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EFFECT OF QUERCETIN ON MEMBRANE-LINKED ACTIVITIES*

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Abstract—The flavonoid quercetin inhibited the Mg^{++}-dependent, Na^+ and K^+ stimulated ATPase.† The effect was dependent on the concentration of Na^+ and K^+. Quercetin, like other known inhibitors which act on both transport ATPase and oxidative phosphorylation, inhibited the mitochondrial ATPase activity. Mitochondrial electron transport was also affected. Sulfhydryl compounds partially reversed the effect of quercetin on ATPase activities and completely eliminated that on electron transport. It is concluded that quercetin shows affinity for membrane-dependent cellular activities.

QUERCETIN, like other flavonoid drugs, is known to inhibit *in vitro* a number of enzymes, presumably through interaction with sulfhydryl and amino groups of proteins.[1] Recently[2] it was found that the positive inotropic activity of quercetin, described earlier,[3] was scarcely affected by reserpine-induced catecholamines depletion or by beta-blocking agents. This result stimulated investigations to study whether the cardiac effect is associated with modification of magnesium-dependent, sodium and potassium-activated ATPase. It will be shown that quercetin, similarly to oligomycin,[4–6] ethacrynic acid[7–9] and tri-*n*-butyltin,[10, 11] inhibits the transport ATPase as well as the mitochondrial ATPase, activities which are dependent on the structural organization of natural membranes. Quercetin inhibits also the mitochondrial electron transport, an effect which was completely reversed by sulfhydryl compounds.

MATERIALS AND METHODS

Quercetin (E. Merck, Darmstadt) was added to the incubation mixture as ethanolic solution. The same amount of ethanol did not affect the reactions under investigation. Oligomycin (a mixture of oligomycins A and B, assumed mol. wt. 410) was kindly supplied by the Upjohn Co. (Kalamazoo, U.S.A.) ATP and NADH were purchased from Sigma; DTT (A grade) from Calbiochem; TTB from Eastman Organic Chemicals. All other reagents were commercial preparations.

Na^+ and K^+-stimulated ATPase was prepared from calf-heart according to Matsui and Schwartz.[11] The enzyme preparation was used at the stage of first DOC treatment. The specific activity in the presence of magnesium alone was 10–12 μmoles ATP split/hr/mg protein at 37°. Upon addition of K^+ and Na^+ it increased to 20–25

* A brief summary of these results was presented at the first joint meeting between the Italian and British Pharmacological Societies, Florence, Sept. 11–13th, 1968. *Br. J. Pharmac.* **34**, 673P (1968).

† *Abbreviations*: ATPase, adenosintriphosphatase; DTT, dithiothreitol (threo-1,4-dimercapto-2,3-butandiol); TTB,4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione; DOC, sodium deoxycholate; DNP, 2,4-dinitrophenol.

μmoles ATP split/hr/mg protein at 37°. Ouabain inhibited the increase by Na^+ and K^+. Mitochondrial contamination was tested by measuring the succinate oxidase and succinate dehydrogenase activities; the specific activity did not exceed in both cases 0·03 μmoles succinate oxidized/min/mg protein at 25°.

Mitochondria were isolated according to Hogeboom[12] from livers of male albino rats which had been starved 12 hr. The mitochondria were washed twice and then resuspended in 0·25 M sucrose to a concentration of about 7·0 mg protein per ml. Keilin–Hartree preparation was obtained from beef-heart as described by King[13] with this exception: 9 min homogenization in the cold, using a Waring Blendor, substituted for mortar and sand.

ATPase assay. Mg^{++}-dependent, Na^+ and K^+-stimulated ATPase was tested in 100 mM NaCl, 20 mM KCl, 2·5 mM $MgCl_2$, 1·0 mM ATP-Tris, pH 7·4, 50 mM Tris-HCl, pH 7·4,0·5–0·1 mg enzyme proteins. Final volume, 2·0 ml; incubation in air 20 min at 37°. Mitochondrial ATPase was tested in a final volume of 2·0 ml containing 80 mM KCl, 45 mM Tris-HCl, pH 7·4, 25 mM sucrose, 2·0 mM ATP-Tris, ph 7·4, 0·6–0·8 mg mitochondrial proteins. Incubation in air, 15 min at 30°. The reactions were stopped by the addition of trichloroacetic acid (final concentration, 6–7%).

