

**Zeitschrift:** Acta Tropica  
**Herausgeber:** Schweizerisches Tropeninstitut (Basel)  
**Band:** 43 (1986)  
**Heft:** 4

**Artikel:** Localization of 3'-nucleotidase and calcium-dependent endoribonuclease in the plasma-membrane of "Trypanosoma brucei"  
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**DOI:** <https://doi.org/10.5169/seals-313641>

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## Localization of 3'-nucleotidase and calcium-dependent endoribonuclease in the plasma-membrane of *Trypanosoma brucei*

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### Summary

We have characterized a 3'-nucleotidase activity of *T. brucei*. The enzyme has a pH optimum of 8.7, is inactivated by chelating agents and stimulated by divalent cations. It is inhibited by Zn<sup>2+</sup>, Mn<sup>2+</sup>, pyrophosphate and the trypanocidal drug suramin for which it has a K<sub>i</sub> of 3 μM. From cell fractionation experiments it is concluded that the enzyme is located in the plasma membrane. Alkaline 3'-endoribonuclease is also located in the plasma membrane of *T. brucei* and this activity shares a great number of properties with the 3'-nucleotidase activity, including its sensitivity to suramin. The possibility that both 3'-nucleotidase and endonuclease activities are catalyzed by the same enzyme cannot be excluded.

**Key words:** *Trypanosoma brucei*; ribonuclease; 3'-nucleotidase; plasma membrane; suramin.

### Introduction

In the last few years several reports have appeared on the presence of an unusual 3'-nucleotidase activity in *Leishmania donovani* (Gottlieb and Dwyer, 1981, 1983; Dwyer and Gottlieb, 1984), African trypanosomes (McLaughlin,

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1982; Gardiner et al., 1982) and the non-pathogenic insect Trypanosomatid *Crithidia fasciculata* (Gottlieb, 1985). In *Leishmania* sp. the 3'-nucleotidase was identified as an ecto-enzyme capable of hydrolyzing extracellular 3'-nucleotides and since in *C. fasciculata* this enzyme could be induced when the organism was grown under conditions of adenosine limitation it was attributed an important role in making available purine nucleosides for transport into the cell and for their subsequent salvage (Gottlieb, 1985).

3'-Nucleotidase activity has been detected by cytochemical techniques in the procyclic insect stage of *Trypanosoma rhodesiense*, but unexpectedly not in the bloodstream forms (Gardiner et al., 1982) although it was detected in the latter by biochemical methods (McLaughlin, 1982). In this paper we report the presence of 3'-nucleotidase activity in the plasma membrane of procyclic insect stages as well as the bloodstream form trypomastigote of *Trypanosoma brucei* and we propose that this enzyme may serve as an excellent marker enzyme in cell fractionation experiments not only for the plasma membrane of *T. brucei* but for that of the Trypanosomatidae in general.

On the basis of a great number of similarities in properties between 3'-nucleotidase and Ca<sup>2+</sup>-dependent endoribonuclease, another nuclease recently described for *T. brucei* (Gbenle and Akinrimisi, 1982), it is suggested that both activities are catalyzed by one and the same protein.

## Materials and Methods

Growth, isolation and purification of the bloodstream and the procyclic insect trypomastigote forms of *Trypanosoma brucei* 427 were done as described before (Oppenoes et al., 1976, 1981).

*Cell fractionation.* Trypanosomes were homogenized by grinding with silicon carbide abrasive grain as previously described (Steiger et al., 1980), except that homogenization buffers containing 0.25 M sucrose, 1 mM imidazole-HCl and 1 mM EGTA (pH 7.0) and 0.25 M sucrose, 25 mM Tris-HCl and 1 mM EDTA (pH 7.2) were used, respectively. Differential centrifugation of the whole homogenate and isopycnic centrifugation of the post-large-granule extract in sucrose/Tris/EDTA or sucrose/imidazole/EGTA medium, respectively, were performed as previously described (Oppenoes and Steiger, 1981).

