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INVOLVEMENT OF SULFHYDRYL GROUPS IN THE ACTION OF INSULIN AND RADIATION ON THYMOCYTE Na^+-DEPENDENT AMINO ACID TRANSPORT

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SUMMARY

1, *p*-Chloromercuribenzene sulfonate concentrations $< 10^{-5}$ M stimulate the uptake by thymocytes of 2-aminoisobutyrate, a non-metabolized amino acid. At concentrations $> 10^{-5}$ M of this reagent, transport is impaired and cell viability is affected. In contrast, 5,5′-dithiobis-(2-nitrobenzoate) between 10^{-4} and 10^{-6} M produces only stimulation of 2-aminoisobutyrate uptake after treating for 10 min.

2. Treatment of thymocytes with 10^{-4} M 5,5′-dithiobis-(2-nitrobenzoate) reveals at least three categories of reactive SH groups. Titration of the most rapidly reacting category, $4 \cdot 10^7$–$7 \cdot 10^7$/cell, activates 2-aminoisobutyrate transport to the same extent as does *p*-chloromercuribenzene sulfonate. Cells treated with 10^{-6} M insulin showed a 30–50 % reduction in the number of sulfhydryl groups that could be titrated with 5,5′-dithiobis-(2-nitrobenzoate). In thymocytes treated with 10^{-6} M *p*-chloro[^{203}Hg]mercuribenzene sulfonate, addition of 10^{-6} or 10^{-9} M insulin before treatment with the sulfhydryl reagent again reduces the number of titrable SH groups by 20 %.

3. Insulin (10^{-10}–10^{-6} M) also stimulates 2-aminoisobutyrate uptake, but the effects of insulin and SH blocker are not additive.

4. Insulin, but not *p*-chloromercuribenzene sulfonate, prevents the impairment of 2-aminoisobutyrate transport caused by γ-irradiation. Treatment of cells with *p*-chloromercuribenzene sulfonate prior to irradiation increases the radiation impairment of 2-aminoisobutyrate transport.

5. γ-irradiation reduces the number of 5,5′-dithiobis-(2-nitrobenzoate) reactive sulfhydryl residues by 37 %.

6. A model for the action of insulin and irradiation on 2-aminoisobutyrate transport is presented.

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INTRODUCTION

Sulfhydryl groups are important to membrane function but the mechanisms by which changes in these groups affect the membrane are not well understood. In addition to possible indirect influences on protein configuration, there may be direct contributions through hydrogen bonding [1]. It has also been postulated that interaction between lipid double bonds and sulfhydryl groups of membrane proteins might be critical [2]. Finally, sulfhydryl groups and lipid double bonds are known to be two of the major radiosensitive sites in biomembranes [3].

We have recently shown that γ-irradiation with 0.05–10 kR impairs the Na^+-dependent transport of 2-aminoisobutyrate but does not affect the transport of two Na^+-independent amino acids. This radiation response paralleled the effects observed for the Na^+-K^+ transport system [5], and is compatible with the hypothesis [6] that Na^+-K^+ transport and 2-aminoisobutyrate transport are coupled and that the coupling factor is radiosensitive. In addition, we have found that insulin could protect the thymocyte 2-aminoisobutyrate transport system against the adverse effects of γ-irradiation [4].

The mechanism by which γ-irradiation effects membrane transport is not well understood, but our evidence [4] as well as that of others [7] implicates radiosusceptible SH groups. We have accordingly explored the effect of SH-blocking reagents upon the action of insulin and radiation on Na^+-dependent amino acid transport by rat thymocytes. Our aims were two-fold: (a) clarification of the possible role of SH groups in thymocyte membrane transport, and (b) definition of the molecular events involved in membrane radiation damage and insulin-membrane interaction.

MATERIALS AND METHODS

Reagents

2-Aminoiso[^{14}C]butyrate (9.89–12 mCi/mol) and [^{3}H]inulin (100 mCi/g) were purchased from New England Nuclear. *p*-Chloro[^{203}Hg]mercuribenzene sulfonate was obtained from Amersham-Searle (Spec. Act. 62 mCi/g). *p*-Chloromercuribenzene sulfonic acid and 5,5′-dithiobis-(2-nitrobenzoate) were purchased from Sigma Chemical Corporation, phosphate buffered saline (PBS: 10X) from Grand Island Biological Co. and D-glucose from Fisher Scientific Corporation. Porcine insulin (26.5 units/mg) was obtained from Schwarz-Mann.

