Preferential Inhibition by Quercetin of Mitogen-Stimulated Thymocyte Glucose Transport [1, 2]

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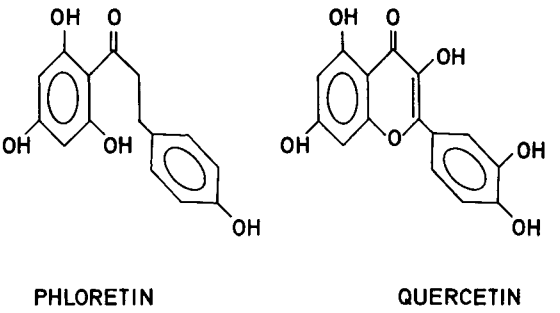
ABSTRACT—The ATPase inhibitor quercetin, which inhibits tumor glycolysis, was shown to be a glucose transport inhibitor like the chemically related compound phloretin. Rat thymocyte glucose transport stimulated by the mitogens concanavalin A or ionophore A 23187 was more sensitive than unstimulated transport to quercetin inhibition. The partial inhibition of Na^+-, K^+-ATPase activity by quercetin observed in tumor cells was confirmed in thymocyte plasma membranes. The specific Na^+-, K^+-ATPase inhibitor ouabain did not mimic the effect of quercetin on mitogen-stimulated glucose transport but did reduce the effectiveness of concanavalin A as a stimulator of mitochondrial pyruvate oxidation. The results support the idea that glycolytic flux and the activity of plasma membrane ATPase are related but suggest that glucose transport, rather than the Na^+-, K^+-ATPase, is the rate-limiting reaction in lymphocytes.—JNCI 62: 1243–1246, 1979.

A relatively consistent characteristic of malignantly transformed mammalian cells is an elevated rate of aerobic glycolysis (*1*). Studies on glucose transport across the plasma membrane of tumor cells indicate that this reaction is stimulated during malignant transformation (*2, 3*). It has been proposed that stimulated glycolysis in tumor cells is a direct consequence of glucose carrier activation (*3*). A contrasting view has been put forward (*4, 5*) suggesting that aerobic glycolysis in tumor cells is regulated by the regeneration of ADP and P_i, a function accomplished primarily by the Na^+-, K^+-ATPase of the plasma membrane. Racker (*4*) suggested that this enzyme is decreased in efficiency in tumor cells to the extent that there is an excess of ATPase activity over that required for ion pumping. In support of his proposal Racker (*4*) investigated the effect of the ATPase inhibitor quercetin, which apparently restores the efficiency of the Na^+-, K^+-pump (by specifically inhibiting the excess ATPase activity without affecting ion pumping). This compound and its derivatives inhibit tumor glycolysis and tumor growth. Racker (*4*) proposed using quercetin derivatives as antitumor agents, because he believes that these inhibitors should not prove to be toxic to nonmalignant cells.

A major weakness of Racker's proposal is that enhanced aerobic glycolysis is not a unique function of malignantly transformed cells but is observed in a number of nonmalignantly transformed, rapidly proliferating cells [e.g., mitogen-stimulated lymphocytes (*6, 7*)]. Glucose transport is also considered to be the rate-limiting reaction that is stimulated in proliferating lymphocytes (*8*). We demonstrated previously that mitogen-stimulated glucose transport in thymocytes is substantially more sensitive than unstimulated transport to the effect of competitive inhibitors (*9*). Quercetin is closely related to phloretin (*see* text-fig. 1), a competitive inhibitor of glucose transport in erythrocytes (*10*)



TEXT-FIGURE 1.—Structural similarity between quercetin and phloretin.

and thymocytes (*9*). In addition, it has been suggested that quercetin specifically inhibits Con A-stimulated Ca^{2+} uptake in rat mast cells (*11*). Ca^{2+} has been implicated in the control of thymocyte glucose transport (*8, 12*). In this paper we show that quercetin is a glucose transport inhibitor that, like phloretin, preferentially inhibits both Con A-stimulated and ionophore A 23187-stimulated glucose transport in thymocytes. This inhibition is not mimicked by the specific inhibitor of the Na^+-, K^+-ATPase, ouabain. In addition to pointing out the nonspecificity of quercetin action on tumor glycolysis, these observations suggest that quercetin does not act in lymphocytes through inhibition of Ca^{2+} transport or the Na^+-, K^+-ATPase.

MATERIALS AND METHODS

Assays of 3-*O*-*methyl*-D-[U-^{14}C]glucose transport (*8, 9*) and [U-^{14}C]glucose metabolism in rat lymphoid tissue (*13*) were detailed. Incubations for the transport assay were carried out in Eppendorf tubes in a volume of 0.25 ml of cells (10^8 rat thymus lymphocytes/ml) in phosphate-buffered saline. The assay was started by addition of 3-*O*-methylglucose (1 μCi·C^{14} 7 mM final). Samples (100 μl) were removed after 10 seconds and 1 minute 10 seconds into a stop mixture (50 μl of 3 mM

ABBREVIATIONS USED: Con A = concanavalin A; cpm = counts per minute; F.R.G. = Federal Republic of Germany.

