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INHIBITION OF GLUCOSE TRANSPORT BY BENZOQUINONE AND THE ADDITION PRODUCT OF BENZOQUINONE AND DITHIOTHREITOL

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Hydroxylated benzene derivatives inhibited transport of D-glucose into calf-thymocyte plasma-membrane vesicles. The relative effectiveness of these was pyrogallol > hydroquinone ≃ catechol ≫ phloroglucinol. The most thoroughly studied of these agents, hydroquinone, produced weak, immediate inhibition when first added to membranes ($K_i > 10$ mM). This was followed by a gradual, time-dependent inhibition of the residual transport activity. The instantaneous inhibition could not be prevented by any agent tested, whereas the time-dependent phase was affected by reducing agents and superoxide dismutase. Several reducing agents (dithiothreitol, glutathione, NADH, ascorbate, bisulfite but not cysteine) prevented, while superoxide dismutase and cysteine potentiated time-dependent inhibition when added to the membrane suspension simultaneously with hydroquinone. NADH and ascorbate also prevented, whereas dithiothreitol potentiated, further time-dependent inhibition when added to membranes 2 h after hydroquinone. In contrast, all three reducing agents arrested time-dependent inhibition when added 2 h after pyrogallol. Numerous agents had no effect on time-dependent hydroquinone inhibition: oxidants (H_2O_2), metal chelators (EDTA, bathophenanthroline disulfonate, Desferral), radical scavengers (benzoate, ethanol), anti-oxidants (butylated hydroxytoluene) and catalase. Benzoquinone, an oxidation product of hydroquinone, was a much more potent inhibitor ($K_i \simeq 1$ mM) than hydroquinone. Several reducing agents (ascorbate, NADH, bisulfite) prevented this effect, while cysteine and dithiothreitol potentiated it. Below 300 μM, benzoquinone had little or no effect on sugar transport with or without glutathione or cysteine. Addition of dithiothreitol to benzoquinone (10–300 μM) resulted in potent inhibition of sugar transport ($K_i \simeq 50$ μM). Maximal inhibition occurred with a 1 : 1 mol ratio of these agents or with excess dithiothreitol. The inhibitory agent from benzoquinone and dithiothreitol lost potency in the presence of air and membranes, but was stable for hours in the presence of either of these alone. DL-*threo*-1,4-bis(2,5-dihydroxyphenylthio)-2,3-butanediol was obtained from the reaction of equimolar quantities of dithiothreitol and benzoquinone in ethanol. The structure of this adduct was established by spectroscopic and chemical methods. This compound exhibited all of the properties of the inhibitor which had been formed from benzoquinone and dithiothreitol in aqueous solution.

Introduction

In a continuing effort to develop methods to identify the glucose transporter of thymocytes, we have attempted to discover agents which affect the rate of sugar transport into isolated plasma-membrane vesicles. When these studies were initiated, there was reason to suspect that glucose transport regulation might involve alteration of the redox state of an essential sulfhydryl group in the membrane [1]. Investigations in our laboratory have confirmed that thiols, sulfhydryl reagents and oxidants stimulate glu-

cose transport in intact rat thymocytes [2]. In contrast to the stimulatory effects of the above redox agents (onset period of 1–2 min), stimulation by other agents (Concanavalin A, A-23187, arsenate, croton oil) required periods of 30 min to 1 h. These observations suggested that effects of redox agents on sugar transport involved one of the final events in transport regulation.

In this study, numerous oxidizing and reducing agents were tested for their ability to affect glucose transport in membrane vesicles, among them several which might interact with membrane sulfhydryls. Of these, quinonoid compounds and quinone-thiol addition products exerted rather strong effects, the significance of which is yet to be determined.