Methods

8- to 10-week-old female Wistar-Lewis rats (Charles River Breeding Laboratory, Wilmington, Ma.) were sacrificed by either chloroform anesthesia or decapitation. Thymuses were removed and thymocytes isolated as described in ref. 4. In most of our thymocyte preparations we found monocyte contamination to be $\sim 1\,\%$ as judged by light microscopy examination of Giesma stained preparations. Efflux studies and transport of 2-aminoisobutyrate were assayed as previously described [4]. Each assay was run at least in triplicate and is expressed in terms of viable (trypan blue negative) cells.

For incubation of thymocytes with *p*-chloromercuribenzene sulfonate, we proceeded as follows. To 9 ml of suspended thymocytes ($2 \cdot 10^7$–$3 \cdot 10^7$ cells/ml) in

glucose buffer, (phosphate buffered saline plus 15 mM α-D-glucose, pH 7.4), 1 ml of *p*-chloromercuribenzene sulfonate solution in glucose buffer was added at room temperature to give final concentrations of *p*-chloromercuribenzene sulfonate ranging between 10^{-4} and 10^{-7} M. Stock solutions of *p*-chloromercuribenzene sulfonate were freshly prepared for each experiment. We allowed the reaction of *p*-chloromercuribenzene sulfonate with the cells to proceed for 10 min at room temperature, then added 3 ml of iced phosphate buffered saline and pelleted the cells at 5000 g · min at 4 °C. The cells were washed once more in cold buffer, centrifuged in glucose buffer and incubated for 10 min at 37 °C before measurement of 2-aminoisobutyrate uptake.

We used *p*-chloro[^{203}Hg]mercuribenzene sulfonate to determine the number of SH groups affected by insulin. A thymocyte suspension was divided into two portions. To one portion we added insulin (final concentration $1.0 \cdot 10^{-6}$ or $1.0 \cdot 10^{-9}$ M) and to the other an equivalent volume of buffer. After incubating for 2 h at 37 °C, the cells were left at room temperature for 30 min. An aliquot of *p*-chloro[^{203}Hg]mercuribenzene sulfonate was added to the control and insulin-treated cells to give a final concentration of $1.0 \cdot 10^{-6}$ M and the reaction was allowed to proceed for 10 min at room temperature before pelleting the cells at $500 \times g$. After removal of the supernatant fluid, the cells were washed once with 3 ml of iced phosphate buffered saline, pelleted and resuspended in 5 ml of buffer. 1-ml aliquots were then placed in 16×100 mm tubes and counted on a Packard Autogamma Counter.

We determined that *p*-chloromercuribenzene sulfonate did not react with insulin by incubating samples of insulin and *p*-chloro[^{203}Hg]mercuribenzene sulfonate at the above concentrations for 2 h, then separating them on a Sephadex G-25 column (1.2 cm × 30 cm). Insulin was isolated within the first void volume and had no detectable radioactivity associated with it. *p*-Chloro[^{203}Hg]mercuribenzene sulfonate was isolated quantitatively after the third void volume.

We also used 5,5′-dithiobis-(2-nitrobenzoate) to determine SH group content in thymocytes [8]. The thymocyte cell suspension was again divided into two portions. To one, $1.0 \cdot 10^{-3}$ M of 5,5′-dithiobis-(2-nitrobenzoate) was added to give a final concentration of $1.0 \cdot 10^{-4}$ M. To the other, we added the same volume of glucose buffer. These reactions were carried out at room temperature. At stated intervals, 1.5 ml aliquots were removed, centrifuged at $500 \times g$ for 2 min and 1-ml samples removed for ultraviolet analysis of the mercaptide formed between 5,5′-dithiobis-(2-nitrobenzoate) and sulfhydryl groups (absorbance maximum 412 nm) on either a Beckman model DB-G Grating Spectrophotometer or a Gilford 2400-S Spectrophotometer. For an ultraviolet blank, we used the supernatant obtained from untreated cells. 5,5′-dithiobis-(2-nitrobenzoate) was added (final concentration $1 \cdot 10^{-4}$ M) after the supernatant had been separated from the pellet. To insure the 5,5′-dithiobis-(2-nitrobenzoate) was not reacting with the insulin, we incubated $1.0 \cdot 10^{-4}$ insulin with $1.0 \cdot 10^{-4}$ M dithiobis-(2-nitrobenzoate) for 2 h at room temperature. Ultraviolet analysis showed no change at 412 nm when compared to a solution of $1.0 \cdot 10^{-4}$ M 5,5′-dithiobis-(2-nitrobenzoate). The remainder of the supernatant was aspirated and the cells resuspended in 1 ml of glucose buffer.