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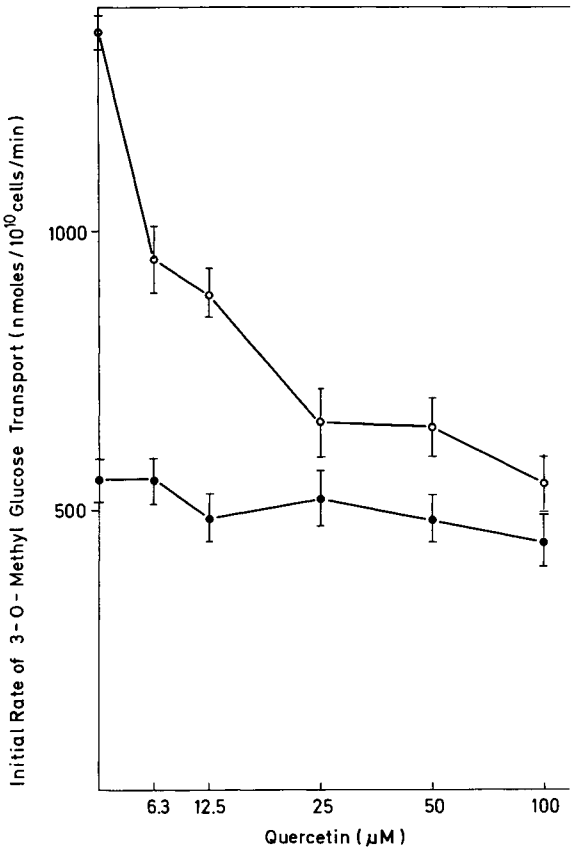
phloretin). The cells were separated from the radioactive supernatant by centrifugation through a bovine serum albumin cushion and assayed for radioactivity (*8*). 3-*O*-methylglucose transport is linear during this period (*8*).

Total Mg^{2+} ATPase activity in purified calf thymocyte plasma membranes (*14*) was assayed by a modification of the technique of Crane and Lipmann (*15*). As observed by Dornand et al. (*16*), ouabain-sensitive ATPase activity was detectable only after the membranes were treated with deoxycholate. The optimum noninhibitory concentration of Na deoxycholate was 0.032% (wt/vol).

The reaction mixture for the ATPase assay contained 100 mM imidazole buffer (pH 7.4), 5 mM $MgCl_2$, 30 mM KCl, 150 mM NaCl, 0.6 mM ATP (0.67 μCi γ-[^{32}P]ATP/μmole), and 10–40 μg membrane protein in a total volume of 1.0 ml. After the mixture was incubated for 10–30 minutes at 37° C, 2 mg of bovine serum albumin was added to facilitate membrane protein precipitation; the reaction was then stopped by addition of 4 ml of trichloroacetic acid (5% wt/vol, 4° C). The extract was centrifuged, and 3 ml of supernatant was adsorbed on approximately 2 g of activated charcoal (Norit A; Serva, Heidelberg, F.R.G.). After centrifugation, 1 ml of extract was mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard Tricarb liquid scintillation counter. All radiochemicals were obtained from the Radiochemical Centre (Amersham, Buckinghamshire, England); phosphate-buffered saline for all cell incubations was from Serva; Con A was from Pharmacia Chemicals (Uppsala, Sweden); A 23187 was a gift from Eli Lilly & Co. (Indianapolis, Ind.); and quercetin and ouabain were from Sigma Chemical Co. (München, F.R.G.).



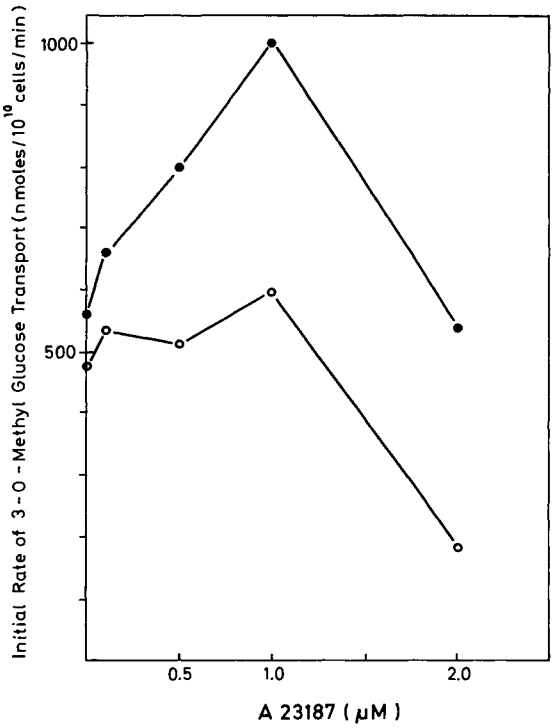
TEXT-FIGURE 2.—Inhibition of Con A-stimulated 3-*O*-methylglucose transport by quercetin. Transport was measured as described in the text. Quercetin (in dimethyl sulfoxide) was added to the cells 1 min prior to Con A. Prior to being assayed, the cells were incubated for a further 30 min. The appropriate amount of dimethyl sulfoxide was added to each control. *Each point* is the average of three expts±SE. Each expt was performed in triplicate. ●, control; ○, addition of Con A (25 μg/ml).