*Determination of enzyme activities.* The determination of ribonuclease activity was essentially as described earlier (Gbenle and Akinrimisi, 1982) except that the reaction mixture contained 25 mM Tris-HCl, pH 8.0, 75 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM CaCl<sub>2</sub> (or 3 mM EGTA), 80 µg poly(A) as substrate, and 10 µg of sample in 0.1% Triton X-100, in a total reaction volume of 200 µl. Incubation was at 37°C for 60 min. Calcium-dependent RNase activity was calculated as the difference between the total activity (using 1 mM CaCl<sub>2</sub>) and the calcium-independent activity (using 3 mM EGTA).

3'-nucleotidase activity was estimated essentially as described by Gottlieb and Dwyer (1983) by measuring the amount of phosphate liberated from 3'-AMP after 30–60 min incubation at 37°C of 25 µg trypanosomal protein in 0.1% Triton X-100 in a reaction mixture containing 10 mM adenosine 3'-monophosphate, sodium salt, 0.1 M Tris-HCl buffer pH 8.7, 0.1 M KCl, 5 mM CoCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> in a total volume of 0.1 ml. The reaction was stopped by adding 0.2 ml of a 12% TCA solution and the amount of inorganic phosphate released was determined in the supernatant. Under these conditions the liberation of inorganic phosphate was linear with time and the amount of protein within the range of 0–30 mg×min.

The activities of *α*-D-mannosidase (Steiger et al., 1980), hexokinase (Steiger et al., 1980), *α*-D-glucosidase (Steiger et al., 1980), aryl acylamidase (Oppendoes and Steiger, 1981), acid phosphatase (Steiger et al., 1980), isocitrate dehydrogenase (Oppendoes et al., 1977) and alanine aminotransferase (Steiger et al., 1980) were determined as described previously.

For latency experiments the RNase, 3'-nucleotidase, aryl acylamidase and alanine aminotransferase assay mixtures were supplemented with 0.25 M sucrose, 5 mM glucose and 0.05% BSA and the reaction stopped after 10 min at 25°C.

Protein was determined fluorometrically (Stein et al., 1973) after diluting and solubilizing samples in 0.2 M carbonate-buffered 1% deoxycholate (pH 11.3) using bovine serum albumin as standard.

*Enzyme kinetics.* The results of kinetic measurements were analyzed on an Apple IIe computer with a program (Barlow, 1983) that fits the data points to a hyperbola and corrects for blank activities. Such corrected hyperbolas were then linearized either to Lineweaver-Burk plots, or by single reciprocal transformation to Dixon plots.

*Presentation of results.* Distribution patterns of the alkaline RNase activities, the 3'-nucleotidase, marker enzymes and protein after isopycnic centrifugations are presented as described previously (Oppendoes et al., 1981; Steiger et al., 1980; Oppendoes and Steiger, 1981).

*Materials.* All chemicals were of the highest purity available. 3'-adenosine monophosphate was purchased from Sigma Chemical Co., St. Louis, Mo, USA. Polyadenylic acid, enzymes, coenzymes and substrates were from Boehringer GmbH, Mannheim FRG. Suramin (Antrypol) was obtained from ICI, Macclesfield, Cheshire, England.

## Results

*3'-nucleotidase.* Fig. 1 shows the pH dependence of the 3'-nucleotidase activity. The enzyme displayed a broad activity profile with maximal activity at pH 8.7. Therefore, all subsequent measurements were carried out at this pH.