0.1 ml aliquots were removed to measure cell number and viability. The remaining cells were allowed to equilibrate at 37 °C for 10 min before addition of $1.0 \cdot 10^{-3}$ M 2-aminoisobutyrate (final concentration $1.0 \cdot 10^{-4}$ M). Amino acid

uptake was allowed to proceed for 15 min before stopping the reaction by addition of 3 ml of ice-cold buffer, pH 7.4.

To evaluate insulin effects, $2 \cdot 10^7$–$4 \cdot 10^7$ thymocytes in buffer were exposed for 2 h to $1.7 \cdot 10^{-6}$–$1.7 \cdot 10^{-10}$ M insulin, before the uptake measurements. To measure the combined effect of *p*-chloromercuribenzene sulfonate and insulin, insulin-treated (2 h incubation) cells were exposed with *p*-chloromercuribenzene sulfonate for 10 min, pelleted and washed with glucose buffer to remove *p*-chloromercuribenzene sulfonate.

Cells were irradiated in 15 ml siliconized plastic culture tubes (Falcon) using a ^{137}Cs source (Radiation Machinery Corporation Model M38 Grammator: dose rate of 510.6±3.5 % rad/min at the center of the radiation chamber). After irradiation, the cells were incubated for 2 h at 37 °C in 95 % air/5 % CO_2, before measuring amino-acid uptake.

RESULTS

Effect of p-chloromercuribenzene sulfonate and dithiobis-(2-nitrobenzoate) on 2-amino-isobutyrate uptake

Table I shows the effects of two sulfhydryl reagents on 2-aminoisobutyrate uptake. At concentrations $< 10^{-5}$ M and 10 min incubation with both *p*-chloromercuribenzene sulfonate and 5,5′-dithiobis-(2-nitrobenzoate) stimulate the uptake of the amino acid. We observe inhibition at concentrations $> 10^{-5}$ M in *p*-chloromercuribenzene sulfonate treated cells while slight activation or no effect occurs in the 5,5′-dithiobis-(2-nitrobenzoate)-treated cells.

To further characterize these two sulfhydryl reagents, we examined their effect on 2-aminoisobutyrate uptake as a function of treatment time. As shown in Fig. 1, when $2.5 \cdot 10^7$–$3.0 \cdot 10^7$ cells/ml are exposed for 10 min to 10^{-6} M *p*-chloro-

TABLE I

EFFECT OF SULFHYDRYL REAGENTS ON THE UPTAKE OF 2-AMINOISOBUTYRATE IN THYMOCYTES

Treatment* (10 min)	Concentration (M)	$v_{relative}$**
p-Chloromercuribenzene sulfonate	10^{-4}	0.32± 0.04
	10^{-5}	1.04± < 0.01
	10^{-6}	1.17± 0.01
	10^{-7}	1.21± 0.01
5,5′-Dithiobis-(2-nitrobenzoate)	10^{-4}	1.04± < 0.01
	10^{-5}	1.04± 0.01
	10^{-6}	1.09± 0.02
	10^{-7}	1.17± 0.04

* Approximately $2 \cdot 10^7$–$3 \cdot 10^7$ cells were treated for 10 min at room temperature with various concentrations of sulfhydryl reagent. 3 ml of iced buffer were added, cells pelleted, washed once with 3 ml of buffer and resuspended in glucose buffer for uptake measurements.

** $v_{relative}$ is the reaction velocity relative to the value found in the absence of added perturbants, in terms of viable (trypan blue negative) cells. The values obtained are the mean ±S.D. of five replicate samples.