RESULTS

Text-figure 2 shows the effect of varying the concentration of quercetin on thymocyte 3-*O*-methylglucose transport in the presence and absence of Con A at its optimally stimulatory concentration (25 μg/ml). Low concentrations of the inhibitor preferentially inhibited Con A-stimulated transport without affecting the control rate. In the presence of 25 μM quercetin the response to Con A was practically abolished, whereas at higher concentrations the control and Con A-stimulated transport rates decreased in parallel. This pattern of inhibition was also observed with the glucose transport inhibitors phloretin and cytochalasin B (*9*); the concentration dependence of the quercetin response was very similar to that observed with phloretin, which had an apparent inhibitor constant (K_i) of 9.0 μM in control thymocytes and 5.1 μM in Con A-treated thymocytes (*9*).

Text-figure 3 shows the effect of varying the concentration of ionophore A 23187 on thymocyte 3-*O*-methylglucose transport in the presence and absence of optimally inhibitory concentrations of quercetin. This experiment was prompted by the finding of Fewtrell and Gomperts (*11*) that quercetin does not block ionophore action in mast cells but interacts chemically with the ionophore molecule itself. As observed with other mitogens, the dose response to A 23187 in thymocytes exhibited an optimum, above which glucose carrier activity returned to the control value. Quercetin preferentially inhibited ionophore-stimulated 3-*O*-methylglucose transport. In addition, the combination of superoptimal ionophore concentration plus quercetin substantially inhibited transport to rates well below the control value.

To investigate further the possibility that the action of quercetin involves an inhibition of the Na^+-, K^+-ATPase, we looked at the direct effect of this compound on Na^+-, K^+-ATPase activity in isolated calf thymus plasma membranes. Neither quercetin nor ouabain (a specific inhibitor of the Na^+-, K^+-ATPase) inhibited total ATPase activity of isolated plasma membranes (data not shown). If the membranes were first treated with deoxycholate (*16*), an ouabain-sensitive ATPase activity was exposed (table 1) that constituted approximately 40% of the total ATPase activity. Ouabain-sensitive ATPase activity was inhibited approximately 50% by 25 μM quercetin. The inhibitory effects of ouabain and quercetin were nonadditive.



TEXT-FIGURE 3.—Inhibition of ionophore A 23187-stimulated 3-*O*-methylglucose transport by quercetin. Transport was measured as described in the text. Quercetin (in dimethyl sulfoxide) was added to the cells 1 min prior to the addition of A 23187. Prior to being assayed, the cells were incubated for a further 30 min. Dimethyl sulfoxide was added to appropriate controls. *Each point* is the average of three expts performed in triplicate. ●, control; ○, addition of 25 μM quercetin.

This observation was consistent with the increase in Na^+-, K^+-pump efficiency observed in tumor cells treated with quercetin (*5*).

Elbrink and Bihler (*17*) suggested that there may be a direct link between Na^+-, K^+-ATPase activity and glucose transport. We therefore tested the effect of ouabain on 3-*O*-methylglucose transport in thymocytes and observed a small but nonspecific inhibition of both control and Con A-stimulated transport (result not shown). We were prompted to investigate further the effects of ouabain by the observation that this compound is an inhibitor of mitogen-induced lymphocyte proliferation (*18*). Table 2 shows the effect of ouabain on the metabolism of [U-^{14}C]glucose by thymocytes. Ouabain caused a nonspecific decrease of 30% in glucose uptake and lactate production in both control and mitogen-treated cells.

TABLE 1.—*Effects of ouabain and quercetin on ATPase activity in deoxycholate-treated isolated thymocyte plasma membrane*[a]

Additions	μmoles ^{32}P released/mg protein/min[b]
None	68.45±3.87
Ouabain (1 mM)	38.34±2.33
Quercetin (25 μM)	54.61±2.84
Ouabain+quercetin	37.04±1.95

[a] For details of the method, *see* text.
[b] Results are from a typical expt. *Each figure* is the mean±SD of eight parallel determinations.

