Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis was conducted according to Ornstein [8] and Davis [9] in 5% gels. Approximately 0.06 units of enzyme were loaded and run at 5 mA per gel. After electrophoresis the gels were either stained with Coomassie Brilliant Blue R-250 or Bio Rad silver nitrate staining kit.

Protein determination. Protein content was measured as described by Bradford [10] using bovine serum albumin as the standard.

Results and discussion

Dystrophic male mice and controls (both 6–7 weeks old) were used to prepare the crude extract, as described under 'Materials and methods'. 5 g of tissue were homogenized in 50 ml final volume of three different buffers: 1) Tris-HCl 50 mM pH 7.6. 2) Tris-HCl 50 mM, pH 7.6 containing 1 mM PMSF. 3) Tris-HCl 50 mM, pH 7.6, containing 3 M KCl. The polyacrylamide gel electrophoretic pattern of the crude extracts from dystrophic muscle did not evidence any significant difference with respect to the controls, whatever the extraction buffer, even though such evidence cannot be conclusive (not shown). The highest content of total units of ATP:NMN adenylyltransferase activity was found when the extraction was conducted in the presence of 3 M KCl (table). NAD is the substrate of specific enzymes. One of these, the ATP:NMN adenylyltransferase, is present in the crude extract of skeletal muscle of dystrophic mice and its activity is about one half of that present in the muscles of normal mice (table).

Extracts prepared in Tris buffer without additions, or in the presence of 3 M KCl, or 1 mM PMSF, show that in the muscles of normal mice an appreciable quantity of ATP is present, in agreement with the results reported by Kushmerick [11], who found 6 and 5 μmoles/g w.wt of mouse extensor digitorum longus and soleus muscles respectively. In contrast, the same nucleotide is almost undetectable in the samples extracted from dystrophic skeletal muscle (figure). This evidence is very important because the NAD level is, at least in part, the result of the reaction catalyzed by ATP:NMN adenylyltransferase, where ATP is one of the substrates. Studies on NAD metabolism and the levels of the enzymes involved in the muscles of dystrophic mice and especially in cell cultures are in progress.

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1 Caplan, A. I., and Rosenberg, M. J., Proc. natl Acad. Sci. USA *82* (1975) 1852.
2 Caplan, A. I., Niedergang, C., Okazaki, H., and Mandel, P., Devl Biol. *72* (1979) 102.
3 Rosenberg, M. J., and Caplan, A. I., Devl Biol. *38* (1974) 157.
4 Natalini, P., Ruggieri, S., Raffaelli, N., and Magni, G., Biochemistry *23* (1986) 3725.
5 Beutler, E., Red Cell Metabolism, p. 8. Grune & Stratton, New York 1975.
6 Stocchi, V., Cucchiarini, L., Magnani, M., Chiarantini, L., Palma, P., and Crescentini, G., Analyt. Biochem. *146* (1985) 118.
7 Kornberg, A., J. biol. Chem. *182* (1950) 779.
8 Ornstein, L., Ann. N. Y. Acad. Sci. *121* (1964) 321.
9 Davis, B. J., Ann. N. Y. Acad. Sci. *121* (1964) 404.
10 Bradford, M. M., Analyt. Biochem. *72* (1976) 248.
11 Kushmerick, M. J., Handbook of Physiology, chapt. 7, p. 189. American Physiol. Society, 1983.

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Disruption of mitochondrial function as the basis of the trypanocidal effect of trifluoperazine on *Trypanosoma cruzi*

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Summary. The tricyclic anti-calmodulin drug trifluoperazine (TFP) inhibited growth and motility of epimastigotes of *Trypanosoma cruzi*, at concentrations lower than 100 μM, and motility and infectivity of the bloodstream trypomastigote form at 200 μM. Electron microscopy of TFP-treated epimastigotes showed that the major effect was at the mitochondrial level, with gross swelling and disorganization. The oligomycin-sensitive, mitochondrial ATPase was completely inhibited by 20 μM TFP, and the same drug concentration caused a 60% decrease in intracellular ATP content. The results suggest that the trypanocidal effect of TFP may be related more to mitochondrial damage than to the well-known anticalmodulin effect of the drug.