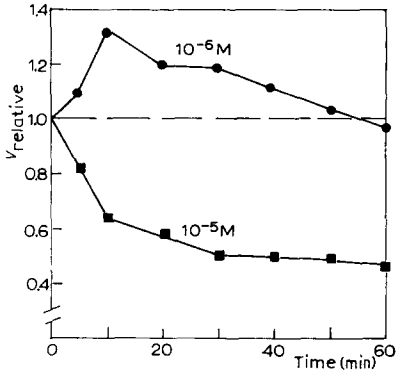

Fig. 1. Time course of *p*-chloromercuribenzene sulfonate effect on 2-aminoisobutyrate uptake in thymocytes. Thymocytes ($3.0 \cdot 10^7$ cells/ml) were treated at room temperature with 10^{-6} M *p*-chloromercuribenzene sulfonate (●). At the indicated times, 1 ml aliquots were removed, 3 ml of ice-cold buffer added and the cells pelleted. The cells were washed once, re-pelleted, suspended in 0.9 ml glucose buffer and allowed to incubate for 10 min at 37 °C before addition of 0.1 ml of $1.0 \cdot 10^{-3}$ M 2-aminoisobutyrate. (■) In this experiment $0.9 \cdot 10^7$ cells/ml were treated with 10^{-5} M *p*-chloromercuribenzene sulfonate and the samples treated in the same manner as the above. $v_{relative}$ is the reaction velocity relative to the value found in the absence of added perturbants at the stipulated time points and is in terms of viable cells. Each point is the mean of triplicate determinations.

mercuribenzene sulfonate, maximum stimulation occurs. Thereafter, uptake falls off and, after 50 min of exposure, the rate of uptake approaches that of control. The effect of *p*-chloromercuribenzene sulfonate also varied with cell density. Using a cell density of $0.9 \cdot 10^7$–$1.4 \cdot 10^7$ cells/ml and a concentration of $1 \cdot 10^{-5}$ M sulfhydryl reagent, an initial sharp drop in transport is seen within the first 10 min (Fig. 1). This is followed by a further gradual decrease in uptake. With cell concentrations

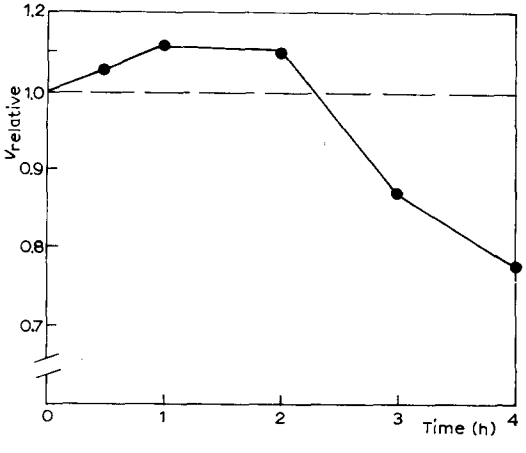

Fig. 2. Effect of 5,5′-dithiobis-(2-nitrobenzoate) on 2-aminoisobutyrate uptake as a function of time. Between $2 \cdot 10^7$ and $3 \cdot 10^7$ cells/ml were used in this experiment and analysis is described in the methods sections. $v_{relative}$ is the reaction velocity relative to the value found in the absence of added 5,5′-dithiobis-(2-nitrobenzoate) at the stipulated time points and with respect to viable cells. Each point is the mean of triplicate determinations.

above $2 \cdot 10^7$ cells/ml and 10^{-6} M *p*-chloromercuribenzene sulfonate, 2-aminoisobutyrate uptake is generally enhanced (2–40 %).

When thymocytes are treated with 10^{-4} M dithiobis-(2-nitrobenzoate), we observed stimulation of 2-aminoisobutyrate uptake for up to 2 h (Fig. 2), followed by inhibition. This result indicates that the SH groups involved in the inhibition of uptake by *p*-chloromercuribenzene sulfonate are accessible to 5,5'-dithiobis-(2-nitrobenzoate). Possibly the different effects of these two reagents on 2-aminoisobutyrate uptake relate to the lower electrophilic character of 5,5'-dithiobis-(2-nitrobenzoate).