Materials and Methods

Chemicals. The sources of the materials used in this study were: pyrogallol (1,2,3-trihydroxybenzene), hydroquinone (1,4-dihydroxybenzene), catechol (1,2-dihydroxybenzene), phloroglucinol (1,3,5-trihydroxybenzene), DL-dithiothreitol, glutathione (reduced form), NADH (Grade III, disodium salt), benzoic acid (sodium salt), butylated hydroxytoluene, 5-thio-D-glucose, β-D-thioglucose and bovine serum albumin from Sigma Chemical Co.; sodium metabisulfite, ethylene diamine tetraacetate, H_2O_2 (30%), D-glucose, tris(hydroxymethyl)aminomethane and *p*-benzoquinone from Fisher Scientific Co.; L-ascorbic acid from Eastman Organic Chemicals; bathophenanthroline disulfonic acid (disodium salt) and dithioerythritol from Aldrich, and Desferral from CIBA Pharmaceutical Co.

Enzymes. Bovine blood superoxide dismutase (Type I) and bovine liver catalase were purchased from Sigma Chemical Co.

Synthesis. (1) Compound I. To 1.39 g (9.0 mmol) of dithiothreitol in 10 ml absolute ethanol, 0.97 g 9.0 mmol) of benzoquinone in 50 ml absolute ethanol was added dropwise with stirring. The solvent was removed with a rotary evaporator at 55–60°C, and the oily residue was redissolved in methanol (1–2 ml). After two recrystallizations from chloroform-methanol, the product formed a white powder, m.p. 91.5–93°C, which was extremely soluble in ethanol and slightly soluble in water: IR (KBr) 3390 and 3300 cm^{-1} (OH) and 1590, 1485 and 1450 cm^{-1} (aromatic); ^{1}H-NMR (acetone-d_6) δ 3.00 (M, CH_2S), 3.73 (M, CHOH), 3.00 (M, CHOH), 6.76 (D × D, J = 8.7 and 0.9 Hz, H-3), 6.64 (D × D, J = 8.7 and 2.4 Hz, H-4), 6.91 (D × D, J = 2.4 and 0.9 Hz, H-6); ^{13}C-NMR (acetone-d_6) δ 39.0 (CH_2S), 71.9 (CHOH), 116.7 (C-3), 116.9 (C-4), 120.4 (C-6), 121.8 (C-1), 150.8 and 151.2 (C-2 and C-5). Elemental analysis was obtained on a sample, m.p. 101–102°C (softening 97°C) which had been dried in vacuo for two days at room temperature. Anal.: Calcd. for $C_{16}H_{18}O_6S_2 \cdot H_2O$: C, 49.46; H, 5.18; S, 16.50. Found: C, 49.36; H, 5.40; S, 16.91.

(2) Compound II. To 0.97 g (9.0 mmol) of benzoquinone in 50 ml absolute ethanol, 0.14 g (0.9 mmol) of dithiothreitol in 5 ml absolute ethanol was added dropwise with stirring. The solution was cooled in ice for 30 min, and the red product was collected by filtration and washed with ethanol. The powder was redissolved in glacial acetic acid, filtered hot and, after addition of water, allowed to crystallize, m.p. 211–213°C. The compound was soluble in acetone, dimethyl sulfoxide and glacial acetic acid: IR (KBr) 3470 (OH), 1660 (C = O), 1640 cm^{-1} (C = C); ^{1}H-NMR (dimethyl sulfoxide-d_6) δ 3.01 (M, CH_2S), 3.72 (M, CHOH), 5.42 (broad S, CHOH), 6.62 (D, J = 2 Hz, H-6), 6.77 (D × D, J = 10 and 2 Hz, H-4), 6.93 (D, J = 10 Hz, H-3); ^{13}C-NMR (dimethyl sulfoxide-d_6) δ 32.7 (CH_2S), 70.4 (CHOH), 125.0 (C-6), 136.2 (C-3), 137.3 (C-4), 151.9 (C-1), 183.9 (C-2 and C-5). The analytical sample m.p. 214.5–215.5°C, was prepared by drying this material in vacuo at 58°C for 24 h. Anal.: Calcd. for $C_{16}H_{14}O_6S_2$: C, 52.44; H, 3.85; S, 17.50. Found C, 52.19; H, 4.06; S, 17.38.