Key words. Trypanosoma cruzi; trifluoperazine; mitochondrial ATPase; trypanocidal drugs.

The American trypanosomiasis, Chagas' disease, caused by the parasitic flagellate *Trypanosoma cruzi*, is a major health problem in Latin America, where there are at least 20 million people infected [1-4]. There are at present only two drugs available which are effective against the acute phase of the disease, nifurtimox and benznidazole. Both are ineffective against the chronic phase of the disease, and have a number of unpleasant side effects [1,4-6], which make it highly desirable to identify new useful drugs. In addition, there is also a need for drugs better than Gentian Violet as trypanocides which could be used to prevent transfusional transmission of the disease [1,7]. Among the 69 compounds found by Hammond et al. [7] to be active in vitro at a concentration of 1 mM or less, most were amphiphilic cationic drugs. Some of these were tricyclic drugs with anti-calmodulin properties [8,9], like chlorimipramine [7,10]. The presence of calmodulin (CaM) in epimastigotes of *T. cruzi* has recently been reported [11], as well as its inhibition by drugs such as chlorpromazine and fluphenazine, which were also able to decrease parasite motility.

We present here a study of the effects of a typical anti-CaM drug, trifluoperazine (TFP) on *T. cruzi*. The results suggest that the main effects of the drug might not be related to its anti-CaM properties, but to the disruption of mitochondrial function.

Materials and methods

Chemicals. TFP dihydrochloride, ATP, oligomycin and an ATP bioluminescence assay kit were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were analytical reagents of the highest purity available.

Source of parasites. Epimastigotes of *T. cruzi*, Tulahuén strain, Tul 0 stock, or RA strain, were grown in complex medium as previously described [12]. Culture trypomastigotes (RA strain) were obtained from infected Vero cells in culture [13]. Bloodstream trypomastigotes (Tulahuén strain, Tul 2 stock) were obtained from infected mice; the infected heparinized blood was used for the assays described below, without parasite purification.

Assay of trypanocidal effect. To test the effect of TFP on growth of epimastigotes, the drug was added (as a 1 mM solution in water sterilized by filtration) to parasite suspensions in the same culture medium (10 ml final volume) which were incubated at 28 °C in 125-ml Erlenmeyer flasks fitted with a lateral tube which allowed growth to be followed turbidimetrically, in a Lumetron colorimeter (Photovolt Company) using a red filter. Preliminary experiments showed that, since this stock of the parasite does not form rosettes to any significant extent, turbidity was proportional to cell number within the range used.

Assay of inhibition of motility. Blood from infected mice containing bloodstream trypomastigotes was incubated at 25 °C for different times and at several TFP concentrations. The number of motile parasites was assessed using a Neubauer hemocytometer. Parasite death was shown by staining with 0.1% eosin. Culture epimastigotes, or tissue culture trypomastigotes, suspended in the appropriate fresh culture medium, were incubated with different concentrations of TFP, and the time for complete immobilization of the parasites was recorded. These studies were performed using a parasite concentration of 5×10^6 ml^{-1}, since it was shown that parasite survival depended not only on drug concentration, but also on the number of parasites.

Determination of infectivity. Infectivity of the TFP-treated parasites was tested by intraperitoneal injection into 30-g Albino Swiss male mice (7.5×10^4 parasites in 0.5 ml). Survival was monitored every day, and parasitemia was checked weekly, by microscopic observation of blood obtained by cutting the tip of the tail.

