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.

Acknowledgments. This work was aided by grants from the Consejo Nacional de Investigaciones Cientificas Y Técnicas de la República Argentina (CONICET). JJC, JAC, SRB, and JLL are members of the Carrera del Investigador Cientifico, and BMFC of the Carrera del Personal de Apoyo a la Investigación y Desarrollo, from CONICET.

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.

0014-4754/91/060612-05$1.50 + 0.20/0

Flavonoids as inhibitors of rat liver cytosolic glutathione S-transferase

M. Merlos*, R. M. Sanchez, J. Camarasa and T. Adzet

Laboratory of Pharmacology and Pharmacognosy, School of Pharmacy, University of Barcelona, Zona Universitaria de Pedralbes, s/n, E-08028 Barcelona (Spain)
Received 4 April 1990; accepted 23 October 1990

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.

Flavonoids form a class of plant-derived phenolic substances present in the diet, which exert many potentially beneficial actions at both organ and biochemical levels[1,2]. The molecular mechanisms underlying the pharmacological effects of flavonoids are insufficiently understood, although inhibition or activation of enzymes has been demonstrated in some cases. Thus, inhibition of cyclic AMP phosphodiesterase[3,4], histidine decarboxylase[5], aldose reductase[6], ATPases[7,8] and lipoxygenases[9,10] has been reported. Similarly, some flavonoids influence enzymes involved in hepatic detoxification pathways, such as monooxygenases[11]. In contrast, the effect of flavonoids on the activity of glutathione (GSH) S-transferase has not been extensively studied. GSH S-transferase(s) (EC 2.5.1.18) are a family of cytosolic multifunctional enzymes with an important role in detoxification processes. They catalyze the conjugation of electrophilic xenobiotics with the sulphydryl moiety of GSH, thus providing less toxic and more water-soluble derivatives[12,13]. Das et al.[14] have reported that quercetin is a strong inhibitor of GSH S-transferases isolated from human tissues and rat liver. Up to now, the effect of other flavonoids on GSH S-transferases has not been studied. Hence, structure/activity relationships cannot be established. Accordingly, in this study we have evaluated the ability of different flavonoids to inhibit GSH S-transferase activity in the cytosolic fraction of rat liver, with special attention to some structural features necessary for inhibition. In addition, the kinetic inactivation of the enzyme with quercetin, the strongest inhibitor found, was examined.

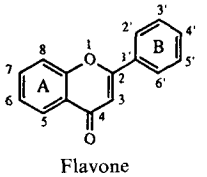
Materials and methods

Livers were obtained from male Wistar rats (180–210 g) and homogenized in 3 vols of ice-cold 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was centrifuged for 30 min at 10,000 × g. The supernatant was further centrifuged at 100,000 × g for 1 h. The resulting supernatant was used as the cytosolic fraction. The GSH S-transferase activities were assayed according to the method of Habig et al.[15]. 1-chloro-2,4-dinitrobenzene (CDNB) was used as electrophilic substrate. The final GSH and CDNB concentrations in the assay were 5 and 1 mM, respectively. Flavonoids were dissolved in DMSO just prior to carrying out the assay, because of their poor water solubility. Aliquots of these solutions were then added to the reaction mixture to yield a final flavonoid concentration ranging from 5 μM to 1 mM. The final DMSO concentration was 1.7%. In a previous study we have examined the effect of this organic solvent on the GSH S-transferase activity. Only concentrations higher than 7% DMSO produced a significant inhibition. Hence, the amount of DMSO used in the assay does not inhibit GSH S-transferase activity. All enzyme activities were measured versus 1.7% DMSO blanks to subtract the possible effect of chemical conjugation. The blanks contained the same inhibitor concentration as in the reaction mixtures but no cytosolic protein was added. The protein concentration was determined by the method of Lowry et al.[16], with bovine serum albumin as standard. The enzyme activity was expressed as μmol of CDNB-GSH conjugate produced/min/mg protein, using the extinction coefficient cited in the literature[15] ($\varepsilon_{340\,nm} = 9.6\ mM^{-1}\ cm^{-1}$). The IC_{50} values were calculated by linear regression of no less than five points in the range of 20–80% inhibition. Each point was the mean of at least three determinations.