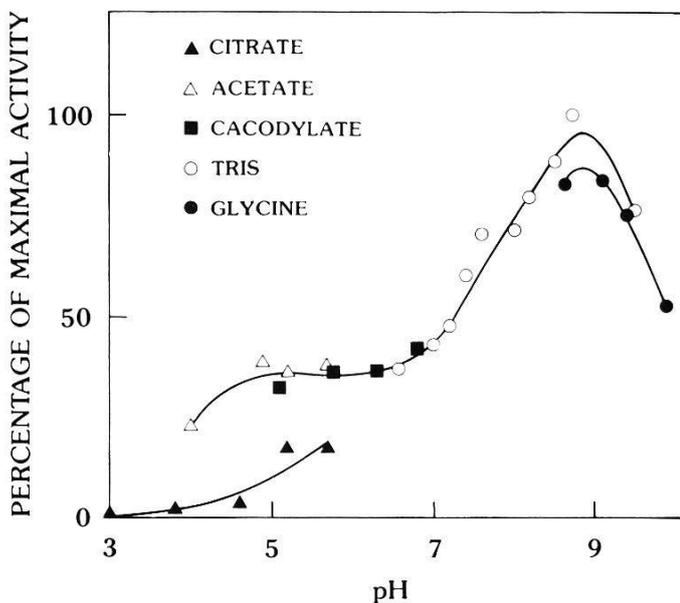


Fig. 1. The influence of pH on the 3'-nucleotidase activity in an homogenate of *Trypanosoma brucei* bloodstream-form trypomastigotes. The activity was measured as described in Materials and Methods except that in different pH ranges different buffers at 0.1 M concentration were used as indicated.

Table 1. The effect of various compounds on the 3'-nucleotidase of *Trypanosoma brucei* bloodstream-form trypomastigotes

Additions	Relative activity (%)
None	100
EDTA	37
CaCl <sub>2</sub>	106±17 (4)
CoCl <sub>2</sub>	171±20 (4)
MgCl <sub>2</sub>	103±12 (4)
MnCl <sub>2</sub>	55 (1)
ZnCl <sub>2</sub>	21 (2)
HgCl <sub>2</sub>	80±2 (3)
Ammonium molybdate	94±26 (4)
NaF	100±6 (4)
Sodium/potassium tartrate	104±12 (4)
Sodiumpyrophosphate	63±13 (3)
Spermine	100±4 (3)
Spermidine	105±6 (3)
Heparin (0.05 mM)	107±8 (3)
Normal assay mixture	172±18 (3)

All additions were 5 mM unless stated otherwise. Values are expressed as mean±standard deviation of the number of experiments indicated in brackets. Values are relative to a suboptimal control containing the standard assay mixture (see Materials and Methods) but without Ca<sup>2+</sup> and Co<sup>2+</sup> ions.

Table 1 shows that the *T. brucei* 3'-nucleotidase displayed a sensitivity towards various compounds which was similar to that of the enzyme described for *Leishmania donovani* (Gottlieb and Dwyer, 1983). Like the *L. donovani* enzyme it was inactivated by EDTA and activated by cobalt ions, and unaffected by tartrate and fluoride. Mn<sup>2+</sup> and Zn<sup>2+</sup> ions had an inhibitory effect. Contrary to the situation in *L. donovani* (Gottlieb and Dwyer, 1983) and *Crithidia luciliae* (Opperdoes and Van Roy, unpublished results) no 5'-nucleotidase activity could be detected in *T. brucei* bloodstream forms, provided that any non-specific acid phosphatase was inhibited by adding an excess of glycerol 2-phosphate.

*Kinetics of suramin inhibition.* Fig. 2A shows the effect of increasing amounts of 3'-adenosine monophosphate and suramin on the velocity of the 3'-nucleotidase reaction. In the absence of inhibitor a K<sub>m</sub> for 3'-AMP of 0.19 mM was measured with a V<sub>max</sub> of 82 nmol/min/mg protein in a total homogenate. The binding of suramin to the enzyme has a strong influence on its affinity for substrate (apparent K<sub>m</sub>) and only a slight effect, if any, on its apparent V<sub>max</sub>. Therefore it is concluded that the inhibition by suramin is competitive in nature or of a mixed type. The apparent K<sub>i</sub> for suramin with the highest 3'-AMP concentration tested (2 mM) was 10 μM. When the data, after correction for a small suramin-insensitive contribution to the total 3'-nucleotidase activity, were replotted as Dixon-plots a K<sub>i</sub> for suramin of 3'-nucleotidase of 3 μM was calculated (Fig. 2B).