Preparation and assay of oligomycin-sensitive Mg^{2+} ATPase. The epimastigotes, harvested and washed as previously described [12] were broken by grinding with glass powder (4 g per g of cells, wet weight) in a mortar. The resulting paste was suspended in 0.25 M sucrose – 5 mM KCl (5 ml per g of cells, wet weight) and the homogenate was centrifuged for 5 min at 3000 × g, at 4 °C. The pellet, containing cell debris and glass powder, was suspended in the same volume of sucrose-KCl solution, and centrifuged again. The pellet was discarded, and the combined supernatants were then successively centrifuged at 5000 × g (10 min), 11 500 × g (10 min) and 43 000 × g (30 min). The precipitates were suspended in the sucrose-KCl solution; the 43 000 × g fraction, which had the highest specific activity of the oligomycin-sensitive Mg^{2+} ATPase, was used for the experiments. The enzyme was assayed as previously described [14] in a reaction mixture containing 150 mM Tris-HCl buffer (pH 7.6), 3 mM ATP, 4 mM $MgCl_2$, 0.5 mM EGTA, and enzyme, which was preincubated for 10 min at 20 °C with or without oligomycin (20 μg · ml^{-1}). Enzyme activity is expressed as nmoles of P_i liberated min^{-1} · mg of protein^{-1}.

Determination of ATP. Suspensions of epimastigotes (2×10^6 cells ml^{-1}) were incubated for 30 min in the presence of different TFP concentrations. Soluble cell contents were extracted and ATP was determined in the supernatants using a Sigma bioluminescence assay kit, according to the manufacturer's instructions.

Electron microscopy. Epimastigotes were fixed by suspension in 2.5% glutaraldehyde/0.5% formaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at room temperature. Following a brief rinse in cacodylate buffer, the parasites were post-fixed for 20 min in 1% OsO_4 dissolved in the same buffer. After rinsing the samples with cacodylate buffer they were dehydrated in graded ethanol solutions and propylene oxide. Each step was followed by centrifugation at 3000 × g for 15 min in order

to obtain a pellet of parasites. Later, the pellets were embedded, cut and stained as previously described[15]. The sections were observed in a Phillips EM 300 electron microscope.

Results

Figure 1 shows that when TFP was added to the culture medium, growth of the epimastigotes was completely inhibited at about 50 µM. When TFP was added to the epimastigotes, a concentration of 50 µM was enough to arrest their motility completely in 12 min, in the case of the Tul 0 stock, or in about 1 h in the case of the RA strain (table 1). The cells became rounded, and, when observed under the electron microscope, gross alteration (swelling) of the kinetoplast-mitochondrion complex was evident (fig. 2b), as compared with the control (fig. 2a), without alteration of the subpellicular or flagellar microtubules (fig. 2c).

Table 2 shows that incubation of the parasites with TFP resulted in a decrease of their intracellular ATP content. The lowest concentration assayed (10 µM) already caused a 50% decrease, and 100 µM elicited a 90% decrease in ATP content.

Figure 3 shows that similar concentrations of TFP inhibited the mitochondrial, oligomycin-sensitive Mg^{2+}-ATPase obtained from epimastigotes; inhibition was complete at 20 µM, whereas the oligomycin-insensitive

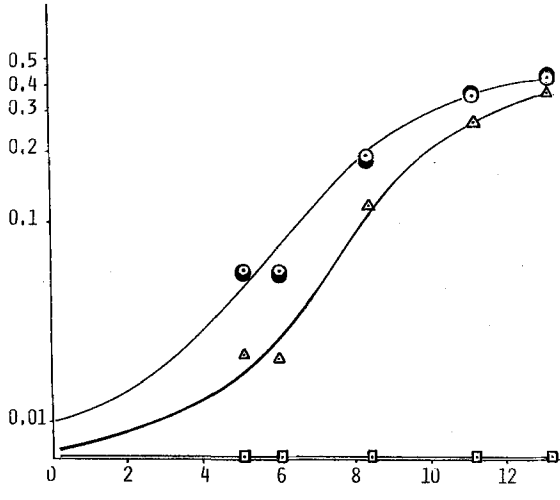

Figure 1. Effect of trifluoperazine on growth of *T. cruzi* epimastigotes in axenic culture. The experiment was performed as described under 'Materials and methods', using 0 (○), 0.5(●), 9.5 (△) or 47.5 (□) µM TFP.