Preparation of plasma membrane for transport experiments. Plasma-membrane vesicles were isolated from frozen calf thymus as previously described [3]. Transport activity was preserved by freezing the purified membrane (2 mg protein/ml) in 10 mM Tris-HCl (pH 7.35) containing either 100 mM sucrose or 15% (v/v) glycerol [4]. To prepare membrane vesicles for transport experiments, thawed membrane (1 ml) was diluted with 10–20 volumes of 150 mM NaCl, 10 mM Tris-HCl, pH 7.35 (buffer) and contrifuged at 30000 × g_{av} for 45 min (2°C). The membrane was then resuspended in 10–20 vol. of buffer and centrifuged as described above. The final pellet was resuspended in buffer to a final protein concentration of 2 mg/ml.

Assay of D-glucose transport. Unless specified otherwise, the standard assay for sugar transport consisted of the following. Membrane vesicles in buffer were added to glass tubes, and the appropriate reagent(s) in buffer added so that the total incubation volume would be 70–300 μl, and the final membrane concentration would be approx. 1 μg protein/μl. Tubes were then capped, and incubated at 37°C for times indicated in the tables. At the appropriate time, samples (30 μl) were transferred to a second tube, cooled to 20°C, and specific D-glucose transport determined as the D-[^{14}C]glucose space in excess of the L-[^{3}H]glucose space [3]. In most instances, uptake was terminated at 0.2 min, the equilibration half-time of the D-glucose-specific space of control vesicles. In some instances, however, the incubations were terminated at 15 min, a time when the D-glucose-specific space of control vesicles was completely equilibrated with D-glucose but not significantly equilibrated with L-glucose. Since variable rates of transport were obtained with the four different membrane preparations used in this study, all values in the tables below have been normalized as the percent of the appropriate control. The 0.2-min specific D-glucose space of control vesicles ranged between 0.5 and 1.5 μl/mg protein and the 15-min specific D-glucose space of control vesicles ranged between 0.9 and 3.0 μl/mg protein. Unless otherwise indicated, values in paretheses represent the number of repetitions of that condition on different days. On a given day, each variable was tested in duplicate or triplicate. The reproducibility of the system can be perhaps be appreciated in a comparison among the six sets of experiments in Table II with 10 mM hydroquinone.

Other methods. Protein was estimated (5) using bovine albumin as the standard. Nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX-90Q pulsed spectrometer operating at 89.55 and 22.5 MHz for ^{1}H and ^{13}C, respectively. ^{13}C-NMR assignments were supported by multiplicities and coupling constants observed in undecoupled spectra. Infrared spectra were obtained with KBr pellets using a Perkin-Elmer 727 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Results

Effects of oxidants and reductants on D-glucose transport

To investigate the redox state of the plasma membrane as a potential regulatory mechanism for sugar transport, we tested several thiols, oxidants and anti-oxidants for their ability to affect D-glucose entry into plasma-membrane vesicles. Of these, only the anti-oxidants were with effect, all being inhibitory. The relative effectiveness of these agents (3–10 mM) was pyrogallol > hydroquinone ≃ catechol ≫ phloroglucinol. Other agents tested which were without effect were: H_2O_2 (10 mM), dithiothreitol (1–3

TABLE I

EFFECTS OF HYDROQUINONE AND PYROGALLOL ON D-GLUCOSE TRANSPORT

Hydroquinone (1,4-dihydroxybenzene) and pyrogallol (1,2,3-trihydroxybenzene) were tested for their effects on 0.2- and 15-min specific D-glucose transport at the concentrations and times shown at the head of each column.

Agents	Specific D-glucose transport (% of control)				
	0.2-min uptake				15-min uptake
	0 min	10 min	60 min	120 min	120 min
Hydroquinone					
1 mM	–	97 (1)	92 (1)	101 (3)	99 (2)
3 mM	–	84 (2)	84 (2)	78 (4)	92 (2)
10 mM	63 (7)	67 (15)	57 (20)	38 (27)	75 (22)
Pyrogallol					
3 mM	–	61 (2)	29 (2)	13 (2)	67 (2)
10 mM	–	29 (2)	3 (2)	0 (2)	31 (2)

TABLE II

EFFECTS OF REDUCING AGENTS AND SUPEROXIDE DISMUTASE ON TIME-DEPENDENT SUGAR TRANSPORT INHIBITION BY HYDROQUINONE

D-Glucose transport was assayed during 0.2-min incubations after treatment of membranes with the agents for the times indicated. In Expt. 1, hydroquinone and the other agent were added simultaneously. In Expt. 2, the reducing agent was added 2 h after the addition of hydroquinone.

