCA insoluble fraction activity.

Of interest are the shifts in $^{14}CO_2$ and intracellular pool ^{14}C-levels of cells under test conditions. The decrease in $^{14}CO_2$ level when the other ^{12}C-substrate is added was relatively larger than that measured for total ^{14}C-assimilated; this was accompanied by an increase in intracellular pool activity. *L. tarentolae* promastigotes exhibited a 128% increase in acid soluble ^{14}C when glucose-^{12}C and proline-^{14}C were present and a 105% increase when proline-^{12}C was added to glucose-^{14}C in the incubation medium. Similar increases were found with *T. scelopori* epimastigotes. Some of the increase in intracellular pool activity may be due to higher labeled glutamate and aspartate activities in the soluble fraction.

Recovery of free ^{14}C amino acids

Dissimilation of ^{14}C into the free amino acid pool of *L. tarentolae* and of *T. scelopori* incubated in proline-^{14}C or glucose-^{14}C was followed using TLC radioautography (Table 2). Most of the ^{14}C-label from L-proline-^{14}C that was recovered in the free amino acid pool was found in proline, alanine, glutamate and aspartate (these 4 amino acids contained ~80% of the total ^{14}C-activity recovered in the free amino acid pool). The most actively labeled amino acids recovered from cells incubated with glucose-^{14}C were alanine, glutamate and

Table 2. Recovery* of the major labeled amino acids in the free amino acid pool of *Leishmania tarentolae* and *L. donovani* promastigotes incubated in L-proline-^{14}C and D-glucose-^{14}C

Substrates** tested	% ^{14}C-label of total recoverable free amino acids□			
	alanine	proline	glutamate	aspartate
Test organism: *Leishmania tarentolae*				
L-proline-^{14}C(2)○△	38.0 ± 0.6	37.5 ± 1.6	3.4 ± 0.7	3.4 ± 0.2
D-glucose-^{14}C(2)△△	66.5 ± 0.1	0	3.6 ± 0.1	3.6 ± 0.6
L-proline-^{14}C + D-glucose(2)	24.2 ± 0.2	23.9 ± 0.9	14.3 ± 0.3	21.8 ± 0.8
D-glucose-^{14}C + L-proline(2)	74.8 ± 0.5	0	2.8 ± 0.1	4.8 ± 0.7
Test organism: *Leishmania donovani*				
L-proline-^{14}C(2)□□	35.6 ± 2.0	19.0 ± 7.3	19.3 ± 3.8	12.7 ± 8.8
D-glucose-^{14}C(2)○○	63.8 ± 6.9	0	4.3 ± 0.5	6.0 ± 0.3
L-proline-^{14}C + D-glucose(2)	17.5 ± 4.1	49.3 ± 7.3	12.7 ± 0.7	8.0 ± 11.3
D-glucose-^{14}C + L-proline(2)	59.0 ± 10.5	0	11.7 ± 3.1	5.5 ± 5.5

* Recovery by TLC. Solvents: 1st dimension-propanol (40 pts): formic acid (2 pts); 2nd dimension – tert-butanol (25 pts): methylethylketone (15 pts): 0.88 M NH_3 (5 pts): H_2O (5 pts)
** Specific activities as in Table 1
○ ( ) = number of determinations
○○ Other ^{14}C-amino acids identified: cystine, glycine and threonine
□ Average total CPM ~8000
□□ Other ^{14}C-amino acids identified: glycine
△ Other ^{14}C-amino acids identified: cystine and threonine
△△ Other ^{14}C-amino acids identified: cystine and glycine

aspartate (these 3 amino acids comprised ~75% of the total ^{14}C-activity recovered in the amino acid pool). Labeled proline was not found in organisms incubated with glucose-^{14}C.