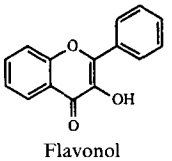
Results and discussion

The IC_{50} values and their 95% confidence limits for the different flavonoids studied in the rat liver cytosolic fraction are summarized in the table. A high degree of linear-

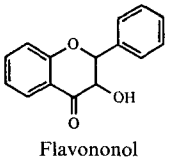
Comparison of the inhibitory potencies of different flavonoids on rat liver cytosolic GSH S-transferase activity using CDNB as substrate



Flavone



Flavonol



Flavononol

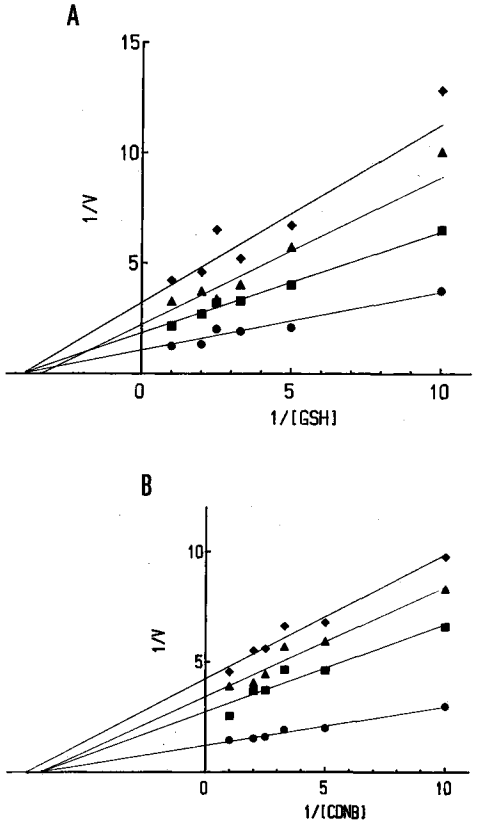
	Substituents 5	7	2′	3′	4′	IC_{50} (mM)	95% confidence limits
Flavones							
Flavone	H	H	H	H	H	0.93	(0.64–1.28)
Luteolin	OH	OH	H	OH	OH	0.042	(0.023–0.077)
7-Glucosylluteolin	OH	Glc	H	OH	OH	0.21	(0.16–0.28)
Apigenin	OH	OH	H	H	OH	39.5% inh. at 0.2 mM	
Acacetin	OH	OH	H	H	OCH_3	22.8% inh. at 0.05 mM	
Flavonols							
Quercetin	OH	OH	H	OH	OH	0.031	(0.022–0.043)
Kaempferol	OH	OH	H	H	OH	0.076	(0.047–0.120)
Morin	OH	OH	OH	H	OH	0.041	(0.038–0.045)
Flavononol							
Taxifolin	OH	OH	H	OH	OH	0.60	(0.42–0.85)

ity was found in the dose-response curves for all the compounds tested, with r^2 values ranging from 0.944 to 0.999. If flavonoids were not soluble enough to provide a concentration causing more than 50% inhibition under the assay conditions used, the inhibition of the highest concentration tested is given as a percentage. The inhibitory values vary over 30-fold, suggesting the existence of some structure-activity relationships. The flavone nucleus alone (IC_{50} about 1 mM) provides a certain degree of inhibition, but the hydroxylation pattern seems to be responsible for increased inhibition. The different hydroxylic substitutions in the tested flavonoids supplied information on the relative importance of each position. Thus, substitution of the 7-hydroxyl group of the ring A system of luteolin by a glucose moiety, to yield the glycoside 7-glucosyl-luteolin produced a 4–5-fold decrease of activity. In general, glycosides are reported to be less potent inhibitors than the corresponding aglycones in a wide range of enzymatic activities[17–19]. On the other hand, the presence of a hydroxyl group at position 3 of the benzopyrone ring, which distinguishes flavonols from flavones, seems to play a less important role in inhibition, if we compare the results for quercetin and luteolin.