Agents	Specific D-glucose transport (% of control)		
Experiment 1	10 min	60 min	120 min
Hydroquinone 10 mM	63 (3)	54 (4)	34 (4)
+1 mM dithiothreitol	60 (3)	66 (3)	54 (3)
+2 mM dithiothreitol	58 (1)	–	61 (1)
+3 mM dithiothreitol	56 (1)	–	71 (1)
Hydroquinone 10 mM	65 (4)	53 (4)	35 (4)
+1 mM glutathione	76 (4)	81 (4)	79 (4)
Hydroquinone 10 mM	65 (2)	52 (2)	34 (3)
+1 mM cysteine	66 (2)	69 (2)	3 (3)
Hydroquinone 10 mM	58 (1)	–	39 (1)
+3 mM NADH	62 (1)	–	64 (1)
Hydroquinone 10 mM	64 (3)	60 (2)	28 (3)
+1 mM ascorbate	70 (2)	72 (1)	62 (2)
+10 mM ascorbate	66 (3)	68 (2)	69 (3)
+1 mM $Na_2S_2O_5$	67 (2)	70 (1)	72 (2)
+10 mM $Na_2S_2O_5$	60 (3)	67 (2)	64 (3)
Hydroquinone 10 mM	68 (5)	61 (5)	35 (5)
+50 μg/ml superoxide dismutase	64 (2)	44 (2)	19 (2)
+100 μg/ml superoxide dismutase	58 (5)	37 (5)	16 (5)
Experiment 2	120 min	150 min	180 min
Hydroquinone 10 mM	43 (2)	35 (2)	28 (2)
+3 mM NADH	37 (1)	42 (1)	37 (1)
+3 mM ascorbate	43 (1)	40 (1)	42 (1)
+3 mM dithiothreitol	19 (2)	17 (2)	13 (2)

mM), glutathione (1 mM), cysteine (1 mM), NADH (3 mM), ascorbate (1–10 mM) and $Na_2S_2O_5$ (1–10 mM).

Time-dependent inhibition by hydroquinone and pyrogallol; interaction of hydroquinone with other agents

Because anti-oxidants (hydroxylated benzenes) were the only agents which markedly affected glucose transport, we tried to characterize their effects further. Table I shows that high doses of hydroquinone and pyrogallol inhibited glucose transport in a time-dependent, progressive manner. Reducing agents added to the reaction mixture simultaneously with hydroquinone (Table II, Expt. 1) did not significantly affect the initial inhibition; these agents did prevent time-dependent, progressive inhibition. Cysteine was an exception, however, in that it potentiated the progressive inhibition. We were not able to alter either the initial or progressive hydroquinone inhibition with H_2O_2 (10 mM), metal chelators (1 mM EDTA, 1 mM bathophenanthroline disulfonate, 500 μg/ml Desferral), anti-oxidants (250–500 μM butylated hydroxytoluene), free-radical scavengers (1% ethanol, 10 mM benzoate) or 10–100 μg/ml catalase (data not shown). Superoxide dismutase (Table II)

potentiated progressive hydroquinone inhibition without affecting sugar transport of the control membranes.

The effects of reducing agents on established hydroquinone inhibitions were also investigated (Table II, Expt. 2). Here, the reducing agents were added to membranes two hours after the addition of hydroquinone. As is seen both NADH and ascorbate prevented further progressive inhibition of sugar transport by hydroquinone. In contrast, dithiothreitol potentiated inhibition. When this experiment was repeated using the same reducing agents, but pyrogallol (3 mM) instead of hydroquinone, no potentiation of pyrogallol's progressive inhibition was seen in the case of dithiothreitol. All reducing agents tested prevented further progressive pyrogallol inhibition (data not shown). This observation suggested that an oxidation product of hydroquinone interacted with dithiothreitol to produce a more potent inhibitor of sugar transport (see below).