Table 1. Effect of trifluoperazine on the motility of axenic culture epimastigotes and cell culture trypomastigotes of *Trypanosoma cruzi*

Concentration of TFP (µM)	0	20	50	70	100	150
	Time for complete immobilization (min)					
Epimastigotes, Tul 0 stock	> 120	18	10	8	6	5
Epimastigotes, RA strain	> 120	90	70	30	35	21
Trypomastigotes, RA strain	> 120	38	14	7	3	0.12

Parasites (5×10^6 cells ml^{-1}) were incubated at 25 °C in fresh culture medium, in multiwell plates, in the presence of the drug concentrations stated, and their motility was judged by microscopy at frequent time intervals.

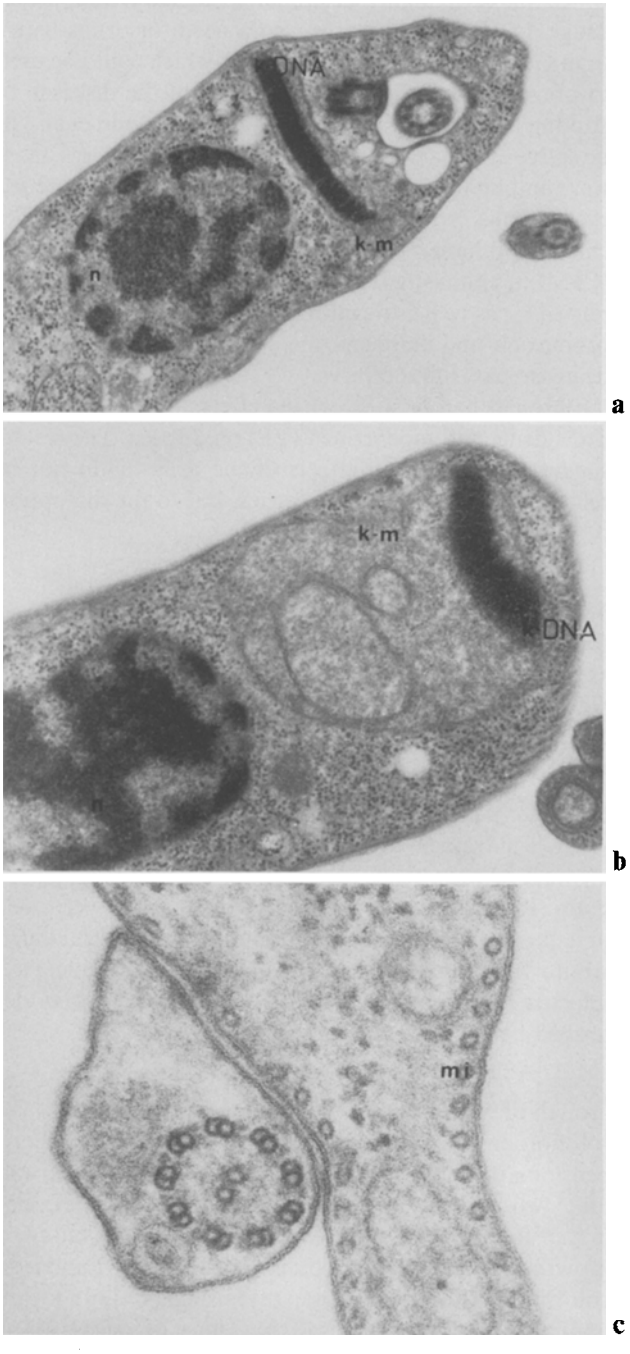

Figure 2. Effect of trifluoperazine on the ultrastructure of *T. cruzi* epimastigotes. Epimastigotes were incubated for 210 min at 28 °C in the absence (*a*) or in the presence of 67 µM TFP (*b, c*), and samples were processed for electron microscopy as described under 'Materials and methods'. Fig. 2a: ×25,200; Fig. 2b: ×26,000; Fig. 2c: ×82,200. k-m, kinetoplast-mitochondrion complex; k-DNA, kinetoplast DNA; mi, microtubules; n, nucleus.