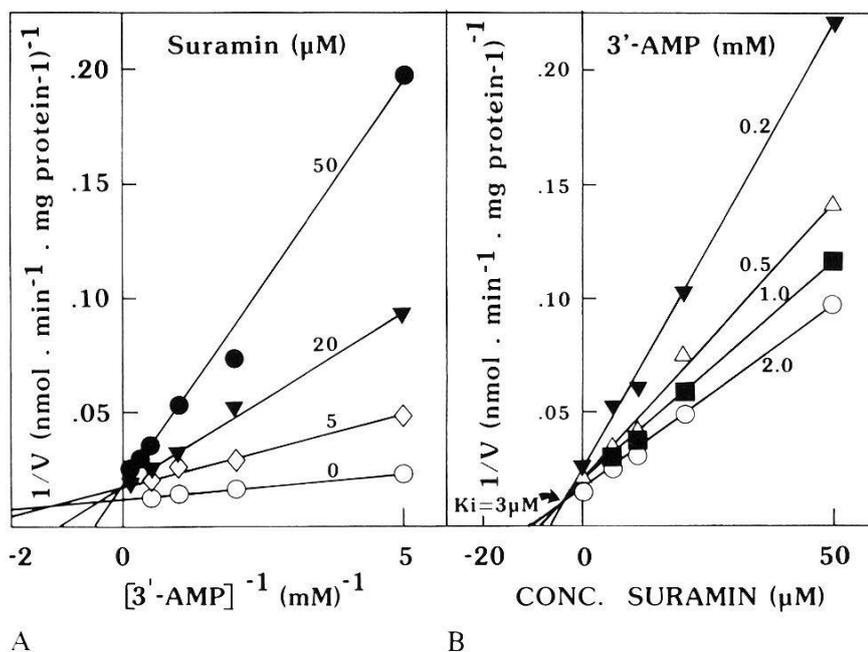


Fig. 2. A. Lineweaver-Burk plots of the dependence of 3'-nucleotidase activity on the 3'-AMP concentration at fixed concentrations of suramin. B. Dixon plots for the inhibition of 3'-nucleotidase by suramin at fixed concentrations of 3'-AMP. All data points were corrected for remaining activity after extrapolation to infinite suramin concentration and then a hyperbolic inhibition curve was fitted to the corrected points. The linearized curves were obtained by reciprocal transformation.

Alkaline endoribonuclease, as measured in the presence of 1 mM  $\text{CaCl}_2$ , also appeared to be highly sensitive to suramin. Since the effect of suramin on this enzyme was only studied at one fixed poly (A)<sup>+</sup> concentration (400  $\mu\text{g}/\text{ml}$ ), only the apparent  $K_i$  for suramin of 20  $\mu\text{M}$ , of the same order of magnitude as that for 3'-nucleotidase (10  $\mu\text{M}$ ), was determined (not shown).

*Localization of 3'-nucleotidase and alkaline ribonuclease activities.* Homogenates of *T. brucei* were first subjected to cell fractionation by differential centrifugation to roughly localize 3'-nucleotidase and total alkaline ribonuclease within the cell. Both enzymes were largely sedimentable (74 and 78%, resp.). The highest relative specific activities for 3'-nucleotidase and the total alkaline RNase were found in the microsomal fraction and their distribution resembled that of  $\alpha$ -glucosidase a marker enzyme for the plasma membrane (not shown).

