suggest that differences in brain dopamine neuron number may underlie some of these strain differences in prolactin levels. Furthermore, since the incidence of mammary cancer among strains of mice is correlated to serum prolactin levels[17], it may be that brain dopamine neuron number can be related to the development of mammary cancer in some strains. In fact, BALB/cJ mice have a much lower incidence of mammary tumors than CBA/J mice[18].

In summary then, we have demonstrated that two strains of mice that differ in the number of dopamine neurons in the brain also differ in blood and pituitary prolactin levels in a manner predicted by their dopamine neuron number.

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1 Ciaranello, R.D., Barachs, R., Kessler, S., and Barchas, J.D., Life Sci. *2* (1972) 565.
2 Ross, R.A., Judd, A.B., Pickel, V.M., Joh, T.H., and Reis, D.J., Nature *264* (1976) 654.
3 Baker, H., Joh, T.H., and Reis, D.J., Proc. natl Acad. Sci. *77* (1980) 4369.
4 Baker, H., Joh, T.H., Ruggerio, D.A., and Reis, D.J., J. Neurosci. *3* (1983) 832.
5 Sved, A.F., Baker, H.A., and Reis, D.J., Brain Res. *303* (1984) 261.
6 Fink, J.S., and Reis, D.J., Brain Res. *222* (1981) 335.
7 Fink, J.S., Swerdloff, A., and Reis, D.J., Neurosci. Lett. *32* (1982) 301.
8 Greenwood, F.C., Hunter, W.M., and Glover, J.S., Biochem. J. *89* (1963) 690.
9 Sinha, Y.N., Selby, F.W., Lewis, U.J., and VanderLaan, W.P., Endocrinology *91* (1972) 1045.
10 Coyle, J.T., Biochem. Pharmac. *21* (1972) 1935.
11 Joh, T.H., Geghman, C., and Reis, D.J., Proc. natl Acad. Sci. *70* (1973) 2667.
12 Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
13 Sinha, Y.N., Salocks, C.B., and VanderLaan, W.P., Endocrinology *97* (1975) 1112.
14 MacLeod, R.M., in: Frontiers in Neuroendocrinology, vol.4, p.169. Eds L. Martini and W.F. Ganong. Raven Press, New York 1976.
15 Gudelsky, G.A., Psychoneuroendocrinology *6* (1981) 3.
16 Maurer, R.A., J. biol. Chem. *255* (1980) 8092.
17 Welsch, C.W., and Nagasawa, H., Cancer Res. *37* (1977) 951.
18 Altman, P.L., and Katz, D.D. (eds), Inbred and Genetically Defined Strains of Laboratory Animals, p.22. FASEB, Bethesda, Maryland 1979.

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Effect of tingenone, a quinonoid triterpene, on growth and macromolecule biosynthesis in *Trypanosoma cruzi*[1]

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Summary. Tingenone and horminone, two natural quinonoid substances, inhibited the in vitro growth of *Trypanosoma cruzi,* 30 μM drug concentration producing total inhibition of growth. Tingenone inhibited total uptake and incorporation of [^{3}H]thymidine, [^{3}H]uridine, L-[^{3}H]leucine into parasite macromolecules. Other quinonoids assayed were either less effective (abruquinone A) or even quite inactive (visminone B and ferruginin B). Investigation of several mechanisms for the cytotoxic action of tingenone pointed to the interaction with DNA as the most likely factor involved. Tingenone also inhibited the growth of *Crithidia fasciculata,* but the drug was significantly less active on this organism than on *T. cruzi.*
Key words. Trypanosoma cruzi; Crithidia fasciculata; tingenone; horminone.

Tingenone (maitenine; fig. 1) is a triterpene quinone-methide, isolated from various plants of the Celastracea and Hyppocrateacea families as a red-orange pigment, displaying antineoplastic activity[3-5]. The size and shape of the tingenone molecule are favorable for its inclusion within the narrow groove of DNA, and hydrogen bonds can be formed between the hydroxyl group of tingenone and the phosphate group of DNA[6]. In the present study we have investigated the action of tingenone and other natural quinonoid substances as inhibitors of growth and macromolecule biosynthesis in *Trypanosoma cruzi,* the agent of Chagas' disease. The same substances were tested on *Crithidia fasciculata,* a nonpathogenic flagellate which has been suggested for in vitro testing of potential trypanocides[7]. Studies on the mode of action of drugs on *T. cruzi* are of permanent interest because the identification of potential targets for chemotherapeutic attack may pave the way for the development of new agents for the treatment of Chagas' disease.