Action of p-chloromercuribenzene sulfonate on 2-aminoisobutyrate efflux

The natural logarithm of the intracellular 2-aminoisobutyrate content varies linearly with time for at least 60 min in both control and *p*-chloromercuribenzene sulfonate-treated cells (10^{-6} M); i.e. efflux behaves as a first order process in both control cells and cells exposed to stimulatory concentrations of SH reagent. We obtain a fractional 2-aminoisobutyrate efflux coefficient of 0.020 ± 0.002 min^{-1} for control thymocytes and 0.022 ± 0.001 min^{-1} for the *p*-chloromercuribenzene sulfonate-treated cells.

The effect of stimulatory concentrations of *p*-chloromercuribenzene sulfonate is thus not due to an increased general permeability of the plasma membrane. At a *p*-chloromercuribenzene sulfonate concentration of 10^{-4} the cells become leaky as judged by trypan blue uptake. Over 40 % of the cells took up the dye compared to 5 % in the untreated cells. Treatment of the cells with 10^{-3} M *p*-chloromercuribenzene sulfonate caused over 90 % of the cells to take up trypan blue.

Effect of p-chloromercuribenzene sulfonate on 2-aminoisobutyrate transport in insulin-treated thymocytes

We had shown earlier [4, 9] that insulin concentrations between 10^{-6} and 10^{-12} M stimulate 2-aminoisobutyrate transport in rat thymocytes. Czech [10] has shown that sulfhydryl groups are involved in the action of insulin on glucose transport in adipocytes. In addition, *N*-ethylmaleimide and *p*-chloromercuribenzene sulfonate exhibit the same concentration dependency on glucose transport in adipocytes [11, 12] as is observed in our system, i.e. stimulation at low concentration and inhibition at high concentration of sulfhydryl reagent. To determine whether insulin and *p*-chloromercuribenzene sulfonate act on a common component of 2-aminoisobutyrate transport, we compared the effect on transport of insulin (2 h incubation), of *p*-chloromercuribenzene sulfonate (10 min treatment) and of pretreatment with insulin for 2 h followed by a 10 min incubation with *p*-chloromercuribenzene sulfonate. Addition of *p*-chloromercuribenzene sulfonate to insulin-treated cells yielded a 32 % increase in uptake. Insulin and *p*-chloromercuribenzene sulfonate alone stimulated uptake by 20 and 40 %, respectively. The two agents thus do not act in an additive fashion, suggesting that they effect 2-aminoisobutyrate transport through a common component.

Effect of p-chloromercuribenzene sulfonate on 2-aminoisobutyric acid uptake in γ-irradiated thymocytes

Since insulin protects 2-aminoisobutyrate transport from the adverse effects of γ-irradiation [4], we investigated the effect of γ-irradiation on *p*-chloromercuribenzene

TABLE II

EFFECT OF INSULIN, *p*-CHLOROMERCURIBENZENE SULFONATE AND γ-IRRADIATION TREATMENT ON 2-AMINOISOBUTYRATE UPTAKE IN RAT THYMOCYTES

First treatment	Second treatment	Total incubation time before uptake measured (h, min)	$V_{relative}$**
None	None	4	1.0
Insulin, 10^{-6} M (2 h, 37 °C)*	None	2	1.12±0.05
p-Chloromercuribenzene sulfonate, 10^{-6} M (10 min, room temperature)	None	2, 10	1.36±0.03
5 krad irradiation (2 h, 37 °C)	None	2	0.82± < 0.01
Insulin, 10^{-6} M	5 krad irradiation	4	1.10± 0.01
p-Chloromercuribenzene sulfonate, 10^{-6} M	5 krad irradiation	2, 10	0.70± 0.03

* The figures in parenthesis indicate the duration of incubation in each perturbant and the incubation temperature. All *p*-chloromercuribenzene sulfonate-treated cells were washed twice before any other treatment. For the irradiated cells the time indicated is the incubation time after irradiation.