Large-granule supernatant fractions (Steiger et al., 1980) from such a differential centrifugation experiment were then subjected to isopycnic sucrose gradient centrifugation. The distribution profiles of three alkaline ribonuclease activities (total,  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent), as well as those of 3'-nucleotidase and the plasma membrane marker  $\alpha$ -glucosidase were almost identical (Fig. 3). This strongly suggests a plasma-membrane localization for both the alkaline RNase activities and 3'-nucleotidase. Such a localization could unambiguously be confirmed by the occurrence of a density shift from 1.20  $\text{g}/\text{cm}^3$  to 1.15  $\text{g}/\text{cm}^3$ , which was previously shown to be typical of the plasma

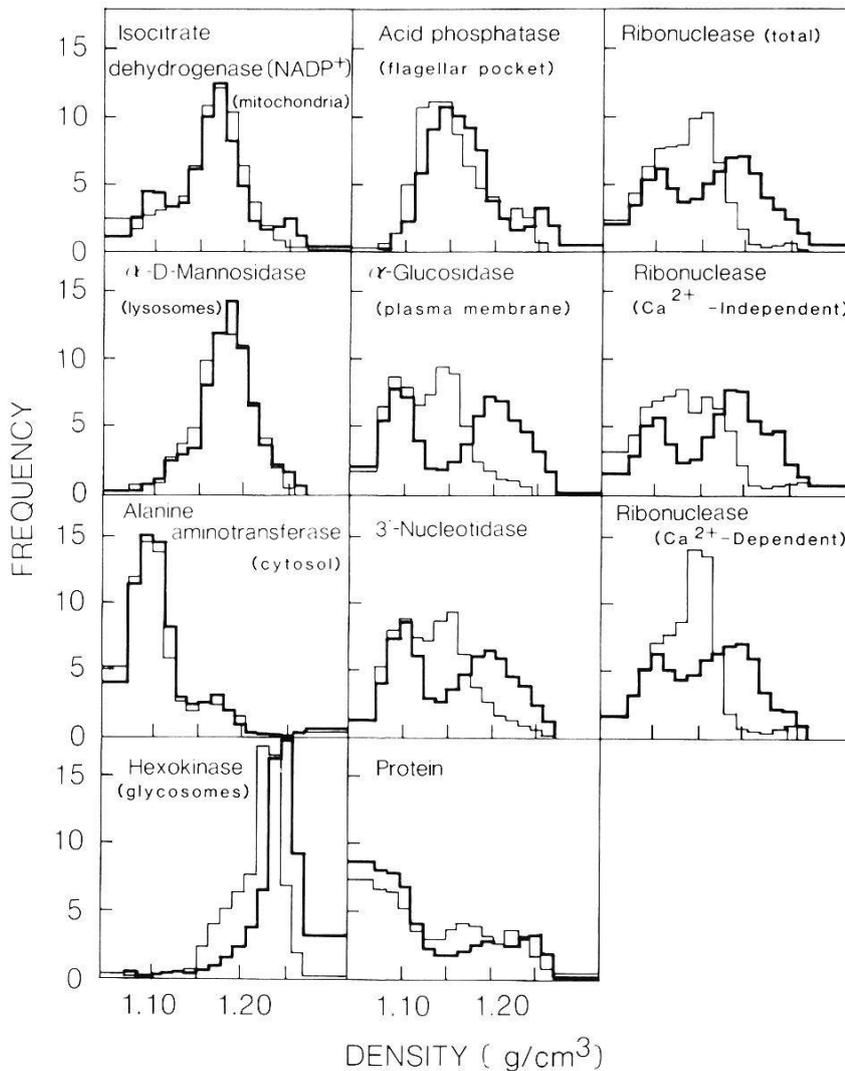


Fig. 3. Distribution profiles of enzymes and organelle markers from post-large-granule extracts of *T. brucei* homogenates after isopycnic centrifugation in linear sucrose gradients. The density scale, divided into 15 normalized fractions of identical increment extends from 1.07 to 1.27. The ordinate represents frequency,  $\Delta Q (\Sigma Q \Delta \rho)$  where  $\Delta Q$  is the amount of constituent present within a section, and  $\Sigma Q$  is the sum of the amounts found in all subfractions. The surface area of each histogram section is equivalent to the fractional amount of constituent present within each normalized fraction. Distribution profiles are delimited on either side by zones representing the density spans 1.04–1.07 and 1.27–1.32; they refer to material recovered below and above the linear gradient limits. Histograms represent mean values of two separate experiments. Thick lines represent the distribution in an imidazole-containing gradient. Thin lines that of a Tris-containing gradient.