Table 2. Effect of trifluoperazine on ATP levels of epimastigote forms of *T. cruzi*

TFP concentration (μM)	% decrease ATP level
0	0
10	50 ± 0.9
20	60 ± 2.0
30	80 ± 1.8
100	90 ± 4.7

ATP levels were determined by the bioluminescence assay as described under 'Materials and methods'. Results are the mean of six experiments per group. ATP levels in the absence of drug were 65 ± 8.1 μmoles/100 mg dry weight.

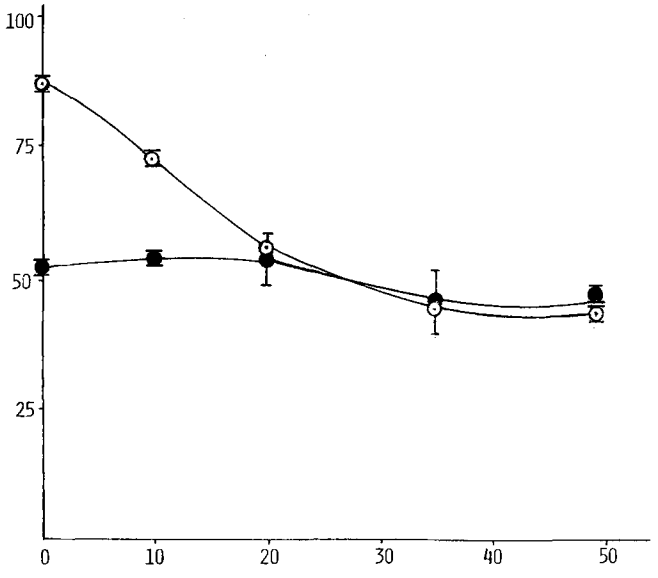

Figure 3. Effect of trifluoperazine on the oligomycin-sensitive (○) and oligomycin-insensitive (●) Mg^{2+}-ATPase activities in *T. cruzi* epimastigotes. ATPase activities were determined as described under 'Materials and methods', using 165 μM of protein (43,000 g fraction) per assay.

Mg^{2+}-ATPase was not affected up to 50 μM. A concentration of 100 μM, however, inhibited the latter enzyme completely (not shown), probably through disorganization of the mitochondrial membranes by the amphiphilic drug.

The trypanocidal effect of TFP was also evident on the trypomastigote form of the parasite. When tissue culture-derived trypomastigotes of the RA strain were incubated with the drug, a concentration of 50 μM TFP was enough to arrest their motility completely in 14 min. In the presence of 150 μM TFP, the effect was almost instantaneous, complete immobilization being attained in about 7 s (table 1). When mouse blood containing trypomastigotes was incubated for 15 min with increasing concentrations of TFP, the parasites lost motility by about 65% at 10 – 20 μM drug, and were completely immobile in the presence of 200 μM TFP. When susceptible mice were injected intraperitoneally with the latter parasites, their survival time increased from 11 – 12 days for the controls, to more than 4 months at which time the experiment was discontinued. Parasites were not observed in the blood of any of the injected mice, thus showing that TFP rendered the parasites completely non-infective.