More differences were found on comparing hydroxylation of the ring B system. Thus, the presence of the 3′, 4′ catechol group was a common feature of both quercetin and luteolin, the most potent flavonol and flavone, respectively. Loss of the 3′ hydroxyl group produced a decrease of activity, as shown in the results for kaempferol and apigenin. Additional 2′ hydroxylation of morin increased the potency compared with that of kaempferol, which possesses only a hydroxyl group in the position 4′ of the ring B. The importance of the 3′, 4′ diol group in the ring B has been emphasized in studies of the inactivation by flavonoids of other enzymes, such as succinoxidase[22] and arachidonate lipoxygenases e.g. plant 15-lipoxygenase[21], rat basophil 5-lipoxygenase[10] or platelet 12-lipoxygenase[22]. In contrast, hydroxylation of ring B reduces the inhibition potency when cyclo-oxygenase activity is measured[19]. Apart from the hydroxylation pattern, a double bond in C-2,C-3 appears to be a very important structural feature for GSH S-transferase inhibition. A 20-fold loss of activity was obtained with the flavononol taxifolin in comparison with its analogous flavonol quercetin. Therefore, the planarity of the benzopyrone system seems to be a requirement for inhibition. Presence of the C-2,C-3 double bond has been reported to be necessary for other flavonoid activities, such as phosphodiesterase[3] and glyoxalase I[23] inhibition. In general, the order of potency found in GSH S-transferase inhibition of flavonoid subtypes was flavonols > flavones > flavononols.

The kinetic inactivation studies of the enzyme with the inhibitor quercetin at different GSH and CDNB concentrations were performed to understand the mechanism of inhibition of the enzyme by flavonoids. When a fixed CDNB concentration (1 mM) was incubated with different concentrations of GSH (0.1–1 mM) in the presence or absence of quercetin, a typical non-competitive Lineweaver-Burk plot was obtained (fig. A), with similar K_m values (between 0.27 and 0.31 mM) and lower V_{max} values at increasing quercetin concentration. The apparent K_i value with respect to GSH was $7.5 \pm 1.4 \times 10^{-5}$ M. When the assay was performed at a fixed GSH concentration (5 mM) and the CDNB concentration varied from 0.1 to 1 mM, a similar kinetic profile was obtained (fig. B). In this case, the apparent K_i value with respect to CDNB was $6.0 \pm 1.3 \times 10^{-5}$ M. Thus it can be concluded that quercetin acts as a non-competitive inhibitor for the nucleophilic substrate GSH and the electrophilic CDNB.

In fact, quercetin does not act as a substrate of GSH S-transferase, as we found in previous studies monitoring GSH disappearance by the Ellman method (data not shown). Non-competitive inhibition has been reported for several flavonoid activities, e.g. taxifolin is known to behave as a non-competitive inhibitor of biphenyl-4-hydroxylase, biphenyl-2-hydroxylase and aminopyrine N-demethylase[24].



Inhibitory effect of quercetin on cytosolic GSH S-transferase activity at variable concentrations of GSH and fixed (1 mM) concentration of CDNB (panel A) and at variable concentrations of CDNB and fixed (5 mM) concentration of GSH (panel B). Reciprocal velocity (1/V) in (μmol of CDNB-GSH conjugate/min/mg cytosolic protein)$^{-1}$ versus reciprocal substrate concentration in mM^{-1} without (●) or with increasing quercetin concentrations, 0.05 (■), 0.08 (▲) and 0.12 (◆) mM.

The flavonoids are usually present in the diet in relatively large amounts, which might be sufficient to achieve pharmacologically significant concentrations in tissues[2]. Thus, flavonoids could interfere with GSH S-transferase in vivo. Nevertheless, discrepances between in vitro and in vivo results have been described for some chemicals, such as propylthiouracil[25] or ellagic acid[26], which are in vitro inhibitors but in vivo activators of GSH S-transferase. Therefore the relevance of our results with respect to the influence of dietary flavonoids on GSH metabolism remains to be ascertained.

In conclusion, we have shown that flavonoids are potent in vitro inhibitors of GSH S-transferase and some structural features necessary for inhibition have been described. Further studies about the in vivo effect of several flavonoids on GSH metabolism are now in progress.

* To whom correspondence should be addressed.