membrane of *T. brucei* (Opperdoes and Steiger, 1981) upon changing the buffer in the homogenization and centrifugation media from 3 mM imidazole-HCl to 25 mM Tris-HCl. Such density shift was identical for all three alkaline RNase activities as well as for  $\alpha$ -glucosidase and 3'-nucleotidase (Fig. 3).

*Latency of 3'-nucleotidase and alkaline RNase.* Gottlieb and Dwyer (1983; Dwyer and Gottlieb, 1984) have reported that in *L. donovani* the 3'-nucleotidase activity is located on the outer surface of the plasma membrane. We have,

Table 2. Latency of 3'-nucleotidase in intact-cell preparations of *Trypanosoma brucei* and *Crithidia luciliae*

Organism	Percentage latency	
	3'-nucleotidase	aryl acylamidase
<i>T. brucei</i> bloodstream form . . . . .	99±3 (6)	69 (1)
<i>T. brucei</i> procyclic form . . . . .	17±3 (3)	70±7 (3)
<i>C. luciliae</i> . . . . .	14±9 (3)	98±1 (3)

Values are expressed as mean±standard deviation of the number of experiments indicated in brackets. The averaged specific activities for 3'-nucleotidase in *T. brucei* bloodstream forms, *T. brucei* procyclics and *C. luciliae* were 37, 40 and 38 nmol×min<sup>-1</sup>×(mg protein)<sup>-1</sup>, respectively. The averaged specific activities for aryl acylamidase in *T. brucei* bloodstream forms, *T. brucei* procyclics and *C. luciliae* were 8, 12 and 44 nmol×min<sup>-1</sup>×(mg protein)<sup>-1</sup>, respectively.

Table 3. Latency of alkaline RNase in intact-cell preparations of *T. brucei* bloodstream forms

Enzyme	Specific activity (nmoles nucleotides released×min <sup>-1</sup> ×mg protein <sup>-1</sup> )		Latency (%)
	Free	Total	
Total RNase . . . . .	0.92	5.4	83
Ca <sup>2+</sup> -independent RNase . . . . .	0.58	3.2	82
Ca <sup>2+</sup> -dependent RNase . . . . .	0.37	2.2	83
Alanine aminotransferase . . . . .	25.3	168.6	85

therefore, measured latency of the enzyme in intact cell preparations of *T. brucei*. Table 2 shows that in the insect Trypanosomatid *C. luciliae* and in the procyclic insect stage of *T. brucei* the enzyme was indeed highly accessible, however, this was not the case in *T. brucei* bloodstream forms. Virtually no activity could be measured with intact cells. Aryl acylamidase, a cytosolic enzyme (Opperdoes and Steiger, 1981), was used to monitor the integrity of the cells.

Latencies of the various alkaline RNases in *T. brucei* bloodstream forms were measured in a separate experiment (Table 3). Total RNase as well as Ca<sup>2+</sup>-independent and the Ca<sup>2+</sup>-dependent RNase were also inaccessible to substrate in intact cell preparations. In this experiment alanine aminotransferase was used to monitor cell integrity.

## Discussion

Our experiments demonstrate that both the alkaline ribonuclease and the 3'-nucleotidase of *T. brucei* are associated with the plasma membrane. For each of the alkaline ribonuclease activities assayed, as well as for the 3'-nucleotidase, the buoyant density increased from 1.15 to 1.20 g/cm<sup>3</sup> when the Tris buffer in linear sucrose gradients was replaced by imidazole buffer. In each case the distribution profiles and the observed density shift were similar to that of the established plasma membrane marker  $\alpha$ -glucosidase (Opperdoes and Steiger, 1981). No significant differences were observed between the distributions of the total, the Ca<sup>2+</sup>-independent, and the Ca<sup>2+</sup>-dependent RNase activities. All three displayed plasma membrane associated as well as soluble activities.