** Between $2 \cdot 10^7$ and $4 \cdot 10^7$ cell/ml were used in this experiment. $v_{relative}$ is the reaction velocity relative to the value found in the absence of added perturbants or treatments. The values obtained are the mean ±S.D. of five replicate samples.

sulfonate-treated cells. Table II shows the results obtained in a typical experiment. *p*-Chloromercuribenzene sulfonate alone does not exert any radioprotective effect. In fact, exposure of thymocytes to 10^{-6} M *p*-chloromercuribenzene sulfonate prior to radiation enhances the inhibitory effects of radiation by 20–30 %. However, when the cells were first irradiated and then treated with *p*-chloromercuribenzene sulfonate, the normal stimulatory effect of this reagent is abolished and the *p*-chloromercuribenzene sulfonate-induced enhancement of radiation damage to amino acid transport disappears.

Effect of insulin and γ-irradiation on SH group content in thymocytes

We have examined the time of exposure to 5,5'-dithiobis-(2-nitrobenzoate) affect on thymocyte SH group content, to determine whether various categories of SH groups could be detected. Three categories of SH groups are revealed (Fig. 3). The first type is almost fully titrated within 10 min after adding 10^{-4} M reagent and accounts for $4 \cdot 10^7$–$7 \cdot 10^7$ SH groups/cell. The second is saturated within 2 h and the third category is not saturated even after 6 h. In heat-treated cells (100 °C, 60 min, 99 % trypan blue positive), complete saturation is obtained within 10 min.

Since we observe stimulation of transport within 10 min after treatment of cells with dithiobis-(2-nitrobenzoate) (Table I), the titration data suggest that the SH group responsible for the stimulation are located in an exposed environment, perhaps at the surface of the membrane. The second category of SH groups represents a less reactive or less accessible type of SH group which apparently plays no role in modifying transport. The third group is apparently involved in the inhibitory affects of

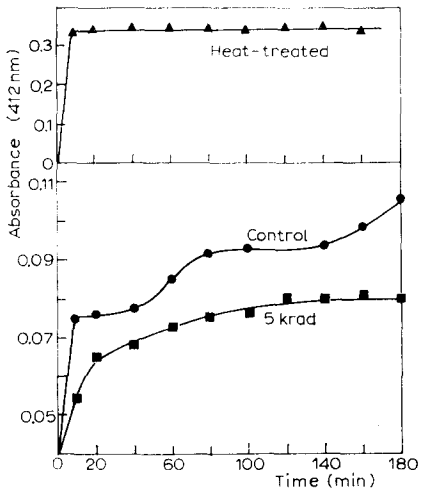

Fig. 3. Titration of thymocyte sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoate). The cell concentration in these experiments was between $4 \cdot 10^7$ and $6 \cdot 10^7$ cells/ml. In the upper panel (▲) cells were incubated at 100 °C for 30 min, pelleted and resuspended in glucose buffer before addition of 5,5′-dithiobis-(2-nitrobenzoate). (>90% of the cells were trypan blue positive.) In the lower panel, the cell suspension was divided into two portions. One portion (■) was irradiated at 5 krad (^{137}Cs) and allowed to incubate for 1.5 h at 37 °C and then 30 min at room temperature before addition of $1.0 \cdot 10^{-3}$ M 5,5′-dithiobis-2(2-nitrobenzoate) (final concentration $1.0 \cdot 10^{-4}$ M). The other portion (●) was also allowed to incubate for 1.5 h at 37 °C and 30 min at room temperature before addition of 5,5′-dithiobis-(2-nitrobenzoate). Analysis of the SH content is described in the Methods section. The data are means of 5 replicates.

5,5′-dithiobis-(2-nitrobenzoate) since we observe inhibition of transport only after the cells have been exposed to 5,5′-dithiobis-(2-nitrobenzoate) for approx. 3 h. SH groups buried within the membrane core and/or intracellular groups probably comprise this category.

After thymocytes are irradiated (5 krad), we find a decreased number of 5,5′-dithiobis-(2-nitrobenzoate) titrable groups (Fig. 3). In addition, the multiphasic character of the kinetic curve is abolished.