TABLE 2.—*Effect of ouabain on* [U-^{14}C]*glucose metabolism by Con A-stimulated thymocytes*[a]

Metabolite	Control	Addition of:		
		Con A	Ouabain	Ouabain+ Con A
O_2 consumption	408±12	443±14	396±12	442±9
CO_2 production	410±21	449±26	367±24	394±13
$^{14}CO_2$ production (cpm×10^{-3})	48.7±5.2	78.4±7.3	43.0±3.0	56.9±4.1
Specific activity of $^{14}CO_2$ (cpm/μmole×10^{-3})	3.38±0.38	5.0±0.35	3.35±0.36	4.00±0.23
Glucose uptake	82.3±9.1	149.2±6.6	50.8±5.7	119.6±11.0
Lactate production	37.9±8.5	153.8±11.7	24.8±8.3	125.6±10.4

[a] Cells (4×10^8 in 3 ml) were incubated in Warburg's flasks at 37° C for 3 hr. Glucose was present at a concentration of 5 mM (6.6×10^4 cpm/μmole). Con A concentration was 50 μg/ml; ouabain, 1 mM. Results are presented as μmoles/10^{10} cells/3-hr incubation ±standard error of the mean (four determinations). The pattern of the results was identical in each expt.

This effect contrasts with the preferential inhibition of Con A-stimulated glucose uptake by quercetin and eliminates the possibility that quercetin acts, in this system, through its effects on the Na^+-, K^+-ATPase. Nevertheless, ouabain did specifically inhibit Con A action at the level of the mitochondria. In addition to its effect on glycolysis, Con A causes a specific increase in pyruvate oxidation, as judged by an increase in the production and specific activity of $^{14}CO_2$ [*see* (*19*)]. This response to Con A was substantially inhibited by ouabain (table 2).

DISCUSSION

Our results suggest that quercetin, like phloretin, is a direct inhibitor of glucose transport and that this is a plausible explanation for its effects on tumors. Thus we support the proposal (*3*) that glucose transport, rather than the Na^+-, K^+-ATPase, is flux generating for tumor glycolysis. However, the proposal that a link exists between the glycolytic rate and the activity of the plasma membrane ATPases (*4*) is not disproved by this study. A 30% inhibition of total ATPase activity induced by ouabain (table 1) does apparently correlate with a 30% decrease in the glycolytic rate (table 2) presumably as a result of the observed inhibition of glucose transport. Coordination of glucose transport and ATPase activities may have regulatory significance in restricting net production of ATP by glycolysis. The absence of a specific effect of ouabain on Con A action demonstrates that the Na^+-, K^+-ATPase stimulation observed in transformed lymphocytes (*18*) is not the direct cause of stimulated glucose transport. Two possibilities remain: *a*) The ATPase and glucose transport activities are controlled independently or *b*) the ATPase activity is controlled directly or indirectly by the rate of glucose transport (*8*). Other work has

suggested that the plasma membrane Ca^{2+}- and Mg^{2+}-stimulated ATPase is also stimulated by Con A during transformation (*16*). This may be relevant to the involvement of Ca^{2+} in the regulation of thymocyte glucose transport (*8, 12*).

The ability of ouabain to interfere specifically with Con A action at the level of the mitochondrion is difficult to explain. We (*19*) presented evidence that Con A stimulates pyruvate oxidation through a Ca^{2+}-dependent activation of mitochondrial pyruvate dehydrogenase. Ouabain, by collapsing the plasma membrane gradient for Na^+ and K^+, may alter the ionic milieu of the cell in such a way that mitochondrial Ca^{2+} uptake is impaired.

The effect of quercetin on Con A-stimulated glucose transport confirms our previous finding that mitogenically stimulated thymocytes are more sensitive to inhibitors of glucose transport than are untreated cells. This finding has now been extended to cells stimulated with the chemically distinct mitogen, ionophore A 23187. Thus quercetin does not seem to cause a specific inhibition of Con A action alone (i.e., a lack of effect on the A 23187 response), as has been observed in the case of the stimulation of histamine release from rat mast cells (*11*). We have suggested that the reason mitogen-stimulated transport is more sensitive to competitive inhibitors is that the active carrier exists in a dimerized form (*9*). It is thus interesting that carrier activity in the presence of superoptimal concentrations of A 23187 is even more sensitive to quercetin (text-fig. 2). Freeze-etch electron microscopy studies have shown that superoptimal concentrations of A 23187 cause a progressive increase in the aggregation of intermembrane particles (integral membrane proteins) in the thymocyte plasma membrane (Bauer HC, Speth V, Ferber E: Unpublished observations). Higher polymers of the glucose carrier may be relatively inactive but still more sensitive to the presence of competitive inhibitors.

ADDENDUM

It has recently been demonstrated that quercetin is an inhibitor of glucose transport in human fibroblasts (*20*).

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