The observation that reducing agents prevented and superoxide dismutase potentiated progressive hydroquinone inhibition, without affecting initial inhibition, suggested that progressive inhibition might be mediated by a product of hydroquinone oxidation; initial inhibition might be caused by hydroquinone per se. Hydroquinone is oxidized by molecular oxygen to its semiquinone and ultimately to 1,4-benzoquinone [6]. These oxidations involve one and two electron transfers, respectively, in which process oxygen is reduced to superoxide anion (single electron) or peroxide (two electrons). The resulting quinones are labile and susceptible to nucleophilic addition by various functional groups, thiols and amines among others. At this point it was reasonable to suspect that benzoquonone and/or benzoquinone-membrane addition products might be involved in progressive hydroquinone inhibition.

Effects of benzoquinone on sugar transport, interactions with reducing agents

If benzoquinone were indeed involved in hydroquinone's progressive inhibition, then benzoquinone could be expected to be a more potent inhibitor of transport than its parent compound. Additionally, it should display properties similar to those seen during hydroquinone's time-dependent inhibition. As seen in Table III, benzoquinone inhibited glucose transport to a greater extent than did hydroquinone (compare Table I and Expt. 1, Table III). Like hydroquinone, benzoquinone displayed dose and time-dependence. The analogy extends further (Table III, Expt. 2) in that reducing agents which antagonized (NADH, ascorbate, bisulfite) or potentiated (cysteine) hydroquinone's progressive inhibition, had a similar effect on benzoquinone. Additionally, dithiothreitol, which potentiated established hydroquinone inhibition (Table II), also potentiated benzoquinone inhibition. All these factors suggest that benzoquinone was the agent responsible for hydroquinone's progressive inhibition.

Because the mixture of dithiothreitol and benzoquinone was extremely effective in inhibiting sugar transport (Table III, Expt. 2), we decided to investigate the synergism of these agents in more detail. To do this, doses of benzoquinone sufficient to inhibit 0–50% of the sugar transport were tested alone or with equimolar amounts of dithiothreitol. As seen in Table III (compare Expts. 1 and 3), the combination of agents was more effective in inhibiting transport than benzoquinone alone. After 10 min exposure of membranes to benzoquinone-dithiothreitol, 50% inhibition of transport occurred at approx. 50 μM agents (data not shown). Neither agent alone affected glucose transport at this concentration, nor did dithiothreitol at doses as high as 3 mM as previously mentioned. The efficacy of equimolar benzoquinone-dithiothreitol was independent of the order in which they were added to membranes (data not shown). Stoichiometry studies were also performed in which a fixed concentration of either dithiothreitol or benzoquinone was combined with increasing concentrations of the other agent. With 50 μM dithiothreitol, increments of benzoquinine up to 50 μM increased transport inhibition to 50% of control, whereas 2- and 4-fold excess benzoquinone reduced inhibition. With 50 μM benzoquinone, increments of dithiothreitol up to 50 μM increased the inhibition to 50% of control, 2- and 4-fold excess thiol being little more effective than equimolar (data not shown).

Another interesting aspect of benzoquinone-dithiothreitol synergism was its reversibility. Whereas these agents effectively eliminated sugar transport at 10 min (150 μM agents), transport activity was restored to 80–100% of control by 120 min in several experiments (Table III, Expt. 3). The role of oxygen in this time-dependent decay of transport inhibi-

TABLE III

SUGAR TRANSPORT INHIBITION BY BENZOQUINONE; EFFECTS OF REDUCING AGENTS ON TIME-DEPENDENT INHIBITION OF SUGAR TRANSPORT BY BENZOQUINONE

Benzoquinone alone or benzoquinone and the other compounds listed were combined and added to membranes to give the final concentrations of reagents shown. D-Glucose transport (0.2-min uptake) was then assayed at the times indicated.