Similar cell fractionation experiments carried out on homogenates of *T. brucei* procyclic insect stages and *C. luciliae* revealed that localization of 3'-nucleotidase in these organisms was identical to that of *T. brucei* bloodstream forms (not shown). However, one important difference was that like in *C. luciliae* and *L. donovani*, the 3'-nucleotidase in procyclic insect stages of *T. brucei* was associated with the outside of the plasma membrane and freely accessible to substrate, whereas in the bloodstream form of *T. brucei* the enzyme exhibited an almost complete latency. This latency was significantly higher (99%) than that observed for the cell integrity indicator aryl acylamidase (69%). This observation suggests that in the bloodstream trypomastigote the 3'-nucleotidase is not simply attached to the inner surface of the plasma membrane but is more likely buried inside the membrane and rendered only fully accessible in the presence of detergent. Indeed we found that even in cell free extracts the 3'-nucleotidase was partly latent and sedimentable, whereas the activity was completely solubilized upon addition of Triton X-100.

Apparently the integration of proteins into the plasma membrane is subject to drastic changes imposed by the transformation of bloodstream-form to insect stage. This interpretation is supported by a previous observation with another plasma membrane enzyme  $\alpha$ -D-glucosidase. In the procyclic form this activity was associated with the cytoplasmic face of the membrane (Opperdoes and Steiger, 1981), whereas in the bloodstream trypomastigote it was found on the external face of the plasma membrane (Steiger et al., 1980). Whether the presence or absence of the surface coat glycoprotein is directly or indirectly responsible for the observed alterations in membrane architecture remains to be tested.

In view of the simplicity of the 3'-nucleotidase assay, the high specific activity of the enzyme and the fact that the activity has now been found in the plasma membrane of several Trypanosomatid species, we propose that 3'-nucleotidase may serve as an easy and universal marker for the plasma membrane of the Trypanosomatidae, provided detergent is added to the fractions before assaying them.

Gottlieb (1985) has recently presented evidence that in *C. fasciculata* 3'-nucleotidase plays an important role in the salvage of exogenous preformed purines. The enzyme would enable the organism to acquire purine nucleosides from the hydrolysis of extracellular 3'-nucleotides. Like all other Trypanosomatidae studied, *T. brucei* is not capable of de novo purine synthesis (Fish et al., 1982). All purine bases and ribonucleotides in this organism are interconverted by active salvage pathways and the order of salvage efficiency has been shown to be adenine>hypoxanthine>guanine>xanthine, where the ribonucleosides are utilized slightly more rapidly than the respective bases (Fish et al., 1982). This is in agreement with the observation (James and Born, 1980) that the rate of uptake of adenosine in *T. brucei* is much greater than that of other purine bases and nucleosides. Adenosine therefore, is probably the most important purine available for *T. brucei* for salvage in vivo. This has raised the question as to the source of the exogenous adenosine for trypanosomes in the infected host (James and Born, 1980) since the concentrations of the latter nucleoside in plasma is very low ( $10^{-8}$  to  $10^{-7}$  M). The demonstration of a 3'-nucleotidase in the plasma membrane of *T. brucei* bloodstream forms may now provide a reasonable explanation as to how this nucleoside is made available to the parasite.

Our present fractionation experiments suggest that the alkaline  $\text{Ca}^{2+}$ -dependent RNase is primarily membrane associated, while this enzyme was previously reported to be soluble (Gbenle and Akinrimisi, 1982; Gbenle, 1985). In these experiments (cf. Gbenle, 1985), however, the enzyme was extracted and purified from a 14,500×g supernatant containing still a considerable amount of membranes, while the activity associated with larger particles was not taken into account.