Succinate or NADH oxidase assay. The conventional Warburg technique was used. Each vessel contained in a final volume of 2·8 ml, 100 mM potassium phosphate buffer, pH 7·4, 500 μg cytochrome *c* (Sigma, from beef heart, Type IV), 40 mM succinate (or 1·8 mM NADH), 1·6 mg Keilin–Hartree preparation proteins. 0·2 ml of 1·8 M KOH on filter paper was placed in the centre well; incubation in air, 20 min with succinate or 5–10 min with NADH. Temperature, 30°.

Mitochondrial ATP–Pi exchange was measured as described previously,[14] succinate dehydrogenase activity according to Ells.[15]

Analytical procedures. Inorganic phosphate was determined by the method of Fiske and SubbaRow.[16] Protein by the biuret method[17] (mitochondria) or by the method of Lowry *et al.*[18] (Keilin–Hartree and calf-heart ATPase preparations). In both cases crystalline serum albumin served as standard. Particulate protein were dissolved by 0·3% (w/v) DOC.

RESULTS

Effect of quercetin on transport ATPase

In Fig. 1 it is shown the effect of quercetin on the calf-heart ATPase preparation in the presence and absence of Na^+ plus K^+. In both cases quercetin strongly inhibited the hydrolysis of ATP even though the concentration needed for 50 per cent inhibition was slightly different: 0·05 mM in the presence and 0·08 mM in the absence of sodium plus potassium, at level of 0·05 mg of enzyme protein. Increasing the protein concentration, the inhibitory effect decreased, moreover the inhibition by quercetin was also relieved by the addition of bovine serum albumin. These results were interpreted as indication of an aspecific binding of quercetin to the proteins of the system.

In Table 1 it is seen that quercetin can actually inhibit the stimulation of ATPase by Na^+ and K^+ and this effect is dependent on the concentration of cations.

The data of experiments 1 and 2 represent the stimulation after addition of sodium and potassium and were obtained by subtracting the values exhibited with addition of Mg^{++} alone to the whole activity. The inhibition by quercetin was enhanced by increasing Na^+ as well as decreasing K^+ concentrations.

In agreement with Matsui and Schwartz,[11] treatment of the enzyme preparation with sodium iodide drastically reduced the ouabain-insensitive ATPase. The residual activity, completely dependent on the addition of Na^+ and K^+, was fully sensitive to quercetin and again the inhibition was enhanced by increasing Na^+ concentration (experiment 3). The inhibition by quercetin was not changed by varying the concentrations of Mg^{++} or ATP.

Interestingly enough, the influence of Na^+ and K^+ on the effect of quercetin resembled that produced on the inhibition by cardiac glycosides.[19]

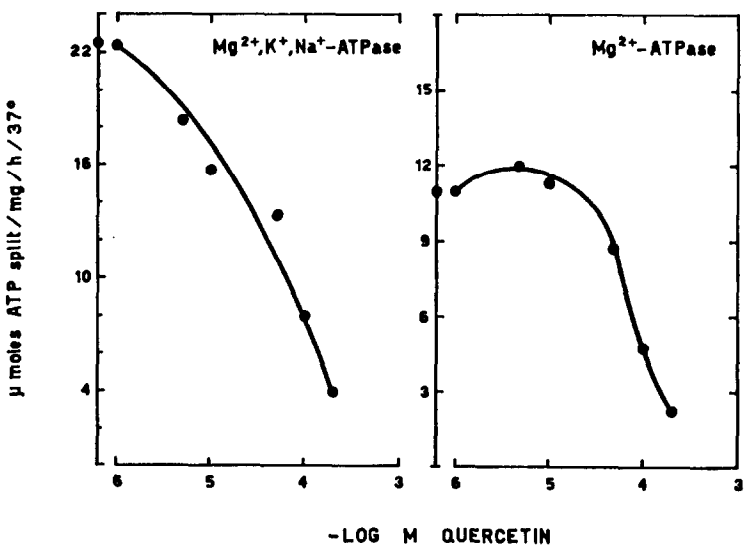

FIG. 1. Effect of quercetin on a calf-heart ATPase preparation in the presence and absence of Na^+ and K^+. Each tube contained in a final volume of 2·0 ml 2·5 mM $MgCl_2$, 1·0 mM ATP-Tris, pH 7·4, 50 mM Tris-HCl, pH 7·4, 0·05 mg enzyme proteins and, where indicated, 100 mM NaCl, 20 mM KCl. 20 min at 37°.