Materials and methods. Organism. T. cruzi (Tulahuen strain) was cultured for 3–4 days, at 28 °C, in a liquid medium composed of brain-heart infusion (Difco), 37 g; hemin, 20 mg, fetal bovine serum, 40 ml and water, to 1 l[8-10]. The cells were collected during exponential growth by centrifugation and resuspended in fresh warm medium at a concentration of 1×10^6 cells/min. Krebs-Ringer was used for resuspending the cells when uptake or incorporation of L-[^{3}H]leucine was to be determined. *Crithidia fasciculata,* anopheles strain ATCC 11745, was cultured as above. After 48 h culture, the cells were collected by centrifugation and washed with 0.154 M NaCl before the experiments.

Heart mitochondria electron transport particles were prepared as described by Turrens and Boveris[11].

Reagents. These were obtained from the following sources: [Methyl-^{3}H]thymidine (5 Ci/mmole), [5,6-^{3}H]uridine (40 Ci/mmol) and L-[4,4-^{3}H]leucine (40 Ci/mmole) (henceforth [^{3}H]thymidine, [^{3}H]uridine and L-[^{3}H]leucine, respectively) from the Radiochemical Centre, Amersham, UK; ascorbic acid, NADH, sucrose, mannitol, EDTA and antimycin, from Sigma Chemical Co, St. Louis, Mo, USA; tingenone[3], abruquinone A[12], ferruginin B[13], vismione B[14] and horminone[15] were

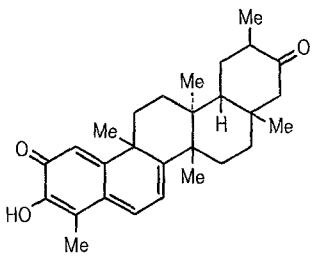

Figure 1. Tingenone: D:A-Friedo-24,30-dinoroleana-1(10),3,5,7-tetraene-2,21-dione, 3-hydroxy,-(20β)-.

isolated as described in the literature. Drugs were added dissolved in ethanol and the control samples received the corresponding volume of solvent.

Growth-inhibition experiments. *T. cruzi* cultures were grown in Erlenmeyer flasks provided with a tube directly adaptable to the photocolorimeter. The quinonoids assayed were added to the medium at the beginning of growth, at the concentration stated in each case and under sterile conditions. Cell concentration during the incubation period (4–5 days) was determined by measuring absorbance of the culture suspension in a photoelectric colorimeter fitted with a blue filter and precalibrated with epimastigote suspensions. The concentration was predetermined by cell counting in the Neubauer chamber. Absorbance and cell concentration values were linearly related[16]. Growth-inhibition experiments with *C. fasciculata* were performed as above, but taking into account the nonpathogenic nature and fast growth of this organism, the effect of tingenone was measured after 24 h culture in conventional Erlenmeyer flasks.

Precursor uptake and incorporation into macromolecules. [^{3}H]Thymidine, [^{3}H]uridine and L-[^{3}H]leucine were assayed simultaneously on the same batch of epimastigotes. Aliquots of epimastigote suspension (10^6/ml) in culture medium were given [^{3}H]labelled precursors to a final concentration of 2 μCi/ml and drugs as indicated. Samples were incubated in a New Brunswick Gyratory Water Bath, Model 45, at 37 °C, for 1, 2 or 3 h. After incubation, 1 ml duplicate samples were withdrawn and immediately filtered on 0.45 μm pore-size Metricel filters. Samples for measuring L-[3H]leucine incorporation into proteins were heated at 100 °C for 10 min before filtering, so as to eliminate *t*RNA complexes. In order to measure total precursor uptake, samples were washed on the filter, 4 times, with 8 ml ice-cold 0.9 (w/v) NaCl (Method A), while samples for measuring precursor incorporation into macromolecules were washed twice with 8 ml ice-cold 0.9% (w/v) NaCl, once with 8 ml 10% (w/v) trichloroacetic acid and once with 8 ml 5% (w/v) trichloroacetic acid (method B). Filters were dried and placed into vials containing 10 ml of scintillation solution (2.5 g 2,5-diphenyloxazole and 0.05 g 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l toluene). Radioactivity was measured in a Tracor 2000 Analytical System, working at 30–35% efficiency.