The role of the alkaline  $\text{Ca}^{2+}$ -dependent RNase in *T. brucei* metabolism is not yet well understood (Gbenle and Akinrimisi, 1982; Gbenle, 1985). Based on its specificity and subcellular localization three possible functions may be proposed. First, it is possible that this enzyme plays an important role in the turnover of cytoplasmic RNA as has been suggested for *E. coli* endoribonuclease and the *E. coli* 3'-exoribonuclease, RNase II (Apirion, 1973; Datta and Niyogi, 1976). Second, since the enzyme is associated with the plasma membrane it may be that, like the 3'-nucleotidase, it has a function in purine salvage, making available oligonucleotides from extracellular RNA for further degradation to nucleotides and nucleosides prior to their uptake by the cell. Third, it cannot be excluded that the  $\text{Ca}^{2+}$ -dependent endoribonuclease activity and the 3'-nucleotidase activity are catalyzed by one and the same enzyme. This is the more likely explanation since both activities 1. hydrolyze the 3'-phosphoester linkage; 2. are activated by divalent cations and inactivated by chelating agents (cf. Gottlieb and Dwyer, 1983; Gbenle and Akinrimisi, 1982); 3. have an alkaline pH optimum; 4. act on purine nucleotide residues in the following order of preference: adenyl>uracyl>guanyl and cytosyl (cf. Gottlieb and Dwyer, 1983; Gbenle and Akinrimisi, 1982); 5. act on ribonucleotide residues but have no activity on the

corresponding deoxyribonucleotides (cf. Gottlieb and Dwyer, 1983; Gbenle and Akinrimisi, 1982); 6. are inhibited by  $Zn^{2+}$ ,  $Mn^{2+}$  and pyrophosphate, but not by fluoride, tartrate and molybdate (cf. Gbenle and Akinrimisi, 1982); 7. both activities are associated with the plasma membrane but not freely accessible to substrate in *T. brucei* bloodstream forms; 8. the molecular weight of the active subunit of 3'-nucleotidase of *L. donovani* which ranges from 44,000–49,000 (Zlotnick et al., 1985), is not too dissimilar from that of the *T. brucei* 3'-endonuclease which was reported to be 50,000–52,000 (Gbenle, 1985) and 9. both activities are strongly inhibited by low concentrations of the trypanocidal drug suramin. The fact that polyamines (spermine and spermidine) and heparin do not interfere with 3'-nucleotidase activity but inhibit the  $Ca^{2+}$ -dependent RNase activity (Gbenle and Akinrimisi, 1982) does not argue against such a possible identity since the latter three compounds may bind to polynucleotides and not to mononucleotides and thus interfere with RNase activity only.

A possible identity of a 3'-nucleotidase and a 3'-ribonuclease is not unprecedented in the literature. There is ample evidence that the 3'-nucleotidase from plants which has been purified may also function as a ribonuclease. Already in 1953 Shuster and Kaplan (1953) reported that their preparations of 3'-nucleotidase completely degraded ribonucleic acid without more than 6% release of inorganic phosphate. Only 5'-nucleotides were liberated. Such findings have since been reported for an increasing number of plant nucleases (see Williamson et al., 1975 and references quoted therein).

An interesting and new observation is the fact that both 3'-nucleotidase and  $Ca^{2+}$ -dependent endoribonuclease are inhibited by low concentrations of suramin known to abolish trypanosome infectivity in vitro (Williamson et al., 1975). Whether either a direct interference of suramin with the supply of extracellular adenosine required for purine salvage, or an interference with intracellular RNA turnover, or both, are amongst the primary effect of this trypanocidal drug remains to be proven.

#### Acknowledgments

We thank Ms. Françoise Van de Calseyde-Mylle for typing the manuscript. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. G.O.G. was the recipient of a WHO travelling fellowship.

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