Treatment of cells with 10^{-6} M insulin for 2 h also lowers the number of titrable SH groups. The loss in reactive SH groups after insulin treatment corresponds to approximately $2 \cdot 10^7$–$3 \cdot 10^7$ SH groups/cell, indicating that 30–50% of the SH groups accessible within 20 min are being modified or lost upon insulin exposure. When insulin-treated thymocytes are irradiated (5 krad) the 5,5′-dithiobis-(2-nitrobenzoate) titration curve parallels the 5 krad titration curve but is approximately 30% lower. This corresponds approximately to the difference between the unirradiated controls and the insulin-treated cells, suggesting that insulin modifies a population of SH groups and protects them against the adverse effects of irradiation.

We also examined the binding of *p*-chloro[^{203}Hg]mercuribenzene sulfonate to thymocytes. At 10^{-6} M of this reagent, we found that 10 min incubation complexes about $1.5 \cdot 10^6$ SH groups/cell. Simultaneous experiments on cells exposed to 10^{-6} M or 10^{-9} M insulin for 2 h showed an approx. 20% reduction of reactive SH groups (in 10 min).

DISCUSSION

Addition of low concentrations ($< 10^{-5}$ M) of *p*-chloromercuribenzene sulfonate to isolated rat thymocytes can stimulate 2-aminoisobutyrate uptake up to 40 % over control levels for about 10 min. This stimulation declines with time and after 60 min uptake is inhibited. Moreover, as the concentration of SH-inhibition is increased from 10^{-5} to 10^{-3} M a dose-dependent depression of uptake occurs shortly after addition of the SH-blocker; at 10^{-4} M the inhibitory effect progresses with time, but, at 10^{-3} M *p*-chloromercuribenzene sulfonate, uptake is fully inhibited immediately.

We ascribe the inhibitory effects of *p*-chloromercuribenzene sulfonate to SH-bearing proteins, whose function is required for normal transport; these could be plasma membrane proteins, glycolytic enzymes, mitochondrial components and others.

We suspect that the stimulatory effects occur at the level of membrane transport for the following reasons. (a) *p*-Chloromercuribenzene sulfonate does not readily permeate biomembranes [11, 13]. (b) Surface membrane sulfhydryl groups are implicated by titration and transport studies with 5,5'-dithiobis-(2-nitrobenzoate) and *p*-chloromercuribenzene sulfonate. (c) 2-Aminoisobutyrate uptake requires production of metabolic energy which is blocked by sulfhydryl inhibitors [12], but stimulation of uptake occurs immediately after addition and only at low concentrations of *p*-chloromercuribenzene sulfonate. (d) Stimulation of uptake is due to enhanced transport, not to a non-specific decrease in membrane permeability.

How might the transport enhancement produced by low concentrations of sulfhydryl reagent relate to the similar transport stimulation effected by insulin? The fact that the stimulatory actions of the two compounds are not additive indicates that they act on a common pathway but, whereas insulin protects 2-aminoisobutyrate transport against radiation damage, *p*-chloromercuribenzene sulfonate enhances the deleterious effects of ionizing radiation. However, the effects of ionizing radiation are mediated by water free radicals and the different actions of *p*-chloromercuribenzene sulfonate and insulin might indicate that the SH blockers expose a critical membrane component(s) to such free radicals whereas insulin does not. One mechanism by which *p*-chloromercuribenzene sulfonate might enhance the effects of irradiation is suggested by the experiments of Myers and Slade [14], showing that peroxidation of erythrocyte lipids was accelerated during post-irradiation incubation when the cells were pretreated with the SH blocker iodoacetamide. This peroxidation of lipids leads to the generation of long-lived radicals which potentiate the initial effect of ionizing radiation.

The progressive impairment to 2-aminoisobutyrate uptake indicates that processes other than the generation of free radicals leads to the oxidation of SH groups. Thus, 1–10 krad γ-irradiation leads to an initial sharp drop (within 10–20 min) in 2-aminoisobutyrate transport [4], which is followed by a slower decrease. The initial drop could be due to the primary effects of ionizing radiation, i.e. reaction of water-free radicals with critical SH groups and/or unsaturated lipids to form longer-lived lipid peroxides. The second stage of the irradiation effect could be due to time-dependent changes in lipid-lipid, lipid-protein, or protein-protein interactions due to the formation of disulfide bridges and/or lipid peroxidation products.