Dissimilation of ^{14}C into the above amino acids when unlabeled substrate was added to the reaction mixture was also followed (Table 2). There was a 36% decrease in alanine-^{14}C recovered from *L. tarentolae* when glucose-^{12}C was added to proline-^{14}C. This decrease in alanine-^{14}C formation was accompanied by a 4-fold rise in glutamate-^{14}C and a 6-fold increase in aspartate-^{14}C in the amino acid pool. Alanine-^{14}C levels in *L. tarentolae* increased by 12% when proline-^{12}C was added to glucose-^{14}C in the incubation medium. Glutamate-^{14}C and aspartate-^{14}C levels in these cells remained unchanged. In *L. donovani*, alanine-^{14}C activity dropped by 51% when glucose-^{12}C was added to proline-^{14}C in the reaction mixture. Labeled glutamate levels also dropped (by 34%) whereas the levels of proline-^{14}C in the pool went up by 159%; the levels of

aspartate-^{14}C decreased by 37%. When proline-^{12}C was added to glucose-^{14}C, alanine-^{14}C-activity in the free amino acid pool dropped by 8%.

Our observations support von Brand's (1973) suggestion that interactions of various environmental conditions modify cell responses to specific factors studied under simplified in vitro conditions. We found changes in assimilation and in the distribution of labeled end products in 3 hemoflagellate species which were dependent upon the nature of the substrate(s) present in the incubation medium. These results probably represent shifts in some of the parasites' metabolic pathways. Chappell et al. (1972) reported changes in the labeling patterns of the free amino acid pool of another hemoflagellate, *Trypanosoma gambiense,* which were dependent upon the labeled compound added to the incubation medium. They pointed out that the relationships between absorption, interconversion and biosynthesis of amino acids by *T. gambiense* are complex. It was not possible for these workers to compare cell responses to the addition of amino acids alone because glucose was required for survival; all of their experiments were therefore done with glucose added to the incubation medium.

Studies with hemoflagellates have shown that the presence of one amino acid can markedly affect the uptake and disposition of another amino acid (see discussion in von Brand, 1973). In one such study, Ruff and Read (1974), working with *T. equiperdum,* found that proline inhibited the uptake of alanine, glutamate and phenylalanine. We did not follow this type of interaction in the present study but it should be of importance in vivo.

Uptake and utilization of ^{14}C-labeled proline by the 3 hemoflagellate species studied in this paper was affected by addition of glucose-^{12}C. Although addition of the sugar resulted in decreased $^{14}CO_2$ evolution, the decrease was much less than that found when 10 mM proline-^{12}C was added to the reaction mixture (see Table 1). This indicated that reduction of $^{14}CO_2$ evolution caused by added unlabeled glucose was not entirely due to a co-substrate dilution. Incorporation of ^{14}C into the TCA-insoluble cell fraction was unaffected by addition of unlabeled sugar. These results suggest that the sugar potentiated utilization of proline by the cells, a view supported by the changes found in ^{14}C-activity of alanine, glutamate and aspartate in the free amino acid pool.

A fascinating result in this study was the rise in TCA soluble (cell pool) ^{14}C when glucose-^{12}C was added to proline-^{14}C in the incubation medium (*L. tarentolae* and *T. scelopori*). There appears to be a change in the flow of carbon atoms from proline when glucose is present. TLC experiments indicated that some of the increased ^{14}C-activity in the cell pool caused by glucose was due to higher levels of glutamate-^{14}C and arginine-^{14}C, at least in the case of *L. tarentolae.*

Glutamate and alanine are important intermediates and/or end products in proline metabolism (Krassner and Flory, 1972). There is a well-known metabolic relationship between glutamate and aspartate via aspartate aminotrans-

ferase (E.C. 2.6.1.1.), an enzyme common to many trypanosomatidae (see discussion in Steiger et al., 1974). Alanine was the most heavily labeled amino acid in our chromatograms. Depending upon the incubation mixture it comprised 24–75% (*L. tarentolae*) and 17–64% (*L. donovani*) of the ^{14}C recovered in free amino acid pools. In *T. gambiense*, alanine was the only labeled amino acid derived from D-glucose-^{14}C, making up ~69% of the ^{14}C-label in the sugar that was added to the medium (Chappell et al., 1972). Alanine is a common end product of amino acid metabolism (see discussion in Krassner, 1969). Promastigotes of *L. tarentolae* and *L. donovani* derived less alanine from proline when glucose was present and the former derived more alanine from glucose when the amino acid was present (see Table 2).