1 Havsteen, B., Biochem. Pharmac. *32* (1983) 1141.
2 Middleton, E. Jr, Trends pharmac. Sci. *5* (1984) 335.
3 Ferrell, J. E. Jr, Chang Sing, P. D. G., Loew, G., King, R., Mansour, J. M., and Mansour, T. E., Molec. Pharmac. *16* (1979) 556.
4 Beretz, A., Stierle, A., Anton, R., and Cazenave, J. P., Biochem. Pharmac. *31* (1982) 3597.
5 Middleton, E. Jr, and Drzewiecki, G., Biochem. Pharmac. *31* (1982) 1449.
6 Varma, S. D., and Kinoshita, J. H., Biochem. Pharmac. *25* (1976) 2505.
7 Kuriki, Y., and Racker, E., Biochemistry *15* (1976) 4951.
8 Fewtrell, C. M. S., and Gomperts, B. D., Nature *265* (1977) 635.
9 Corvazier, E., and Maclouf, J., Biochim. biophys. Acta *835* (1985) 315.
10 Yoshimoto, T., Furukawa, M., Yamamoto, S., Horie, T., and Watanabe-Kohno, S., Biochem. biophys. Res. Commun. *116* (1983) 612.
11 Beyeler, S., Testa, B., and Perrisoud, D., Arzneim. Forsch. (Drug Res.) *33* (1983) 564.
12 Bears, A. J., and Breirien, D. D., Ann. Biol. clin. *38* (1980) 49.
13 Kaplowitz, N., Am. J. Physiol. *239* (1980) G439.
14 Das, M., Bickers, D. R., and Mukhtar, H., Biochem. biophys. Res. Commun. *120* (1984) 427.
15 Habig, W. H., Pabst, M. J., and Jakoby, W. B., J. biol. Chem. *249* (1974) 7130.
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. S., J. biol. Chem. *193* (1951) 265.
17 Beretz, A., Cazenave, J. P., and Anton, R., Agents Actions *12* (1982) 382.
18 Gryglewski, R. J., Korbut, R., Robak, J., and Swies, J., Biochem. Pharmac. *36* (1987) 317.
19 Moroney, M. A., Alcaraz, M. J., Forder, R. A., Carey, F., and Hoult, R. S., J. Pharm. Pharmac. *40* (1988) 787.
20 Hodnick, W. F., Kung, F. S., Roettger, W. J., Bohmont, C. W., and Pardini, R. S., Biochem. Pharmac. *35* (1986) 2345.
21 Wurm, G., Baumann, J., and Geres, U., Dt. Apoth. Z. *122* (1982) 2062.
22 Welton, A. F., Tobias, L. D., Friedler-Nagy, C., Anderson, W., Hope, W., Meyers, K., and Coffey, J. W., Prog. clin. biol. Res. *213* (1986) 231.
23 Klopman, G., and Dimoyuga, M. L., Molec. Pharmac. *34* (1988) 218.
24 Beyeler, S., Testa, B., and Perrissoud, D., Biochem. Pharmac. *37* (1988) 1971.
25 Kariya, K., Lee, E., and Takamura, Y., Xenobiotica *14* (1984) 349.
26 Das, M., Bickers, D. R., and Mukhtar, H., Carcinogenesis *6* (1985) 1409.

0014-4754/91/060616-04$1.50 + 0.20/0

Long-term depressor effects of catecholamine neuronal grafts in the third ventricle of the brain in normotensive rats

R. Hashimoto and F. Kimura

Department of Physiology II, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236 (Japan)
Received 9 July 1990; accepted 30 October 1990

Summary. Neuronal tissue containing A-6 group noradrenalin (NA) neurons of the locus ceruleus, or A-10 group dopamine (DA) neurons of the substantia nigra, was grafted into the third ventricle at the level of the preoptic-anterior hypothalamic region, in normotensive male rats. A significant and long-lasting depressor effect was shown in rats with either graft. In rats with an NA neuron-rich graft, plasma concentrations of arginine-vasopressin (AVP), plasma renin activity (PRA), and corticosterone (CS) decreased significantly, whereas in rats with a DA neuron-rich graft, AVP and PRA concentrations also decreased significantly but CS showed no significant change. Neither NA nor adrenalin in plasma changed significantly in rats with either graft.
Key words. Blood pressure; catecholamine; brain grafting; central cardiovascular regulation; rat.

With the help of anatomical and morphological studies to map the localization of catecholaminergic neurons and their receptors in the central nervous system (CNS)[1,2], physiology and pharmacology have accumulated evidence supporting the involvement of the CNS in cardiovascular regulation[3-11].

The anterior hypothalamic area (AHA) including the anteroventral third ventricle (AV3V) region[3-6,8], in cooperation with the nucleus of the solitary tract (NTS)[5,6], participates in body fluid and cardiovascular regulation mechanisms, mainly mediated by catecholaminergic neurons[5-11]. We have already reported that a long-term