Results and discussion. Tingenone inhibits growth of *T. cruzi* in vitro. The half-maximal inhibitory concentration (I_{50}) was 12 μM, while 20 μM tingenone produced total inhibition of growth. Thus, tingenone was, in vitro, as effective as nifurtimox, but more effective than benznidazole, the two drugs most frequently used for the treatment of Chagas' disease (I_{50}, 12 and 40 μM, respectively[8,10]). Furthermore, tingenone was more effective than many alprenol and diphenylpyraline derivatives recently assayed on *T. cruzi* by Hammond et al.[17]. Table 1 allows one to compare the effect of tingenone and other natural quinonoids on parasite growth, at fixed drug concentration. The activity of the quinonoids may be classified as follows; a) highly active: tingenone and horminone; K b) less active: abruquinone A; c) inactive: vismione B and ferruginin B. In this connection it should be noted that the highly active quinonoids possess a polycyclic angular hydrocarbon skeleton; vismione B and ferruginin B are prenyleted anthranoids, whereas abruquinone A is a benzoquinone[12].

DNA, RNA and protein biosynthesis constitute suitable parameters for the assay of cytotoxic drugs, since suppression of macromolecular synthesis in vitro may predict the biological event in vivo[18–21]. DNA, RNA and protein synthesis can be monitored in living cells by measuring the incorporation of radioactive thymidine, uridine and L-leucine, respectively[18–24] and this procedure was used to investigate the mode of action of tingenone on *T. cruzi*. The results presented in figure 2 show that, in contrast to agents that prevent cell growth by selectively inhibiting DNA synthesis[21,24], tingenone also affected RNA and protein. In this connection, our observations are in good agreement with those of Angeletti and Marini-Bettolo[25] in mouse fibroblasts. The effect of tingenone in *T. cruzi* varied with drug concentration, precursor and incubation time. Thus, with 4.7 μM tingenone, [^{3}H]thymidine and L-[^{3}H]leucine incorporation was more strongly inhibited than that of [^{3}H]uridine. Furthermore, with [^{3}H]uridine and L-[^{3}H]leucine, the inhibition kinetics showed an initial lag, which was not observed with [^{3}H]thymidine. On the other hand, after 2–3 h incubation with 19 μM tingenone (a concentration near to that totally inhibiting growth), more homogeneous kinetics and greater inhibitions were observed (fig. 2).

The decreased incorporation of precursors into the acid-stable macromolecule might reflect interference with biosynthetic processes, but could also result from some other mechanism, e.g. decreased uptake into cells[22]. To elucidate this latter possibility, the effect of tingenone was simultaneously assayed on a) precursor uptake into cells and b) precursor incorporation into macromolecules. Table 2 summarizes the results of three experiments, using different epimastigote samples. It can be seen that 4.7 μM tingenone inhibited uptake and incorporation to about the same extent and, accordingly, precursor transport

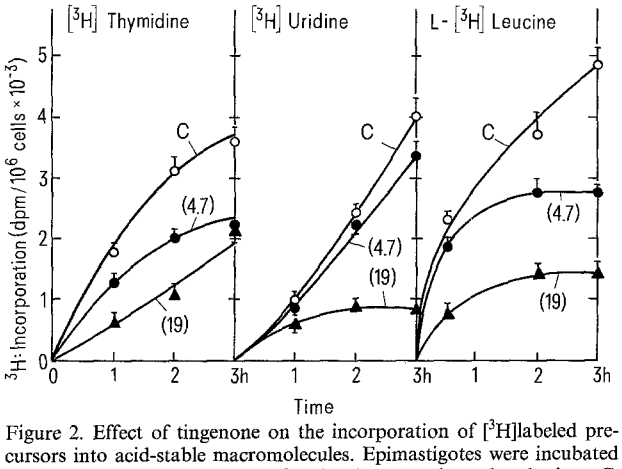

Figure 2. Effect of tingenone on the incorporation of [^{3}H]labeled precursors into acid-stable macromolecules. Epimastigotes were incubated with precursors and tingenone for the time stated on the abscissa. C, control epimastigotes; in parentheses, tingenone concentration (μM). Other experimental conditions were as described under 'materials and methods'. The points represent the mean from three experiments; the bars over the points represent SD.

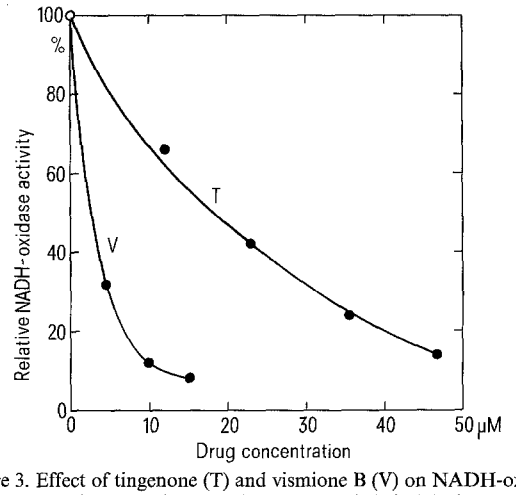

Figure 3. Effect of tingenone (T) and vismione B (V) on NADH-oxidase activity. Submitochondrial particles, 0.2 protein/ml; 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 30 mM Tris-HCl, pH 7.5, 0.5 mM NADH; temp. 30 °C. The rate of NADH oxidation was measured at 340 nm in a Gilford Model 2000 spectrophotometer. Other conditions as described under 'materials and methods'.