TABLE 1. EFFECT OF QUERCETIN ON Na^+, K^+-STIMULATED ATPASE

Exp. No.	Enzyme	Na^+ (mM)	K^+ (mM)	μmoles ATP split/mg/hr/37° without quercetin	μmoles ATP split/mg/hr/37° 0·1 mM quercetin	inhib. %
1	DOC-enzyme	100	20	12·5	8·0	36·0
		300	20	13·0	5·0	61·5
		500	20	10·2	2·7	73·5
2	DOC-enzyme	100	1	8·0	2·5	68·8
		100	20	14·0	8·0	42·9
3	DOC-NaI-enzyme	5	20	4·0	2·7	32·5
		100	20	10·8	4·8	55·6
		300	20	11·8	3·6	69·5

DOC, sodium deoxycholate. Treatment of enzyme with NaI performed as described by Matsui and Schwartz.[11] Experimental conditions as in Fig. 1. The concentrations of Na^+ and K^+ were those indicated. The values are the increment of ATPase produced by addition of Na^+ and K^+.

Effect of quercetin on mitochondrial-ATPase activity

The inhibition by quercetin of Na^+ and K^+ independent ATPase prompted an investigation to study whether other ATPase activities were influenced. In Table 2 it is shown the effect of quercetin on the mitochondrial ATPase stimulated under various conditions. Although less active than the well known inhibitor oligomycin,[4] quercetin inhibited mitochondrial ATPase in any circumstances. Oligomycin and quercetin were different in respect to the unstimulated mitochondrial ATPase which was

TABLE 2. EFFECT OF QUERCETIN AND OLIGOMYCIN ON MITOCHONDRIAL ATPASE ACTIVITY

Additions	μmoles ATP split/mg protein/30°		
	No inhibitors	0·2 mM quercetin	2 μM oligomycin
—	0·36	0·54	0·12
2 mM Mg^{++}, 1 mM DOC	2·55	0·66	0·36
0·1 mM DNP	3·25	1·94	0·30
5 mM Ca^{++}	1·57	0·49	0·24
0·05 μg Valinomycin*	3·12	0·49	0·36

* We are indebted to Dr. B. C. Pressman for the generous gift of valinomycin.
DOC, sodium deoxycholate; DNP, 2,4-dinitrophenol. Each tube contained in a final volume of 2·0 ml, 80 mM KCl, 45 mM Tris-HCl, pH 7·4, 25 mM sucrose, 2·0 mM-ATP-Tris, ph 7·4, 0·8 mg mitochondrial protein. 15 min at 30°.

inhibited by the former and slightly stimulated by the latter. The inhibition of mitochondrial ATPase activity by quercetin indicated that other partial reactions of oxidative phosphorylation could be affected by the drug.

Accordingly, mitochondrial ATP-Pi exchange was found sensitive to quercetin (50 per cent inhibition with 0·2 mM quercetin at level of 4·5 mg mitochondrial protein).

Effect of quercetin on mitochondrial electron transport

Figure 2 shows that quercetin, similarly to known inhibitors of mitochondrial electron transport, strongly affected the succinate oxidase as well as the NADH oxidase activities of Keilin–Hartree preparation. These results are in agreement with the findings of Bartlett[20] on 2'3,4-trihydroxychalcone and with previous report[2] on the effect of quercetin on intact mitochondria.

Relationship between quercetin and sulfhydryl compounds

Sulfhydryl compounds, DTT and cystein, differently influenced the effect of quercetin on the ATPase activities and electron transport. Table 3 shows that 1 mM DTT had limited antagonistic effect on the inhibition of transport ATPase by quercetin, whereas the restoration was of higher extent for the mitochondrial DNP-stimulated ATPase. In both cases cystein had little and not reproducible effect (not shown in the Table). With mitochondrial electron transport, low amount of cystein were sufficient to completely reverse quercetin inhibition. The other inhibitors tested were unaffected by cystein or DTT.