Agents	Specific D-glucose transport (% of control)		
Experiment 1	10 min	60 min	120 min
Benzoquinone			
150 μM	88 (3)	–	85 (3)
300 μM	83 (3)	84 (1)	84 (3)
600 μM	80 (2)	63 (1)	44 (2)
1 mM	55 (2)	40 (2)	28 (2)
5 mM	28 (1)	9 (2)	5 (1)
10 mM	12 (1)	3 (1)	0 (1)
Experiment 2	10 min	60 min	120 min
Benzoquinone 600 μM	75	63	44
+NADH 600 μM	91	90	92
+ascorbate 600 μM	95	101	98
+$Na_2S_2O_5$ 600 μM	100	105	109
+cysteine 600 μM	86	17	10
+dithiothreitol 600 μM	0	0	17
Experiment 3	10 min	120 min	
Benzoquinone +dithiothreitol			
150 μM	12 (3)	92 (3)	
300 μM	8 (3)	63 (3)	
600 μM	0 (2)	13 (2)	

tion by benzoquinone-dithiothreitol was investigated. It was found that replacing air in the system with nitrogen prevented reversal of inhibition. In duplicate experiments using 150 μM agents, specific D-glucose transport at 120 min was 16 and 18% of control when nitrogen was present. Nitrogen had no effect on control membranes under these circumstances. The inhibitory product formed from benzoquinone-dithiothreitol appeared to be quite stable in air at 37°C in the absence of membranes (data not shown).

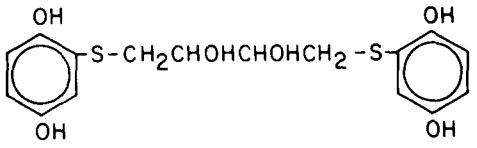
Other thiols besides dithiothreitol were tested for synergism (50–300 μM agents) with benzoquinone at 10 min. Of these (cysteine, glutathione, 5-thio-D-glucose, β-D-thioglucose and dithioerythritol) only dithioerythritol inhibited (data not shown).

Isolation and identification of the benzoquinone-dithiothreitol synergistic inhibitor

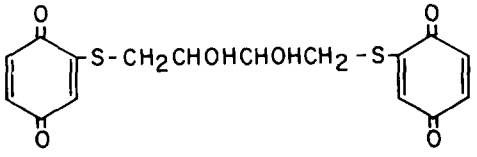
As mentioned, benzoquinone is susceptible to nucleophilic attack and is capable of undergoing extensive modification as a consequence of the resulting addition reactions. (For a more detailed discussion of the chemistry of benzoquinone, see Ref. 7). It was reasonable to suspect that benzoquinone-dithiothreitol-mediated transport inhibition resulted from nucleophulic addition of the thiol to the quinoid ring. To determine the identity of the actual inhibitor, benzoquinone and dithiothreitol were combined at stoichiometric ratios which maximized (molar equivalence) or minimized (4–10-fold molar excess of benzoquinone) sugar transport inhibition. The products of these reactions were then isolated, crystallized, and their structures determined by elemental analysis, proton, carbon NMR, and infrared spectra.

With benzoquinone and dithiothreitol at molar equivalence, compound I was obtained. This substance (DL-*threo*-1,4-bis(2,5-dihydroxyphenylthio)-2,3-butanediol) potently inhibits sugar transport ($K_i \simeq 40$ μM), and displayed the property of time-dependent decay of inhibition in air as described

above.



With a 4–10 mol ratio of benzoquinine to dithiothreitol, compound II was isolated. Compound II (in dimethylsulfoxide, 0.4% (v/v)) had no effect on sugar transport.



Discussion

These studies were undertaken in anticipation of demonstrating effects of oxidizing and/or reducing agents on glucose transport in membrane vesicles. As it turned out, no single agent except hydroxybenzenes and their oxidation products affected transport significantly, and these agents were inhibitors.