inhibition might be a cause of the effect of tingenone on macromolecular synthesis. Nevertheless after incubation for 3 h with 19 μM tingenone, the uptake of [^{3}H]thymidine was less inhibited than its incorporation, in good agreement with the assumption that tingenone, in addition to other possible actions, selectively inhibited DNA synthesis. Taking into account the leading role of DNA replication for cell growth and multiplication, this inhibition may be of special importance for the effect of tingenone on *T. cruzi*.

Quinone redox-cycling in *T. cruzi* (i.e. lapachones) can generate superoxide anion, which, after dismutation to hydrogen peroxide, originates the highly cytotoxic hydroxyl radical[26, 27]. Since *T. cruzi* is an organism that is deficient in protective mechanisms against oxygen radicals[28], it seemed of interest to establish whether tingenone was capable of generating such radicals. Accordingly, the drug was assayed for redox-cycling in the presence of a) ascorbate[29] and b) mitochondrial particles supplemented with antimycin[30]. In both assays tingenone was virtually ineffective (experimental results omitted). In contrast to these results, tingenone proved to be an inhibitor of mitochondrial electron transport (fig. 3), but this did not appear to contribute to the overall action of the drug on *T. cruzi*. In fact, horminone, which, as an inhibitor of electron transport, was 10 times as active as tingenone (I_{50}(μmoles/mg of protein): 2 (tingenone) and 0.2 (horminone)), did no inhibit *T. cruzi* growth (table 1).

Tingenone and abruquinone A were also effective on the growth of *C. fasciculata*. Percentage inhibition values with 15 and 30 μM drug concentration were 37 and 58% with tingenone, and 10 and 18% with abruquinone A, respectively (experimental data omitted). Comparison with the corresponding effects on *T. cruzi* (table 1) demonstrates the lesser sensitivity of *C. fasciculata* to the drugs assayed. These differences show that the cytotoxic effects on *C. fasciculata* cannot validly be extrapolated to other protozoa, as a test for the evaluation of trypanocidal drugs.

Table 1. Effect of tingenone and other natural quinonols on growth of *T. cruzi*[a]

Substance	Concentration (μM)	Inhibition of growth (%)
Tingenone	15	65
	30	100
Horminone[b]	15	75
	30	100
Abruquinone A[c]	30	77
Vismione B[d]	30	0
Ferruginin B[e]	30	0

[a] Experimental conditions were as described under 'materials and methods'. [b] 1,4-Phenanthrenedione, 4b, 5, 6, 7, 8, 8a, 9, 10-octahydro-3, 10-dihydroxy-4b, 8, 8-trimethyl-2-(1-methylethyl)-, [4bS-(4bα, 8aβ, 10β]. [c] 2, 5-Cyclohexadiene-1, 4-dione, 5-(3, 4-dihydro-6, 7-dimethoxy-2H-1-benzopyran-3-yl)-2, 3-dimethoxy-, (S)-; (6, 7, 2', 3'-methoxy-isoflavanquinone). [d] 11H-Anthra[1, 2-b]pyran-11-one, 2, 8, 9, 10-tetrahydro-9, 12-dihydroxy-5-methoxy-. [e] 1(4H)-Anthracenone-3, 8, 9-trihydroxy-6-methyl-2, 4, 4-tris(3-methyl-2-butenyl)-.

Table 2. Effect of tingenone on the total uptake of radioactive precursors into *T. cruzi* epimastigotes and on their incorporation into cellular macromolecules[a]

	Tingenone (μM)	Macromolecule precursor [^{3}H] Thymidine (%)	[^{3}H] Uridine (%)	L-[^{3}H] Leucine (%)
Uptake into cells (%)	0	100 ± 8	100 ± 7	100 ± 6
	4.7	68 ± 10	85 ± 7*	57 ± 10
	19	53 ± 8	37 ± 5	34 ± 6
Incoporation into macromolecules (%)	0	100 ± 12	100 ± 9	100 ± 9
	4.7	62 ± 5	87 ± 7*	58 ± 9
	19	37 ± 5	34 ± 6	38 ± 5

[a] The experimental conditions were as described under 'materials and methods'; incubation time: 3 h. Results represent the average values from three experiments and are expressed as percentages of control ± SD of the mean. The percentage incorporation values were 63 ± 8 ([^{3}H]thymidine), 80 ± 7 ([^{3}H])uridine) and 62 ± 6 (L-[^{3}H]leucine) of the corresponding uptake values. The Student's t-test on the figures for tingenone inhibition yielded $p < 0.05$, except when indicated by *.