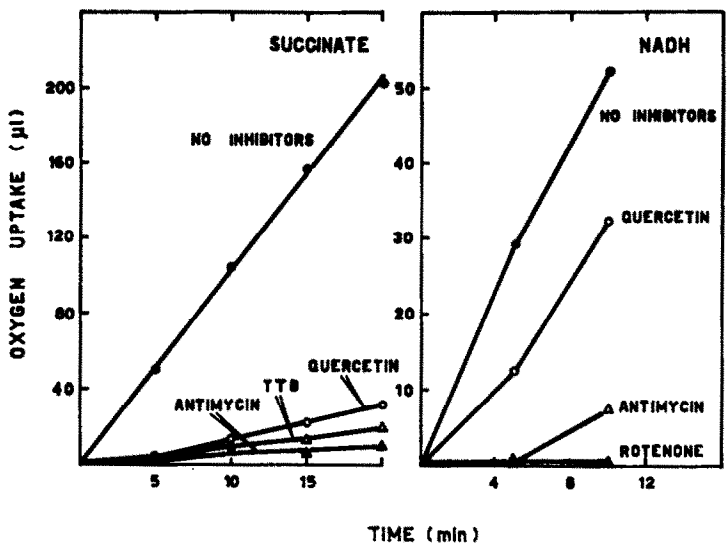

FIG. 2. Effect of inhibitors on the oxidation of succinate or NADH by a Keilin–Hartree preparation. Each tube contained in a final volume of 2·8 ml 100 mM potassium phosphate buffer, pH 7·4, 500 µg cytochrome *c*, 40 mM succinate or 1·8 mM NADH and 1·6 mg Keilin–Hartree preparation proteins, Temperature 30°. 0·2 mM quercetin; 0·1 mM TTB; 1 µg antimycin; 0·013 mM rotenone.

TABLE 3. EFFECT OF SULFHYDRYL COMPOUNDS ON THE INHIBITION OF ATPASE AND ELECTRON TRANSPORT BY QUERCETIN

System	Sulfhydryl compounds	% inhibition by		
		0·2 mM quercetin	2 µM oligomycin	0·1 mM TTB
Mg^{++}, Na^{+}, K^{+} ATPase	—	83·2		
	1 mM DTT	70·6		
Mitochondrial DNP-stimulated ATPase	—	68·0	86·6	
	1 mM DTT	29·5	84·2	
Succinate oxidation	—	83·7		88·7
	1 mM cystein	0·0		87·0
NADH oxidation	—	55·0		
	1 mM cystein	7·0		

DTT, dithiothreitol; DNP, 2,4-dinitrophenol. Experimental conditions as in Figs. 1 and 2 and Table 2.

DISCUSSION

Transport ATPase, oligomycin-sensitive mitochondrial ATPase and electron transport are activities dependent upon a definite structural arrangement of biological membranes. Resolution on these multienzyme systems in the individual components results either in loss of the overall activity or of some important properties, which, only under well controlled conditions, can be regained after recombination.[21, 22]

Low amounts of quercetin (0·5–2·0 µmoles/mg protein) are able to inhibit membrane-linked activities and this indicates the remarkable affinity of the drug for membrane-bound or membrane-constituent enzymes.

The broad spectrum of activity is a demonstration that the effect of quercetin is

rather aspecific. Interaction with sulfhydryl groups is involved, even though the extent of inactivation is apparently different in the various cases. The partial reversal of the inhibition of both ATPase activities by sulfhydryl compounds in contrast to the complete restoration of succinate and NADH oxidation, indicates that in the former case other mechanisms are to be invoked to explain the effect of quercetin. Although not supported by the available evidence, the chelating activity of quercetin could play a role.

The interaction between quercetin and enzyme systems localized in membranes could be relevant for the effect of the drug on isolated organs.[2]

Like in the case of other drugs,[23–25] the positive inotropic activity of quercetin is associated with inhibition of transport ATPase. In this context it might be of interest to note that the concentration of quercetin required to stimulate the activity of isolated guinea-pig atria (0·15 mM, Ref. 2) is in the same order of that active on transport ATPase. Moreover a structural analog of quercetin, morin, devoid of cardiostimulant activity, was found also to be a very poor inhibitor of ATPase activity. However, the absence of data on the influence of quercetin on the sodium and potassium movements in the intact myocardium does not allow conclusions on this point.

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