Inhibition by catechol, hydroquinone and pyrogallol progressed with duration of exposure to the vesicles. The effect of several reducing agents, including dithiothreitol, to retard or prevent the progressive inhibition by hydroquinone, suggested that a product of hydroquinone oxidation was a more potent inhibitor than hydroquinone. Cysteine's action to accelerate hydroquinone's progressive inhibition was a paradox which we have not explored further. Superoxide dismutase also promoted progressive inhibition which indicated that superoxide was not the time-dependent inhibitor, and supported the view that hydroquinone oxidation gave rise to the inhibitor. Catalase and free radical scavengers had no effect, so we concluded that no member of the superoxide-peroxide-hydroxyl-radical sequence [8–11] mediated the inhibition. Oxidized hydroquinone (1,4-benzoquinone) and oxidized pyrogallol were likely candidates for mediating progressive inhibition. Several reducing agents added after 2-h exposure of vesicles to hydroquinone or pyrogallol arrested, but did not reverse, the established time-dependent inhibition. This effect was expected from the actions of reducing agents to prevent time-dependent inhibition. However, dithiothreitol was the exception. Although this agent could prevent time-dependent inhibition, and arrested pyrogallol's established inhibition, it immediately potentiated hydroquinone's established inhibition.

Benzoquinone turned out to be about ten times more potent than hydroquinone in glucose transport inhibition, and could account for hydroquinone's progressive inhibition. Consistent with this view are the observations that several reducing agents which prevented hydroquinone's progressive inhibition also prevented benzoquinone inhibition. Additionally, agents which promoted hydroquinone's progressive (cysteine) or established (dithiothreitol) inhibition had analogous effects on benzoquinone.

Stoichiometric combinations of benzoquinone and dithiothreitol were at least 10-times more potent than benzoquinone and at least 100-times more potent than hydroquinone. Inhibitory potency was indifferent to the order of combining benzoquinone, dithiothreitol and vesicles. Since thiols are known to add readily to benzoquinone, this indifference to order of combination indicated that a dithiothreitol-benzoquinone addition product was the potent inhibitor. Of several thiol compounds tested, only dithiothreitol and dithioerythritol combined with benzoquinone to form a potent inhibitor. In particular, polyols with one thiol group failed to potentiate benzoquinone's inhibition. This was disappointing, because we suspected the polyol character of dithiothreitol rather than its dithiol character was responsible for the synergistic interaction with benzoquinone.

The benzoquinone-dithiothreitol adduct was stable when exposed to air in aqueous solution at 37°C for hours or when exposed to vesicles under nitrogen at 37°C, but its inhibitory potency decayed when it was exposed to both air and vesicles. The vesicles apparently catalyzed oxidation to a non-inhibitor. The presence of excess benzoquinone (an oxidant) in the benzoquinone-dithiothreitol mixture reduced inhibitory potency of the product. Excess dithiothreitol slightly favored formation of the inhibitory product.

The above properties suggested that the inhibitor might be a one-to-one stoiciometric combination of benzoquinone with dithiothreitol, the thiol being added to the number 2 carbon of the benzene ring with concomitant change of the redox state of the

quinone to the hydroquinone. However, chemical and physical analyses of addition products formed in ethanol indicated that the inhibitor (see compound I above) consisted essentially of hydroquinone attached via carbon to both sulfurs of dithiothreitol. The non-inhibitory product formed with excess benzoquinone was similar except that the hydroquinone groups at each end were oxidized. The crystallized (hydroquinone)$_2$-dithiothreitol compound exhibited the same potency as did the product formed in water or buffer. The inhibitory benzoquinone-dithiothreitol adduct is, therefore, analogous in some respects to known glucose-transport inhibitors such as diethylstilbesterol and phloretin.

In our vesicle system, the adduct ($K_i \simeq 40$ μM) is more potent than phloretin ($K_i \leqslant 200$ μM), but much less potent than cytochalasin B ($K_i \leqslant 2$ μM). It is much more water soluble than either of these compounds and may have some distinctive uses in biochemical or physiological studies.

It should not be overlooked, that agents which prevented benzoquinone inhibition did not reverse it. Since these agents formed non-inhibitory adducts with benzoquinone, this indicates that benzoquinone's inhibition is irreversible. We have confirmed this conclusion in experiments (not shown) in which benzoquinone's inhibition persisted after thorough washing with buffer containing or lacking mercaptoethanol. Benzoquinone is, therefore, the most potent irreversible glucose-transport inhibitor yet described. This property may have useful biochemical applications.

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