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3 Delle Monache, F., Marini Bettolo, G.B., Gonçalves de Lima, O., d'Albuquerque, L.I., and de Barros Coelho, J.S., J. chem. Soc. Perkin *1* (1973) 2725.
4 Brown, P.M., Moir, M., Thomson, R.H., King, T.J., Krishnamoorthy, V., and Seshadri, T.R., J. chem. Soc. Perkin *1* (1973) 2721.
5 Melo, A.M., and Jardim, M.L., Rev. Inst. Antibiot., Recife *14* (1974).
6 Campanelli, A.R., D'Alagni, M., and Marini-Bettolo, G.B., FEBS Lett. *122* (1980) 256.
7 Shapiro, A., Hutner, S.H., Katz, L., and Bacchi, C.J., J. Protozool. *28* (1981) 370.
8 Docampo, R., and Stoppani, A.O.M., Archs Biochem. Biophys. *197* (1979) 317.
9 Docampo, R., Moreno, S.N.J., Stoppani, A.O.M., Leon, W., Cruz, F.S., Villalta, F., and Muniz, R.P.A., Biochem. Pharmac. *30* (1981) 1947.
10 Moreno, S.N.J., Docampo, R., Mason, R.P., Leon, W., and Stoppani, A.O.M., Archs Biochem. Biophys. *213* (1982) 585.
11 Turrens, J.F., and Boveris, A., Biochem. J. *191* (1980) 421.
12 Lupi, A., Delle Monache, F., Marini-Bettolo, G.B., Barros Costa, D.L., and d'Albuquerque, I.L., Gazz. chim. ital. *109* (1979) 9.
13 Marini-Bettolo, G.B., Delle Monache, F., and Marquina Mc Quahe, M., Atti Accad. naz. Lincei *65* (1978) 302.
14 Delle Monache, F., Ferrari, F., Marini-Bettolo, G.B., Maxfield, P., Cerrini, S., Fedeli, W., Gavuzzo, E., and Vaciago, A., Gazz. chim. ital. *109* (1979) 301.
15 Janot, M.M., and Poitier, P., Annls pharm. fr. *22* (1964) 387.
16 Docampo, R., Boiso, J.F., and Stoppani, A.O.M., Rev. argent. Microbiol. *5* (1974) 117.
17 Hammond, D.J., Cover, B., and Gutteridge, W.E., Trans. R. Soc. trop. Med. Hyg. *78* (1984) 91.
18 Polak, A., and Richle, R., Ann. trop. Med. Parasit. *72* (1978) 45.
19 Sims, P., and Gutteridge, W.E., Biochem. Pharmac. *27* (1978) 2815.
20 Gugliotta, H.B., Tanowitz, H.B., Wittner, M., and Soeiro, R., Exp. Parasit. *49* (1980) 216.
21 Fricker, S.P., and Klein, R.A., Biochem. Soc. Trans. *11* (1983) 366.
22 Woynarowski, J.M., and Konopa, J., Molec. Pharmac. *19* (1981) 97.
23 Chen, S.-L., Snoga, J.R., and Croft, H., Clin. Chem. *28* (1982) 1852.
24 Yamaki, H., Suzuki, H., Choi, E.C., and Tanaka, N., J. Antibiot. *35* (1982) 886.
25 Angeletti, P.U., and Marini-Bettolo, G.B., Farmaco *19* (1974) 569.
26 Boveris, A., Docampo, R., Turrens, J., and Stoppani, A.O.M., Biochem. J. *175* (1978) 431.
27 Boveris, A., Stoppani, A.O.M., Docampo, R., and Cruz, F.S., Comp. Biochem. Physiol. *61C* (1978) 327.
28 Boveris, A., Sies, H., Martino, E.E., Docampo, R., Turrens, J.F., and Stoppani, A.O.M., Biochem. J. *188* (1980) 643.
29 Pethig, R., Gascoyne, P.R.C., McLaughlin, J.A., and Szent-Gyorgyi, A., Proc. natn Acad. Sci. USA *80* (1983) 129.
30 Scarpa, M., Stevanato, R., Viglino, P., and Rigo, A., J. biol. Chem. *258* (1983) 6695.

0014-4754/85/050646-03$1.50 + 0.20/0