Discussion

In good agreement with previous studies by Hammond et al.[7], TFP exhibited trypanocidal activity against *T. cruzi* in vitro. As judged by loss of motility, the drug was effective at concentrations of 20 μM and higher (table 1), although complete prevention of epimastigote growth was attained at 50 μM (fig. 1). Epimastigote motility was completely arrested by the same drug concentration in about 10 min (Tul 0 stock) or in about 70 min (RA strain); this strain dependence of the drug effect was quite reproducible in different experiments. Preliminary experiments indicated that the effect of TFP also depended on the number of parasites used, so all results compared here were obtained at the same parasite concentration. Trypomastigotes (RA strain) were considerably more sensitive than epimastigotes to TFP; thus, complete immobilization was attained in 14 min at 50 μM TFP (table 1).

These results also fit in well with those of Barioglio et al.[10] and Doyle and Weinbach[16] using chlorimipramine and derivatives, and also with those of Téllez-Iñón et al.[11], who showed that 100 μM chlorpromazine or fluphenazine inhibited epimastigote motility by 50% in 5 min. TFP was more effective than the latter drugs, and this could be related to their efficiency to inhibit calmodulin activity. In fact, the IC_{50} (concentration of drug needed to inhibit 50% of calmodulin activity) has been reported to be 17 μM for TFP, and 40 μM for chlorpromazine[8,9]. The value for chlorimipramine is close to that of chlorpromazine, 42 μM. However, the correlation between the trypanocidal effect observed for some anticalmodulins included in the study of Hammond et al.[7] and their anticalmodulin effectiveness seems to be far from complete. For instance, haloperidol, papaverine or diazepam did not evidence any trypanocidal effect at all[7], despite the fact that their IC_{50} values (65, 130 and 140 μM, respectively) are of the same order as those of chlorpromazine, chlorimipramine or imipramine (40, 42 and 125 μM, respectively), all of which are effective.

This lack of correlation suggests that the anticalmodulin activity might not be the only cause of the trypanocidal effect, and the two might indeed be completely unrelated. Our results point to a strong involvement of mitochondrial function in the killing effect of TFP. In fact, a) electron microscopy indicates the kinetoplast-mitochondrion complex as the main subcellular structure damaged by the drug; b) low concentrations of TFP decreased the intracellular ATP content, and selectively inhibited the mitochondrial oligomycin-sensitive Mg^{2+}-ATPase. There are reports in the literature showing deleterious effects of anticalmodulin drugs on liver mitochondria[17-21]; these include inhibition of the oligomycin-sensitive ATPase and decrease in ATP content[18-20], as shown for *T. cruzi* in the present work. The

mechanism of TFP effects on mitochondria from animals is still not clear, but it has been reported to be CaM-independent[17-21]. To the best of our knowledge, even the presence of CaM in the mitochondrial matrix can still be regarded as a debatable point[20,22-24].

The decrease in ATP content observed in the case of *T. cruzi* seems to consist of two phases: low concentrations of TFP (10 μM) already caused a 50% decrease, whereas higher concentrations, up to 100 μM, were required to bring the decrease to 90%. This suggests that both mitochondrial and glycosomal (glycolytic) generation of ATP are affected, to different extents. The former would be affected at the lower concentrations, which is in good agreement with the sensitivity of the ATPase. The suggested effect on glycolysis might be either direct, due to the drug inhibiting some glycolytic enzyme(s), or indirect, through membrane (glycosomal and/or plasma membrane) damage which would lead to loss of glycolytic intermediates and coenzymes to the medium, or simply to inhibition of substrate uptake. Plasma membrane disruption, including the inhibition of substrate transport into the cell, has been proposed as the mechanism of the effect of imipramine and chlorimipramine on *Leishmania* parasites[25].

Although TFP may not be useful either for patient treatment, or as an additive to blood from blood banks for the prevention of transfusional Chagas' disease, this and other[10] studies suggest the appropriateness of further trials with tricyclic drugs. Our results suggest that, in such further trials, not only the anticalmodulin effectiveness of the drugs or their amphiphilic character should be taken into account, but also their ability to disrupt mitochondrial structure and function.