The inhibitory effects of *p*-chloromercuribenzene sulfonate (Fig. 1) suggest

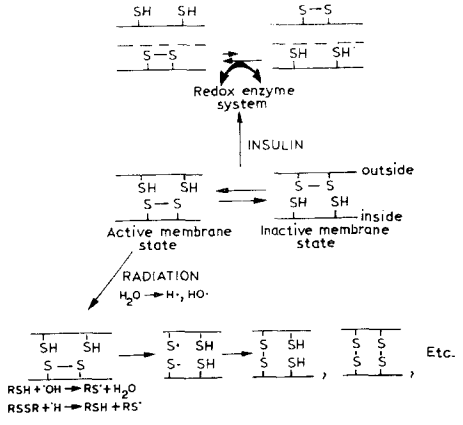

Fig. 4. Proposed model describing the action of insulin and radiation on plasma membrane sulfhydryl groups. This model assumes that the amino acid transport system or a regulatory component thereof exists in two states, an active and an inactive state. We further assume that the rate of transport in the absence of any perturbants depends on the steady states of two categories of SH groups, one lying at or near the external surface of the plasma membrane and the other buried within the membrane core. The active state is hypothetically characterized by the presence of a disulfide linkage(s) of the buried groups and the inactive state by the reduced form of this moiety. According to this scheme, the binding of insulin to its receptors decouples the normal pathway by which the active state is generated and now couples the formation of the inactive to active membrane state (i.e. –SH → S–S–) to a more efficient enzymatic redox system. Radiation causes the generation of water free radicals which impairs transport by causing extensive cross-linking of SH groups involved in transport as well as other cellular functions.

that SH groups are involved in both stages of the irradiation effect. After irradiation we observe an initial sharp drop in uptake (within 10 min) followed by a more gradual decrease [4]. This is relevant to the observation [8] that the sulfhydryl groups controlling cation permeability in erythrocytes react with *p*-chloromercuribenzene sulfonate, and it is these groups that are altered by X-ray-produced water free radicals. Insulin presumably stimulates and protects transport by modifying the reactivity or environment of SH groups critical for transport. Importantly, our data show that, for every molecule of insulin bound, approx. 100–1000 SH groups are modified. Such amplification is observed not only in thymocytes but also in muscle [15] and adipocytes (Pohl, S., private communication). Approx. 20–30 % of the surface sulfhydryl groups are modified when treated with insulin. In addition, preliminary evidence indicates that insulin-treated cells protect this population of SH groups even after 5 krad of irradiation.

We propose the following working model to explain the role of SH groups on 2-aminoisobutyrate transport (Fig. 4). This model is an extension of the hypothesis proposed by Czech et al. [10]. We propose that transport rate depends on the steady states of two categories of SH groups, lying at or near the external surface of the plasma membrane and buried within the membrane core respectively. We further propose that the peptide segments bearing the buried SH groups are arrayed so as to permit intra or interchain S-S formation and that the S-S linkages are limited by disulfide interchange between buried and superficial peptide segments. Low levels of *p*-chloromercuribenzene sulfonate titrate the superficial SH groups allowing a greater

proportion of the deep groups to form the S-S linkages necessary to activity transport and maintaining the active membrane state in a "frozen" configuration. High concentrations of *p*-chloromercuribenzene sulfonate and/or long reaction times inactivate also the buried groups, as well as intracellular enzymes involved in energy generating systems.

The reaction of *p*-chloromercuribenzene sulfonate with external membrane SH groups alters membrane structure, making either the buried SH residues and/or lipid components more accessible to water free radicals.The interaction of insulin with its receptor is to decouple the two classes of SH groups. This might be by (a) producing a structural change which opposes disulfide interchange and changing the environment of the SH groups and (b) coupling the membranes to an enzymatic redox system [8] that acts as an efficient agent for the maintenance of the active membrane state. (Such a system would also reduce water free radicals and/or disulfides generated by ionizing irradiation.)

Although our data fit the concept that a sulfhydryl-disulfide interchange plays a role in the control of Na^+-dependent amino acid transport in thymocytes, other possibilities are not excluded. However, our working model can explain most of our data on the regulation of amino acid transport by insulin and SH reagents. It also provides a basis for further studies designed to determine whether the postulated critical component controls the transport system and accounts for the pleiomorphic actions of insulin and the radiation effects on membrane transport.

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