Glucose catabolism in the 3 hemoflagellate species that we studied yielded carbon atoms to at least 5–6 amino acids (see Table 2 and Sylvester et al., 1974). This supports the contention that in the hemoflagellates, the Kreb's cycle operates as a source of carbon for biosynthesis of amino acids (de Boiso and Stoppani, 1973). Details of the mechanisms leading carbon atoms to amino acids were not examined in the present study. von Brand (1973) suggests reductive amination as the basis for the appearance of labeled glucose carbon in amino acids because keto acids are common intermediates of carbohydrate utilization. Transamination also plays a role in the flow of carbon atoms in amino acid synthesis. Although its role is unclear, von Brand (1973) feels that CO_2 may also be important for amino acid synthesis.

Our results on recovery of ^{14}C-activity in the different fractions from *T. scelopori* are at variance with those reported earlier by Sylvester et al. (1974). This is because an invalid method was used for analysis in the latter study. After completion of incubation, cells were killed by tipping in formalin from a side arm of the respirometer flask and the mixture allowed to set for 30 min to permit complete absorption of $^{14}CO_2$ into the hyamine hydroxide in the center well. Because of the small size of the respirometer flask, sufficient formalin evaporated from the side arm to kill the cells in about 45 min. In addition, formalin damaged the cell membrane permitting internal ^{14}C-label to leak out of the cell during equilibration. The invalid method also compromised results found in time course ^{14}C-labeled proline, arginine and ornithine utilization studies with *L. tarentolae* (Wagner and Krassner, 1976). Corrected values for $^{14}CO_2$ evolution are listed in Table 3.

In the current study the 3 test organisms responded differently to the various test conditions although *L. tarentolae* and *T. scelopori* acted in a similar fashion and were not like *L. donovani.* This and other (e.g. Krassner and Flory, 1971) studies supports the view that proline plays a central role in the metabolism of *L. tarentolae* and *T. scelopori.*

The amino acid appears to play a lesser role in *L. donovani* metabolism; glucose seems to exert a more significant modulating effect on the metabolism of this organism. Janovy (personal communication) and Berens et al. (1976)

Table 3. Evolution of $^{14}CO_2$ from *Leishmania tarentolae* promastigotes incubated in L-proline-^{14}C, L-arginine-^{14}C and L-ornithine-^{14}C

Substrate and incubation time	Percent of total□ recovered ^{14}C-activity in evolved CO_2
L-proline-^{14}C (4)*	
2 h	67.0 ± 0.0
8 h	70.0 ± 0.0
L-arginine-^{14}C (4)**	
2 h	16.0 ± 0.0
8 h	36.0 ± 4.8
L-ornithine-^{14}C (4)***	
2 h	4.5 ± 1.7
8 h	19.8 ± 10.7

* UL 0.1 mCi/ml (spec. activ. 25/mCi/mM)
** UL 0.1 mCi/ml (spec. activ. 305 mCi/mM)
*** UL 0.1 mCi/ml (spec. activ. 203 mCi/mM)
□ Average total recovered CPM ~60,000 for proline, ~200,000 for arginine and ~200,000 for ornithine

have found that *L. donovani* can consume all available culture medium glucose before the end of exponential growth. This species therefore differs from many other trypanosomatids by its ability to rely on glucose catabolism for energy before the end of exponential growth. *T. brucei* subgroup forms (Evans and Brown, 1972), *Crithidia fasciculata* (Marr, 1974) and *L. tropica* (Mukkada et al., 1974) all exhibit delayed glucose utilization in culture. They do not utilize exogenous glucose to any great extent until late in the exponential or early in the stationary growth phase. The present study was done with cells harvested during exponential growth. It would be of great interest to repeat our experiments with cells harvested during the stationary growth phase.

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