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1 Pinto Dias, J. C., Enfermedad de Chagas. Epidemiologia, Clínica, Terapéutica, pp. 9–106. Programa de Salud Humana, Buenos Aires, 1984.
2 Bloom, B. R., Nature *279* (1979) 21.
3 Gutteridge, W. E., Intl J. Parasit. *17* (1987) 121.
4 Docampo, R., and Moreno, S. N. J., Rev. Biochem. Toxic. *7* (1985) 159.
5 Castro, J. A., and Díaz de Toranzo, E. G., Biomed. envir. Sci. *1* (1988) 19.
6 Boainain, E., Rev. Goiana Med. *25* (1979) 1.
7 Hammond, D. J., Cover, B., and Gutteridge, W. E., Trans. R. Soc. trop. Med. Hyg. *78* (1984) 91.
8 Prozialeck, W. C., and Weiss, B., J. Pharmac. exp. Ther. *222* (1982) 509.
9 Weiss, B., Prozialeck, W. C., and Wallace, T. L., Biochem. Pharmac. *31* (1982) 2217.
10 De Barioglio, S. R., Lacuara, J. L., and De Oliva, P. P., J. Parasit. *73* (1987) 451.
11 Téllez de Iñón, M. T., Ulloa, R., Torruella, M., and Torres, H. N., Molec. Biochem. Parasit. *17* (1985) 143.
12 Cazzulo, J. J., Franke de Cazzulo, B. M., Engel, J. C., and Cannata, J. J. B., Molec. Biochem. Parasit. *16* (1985) 329.
13 Andrews, N. W., and Colli, W., J. Protozool. *29* (1982) 264.
14 Frasch, A. C. C., Segura, E. L., Cazzulo, J. J., and Stoppani, A. O. M., Comp. Biochem. Physiol. *60B* (1978) 271.
15 Bernacchi, A. S., De Castro, C. R., Díaz de Toranzo, E. G., and Castro, J. A., Exp. molec. Path. *45* (1986) 245.
16 Doyle, P. S., and Weinbach, E. C., Exp. Parasit. *68* (1989) 230.
17 Ruben, L., Goodman, D. B. P., and Rasmussen, H., Annls N. Y. Acad. Sci. *356* (1980) 427.
18 Ruben, L., and Rasmussen, H., Biochim. biophys. Acta *637* (1981) 415.
19 Vale, M. G. P., Moreno, A. J. M., and Carvalho, A. P., Biochem. J. *214* (1983) 929.
20 Pedersen, P. L., and Hullhen, J., J. biol. Chem. *259* (1984) 15148.
21 Bodden, W. L., Palayoor, S. T., and Hait, W. N., Biochem. biophys. Res. Commun. *135* (1986) 574.
22 Gazzotti, P., Gloor, M., and Carafoli, E., Biochem. biophys. Res. Commun. *119* (1984) 343.
23 Gazzotti, P., Flura, M., and Gloor, M., Biochem. biophys. Res. Commun. *127* (1985) 358.
24 Hatase, O., Doi, A., Itano, T., Matsu, H., and Ohmura, Y., Biochem. biophys. Res. Commun. *132* (1985) 63.
25 Zilberstein, D., and Dwyer, D. M., Proc. natl Acad. Sci. USA *82* (1985) 1716.

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Flavonoids as inhibitors of rat liver cytosolic glutathione S-transferase

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Summary. The inhibitory potencies of different flavonoids for rat liver cytosolic glutathione S-transferase activity varied over 30-fold, depending on the pattern of hydroxylation, the presence of a C-2, C-3 double bond and the substitution of a hydroxyl group with a sugar moiety. Kinetic inactivation studies of the enzyme with the inhibitor quercetin revealed a non-competitive profile versus both glutathione and 1-chloro-2,4-dinitrobenzene.
Key words. Flavonoids; glutathione S-transferase; quercetin; rat liver cytosol.