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Alloxan stimulates *p*-aminohippurate uptake in renal basal-lateral membranous vesicles

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In renal basal-lateral membranous vesicles, the probenecid-sensitive *p*-aminohippurate uptake was stimulated by alloxan. This stimulation of uptake was observed only after a lag period of 15 seconds, and it reached a maximal value after one minute. Stimulation was increased by 1 mM to 5 mM alloxan in a linear fashion. The effect was maximal and constant between 5 mM and 20 mM alloxan. Alloxan affected neither the glucose space of the vesicle nor the rate of transport or diffusion of glutamate, another organic anion. The mechanism of stimulation by alloxan was not clear. Its effect was blocked by the sulfhydryl reagent *N*-ethylmaleimide and weakly mimicked by H_2O_2, an oxidizing reagent. However, ninhydrin, a structural analogue of alloxan which reacts with sulfhydryl groups, and glucose, a neutral structural analogue of alloxan, failed to stimulate probenecid-sensitive uptake.

Introduction

During the past decade several methods have been developed to obtain enriched basal-lateral membranous vesicles from mammalian renal proximal tubules [1–3]. These vesicles contain a specific transport system for the organic anion, *p*-aminohippurate [1,3,4]. Also, these vesicles maintain transport characteristics qualitatively similar to those observed in slices of kidney and in isolated proximal tubules, but because the vesicles are free of metabolic functions and devoid of intracellular organelles, the molecular mechanism underlying the secretion of *p*-aminohippurate by proximal tubules can be studied more directly. However, the mechanism of transport of organic anions is not known. In previous publications [5,6], we have suggested that the *p*-aminohippurate transport system in renal basal-lateral membranous vesicles prepared from rabbit renal cortex contains sulfhydryl groups which are essential for maintaining uptake activity. When vesicles were incubated with organic mercurials such as mersalyl acid, *p*-chloromercuriphenylsulfonate, mercury-containing derivatives of thiosalicylate [6] and 5,5′-dithiobis(2-nitrobenzoate)(DTNB) [5], the *p*-aminohippurate uptake activity decreased to 20–50% of its control value. On the other hand, several neutral and hydrophilic sulfhydryl reagents such as *N*-ethylmaleimide, iodoacetamide and diamide had little or no effect on the uptake of *p*-aminohippurate [5]. Taken together, these results suggest that only certain specific membrane sulfhydryl groups play a role in the translocation of *p*-aminohippurate across the basal-lateral membrane. Because the interaction of sulfhydryl groups of membrane proteins could be important in conformational changes which could mediate transport, we have further studied the effect of certain sulfhydryl reagents on

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transport of *p*-aminohippurate. Interestingly, alloxan, an unstable sulfhydryl reagent with some selectivity [7], was found to stimulate the total and probenecid-sensitive *p*-aminohippurate uptake in a time-dependent and concentration-dependent manner.

Materials and Methods

Preparation of Membranes. Renal basal-lateral membranous vesicles were prepared from New Zealand white rabbits using a combination of differential centrifugation and sucrose gradient centrifugation in a zonal rotor as described previously [3,5]. This procedure yielded preparations with an average 12-fold enrichment in the specific activity of the basal-lateral enzyme marker, $Na^+ + K^+$-ATPase. Membranes so prepared exhibit transport characteristics of basal-lateral membranes and they do not contain functions characteristic of brush borders. The membranes were suspended in 50 mM mannitol, 6.9 mM Hepes-Tris (pH 7.0) and kept at 4°C overnight for use the following day. Transport activity is stable during this period of storage.

Transport studies. Transport of *p*-aminohippurate was measured using tritiated *p*-aminohippurate by the method of rapid filtration [3,5]. The uptakes were carried out at 37°C in the presence of 0.1 mM *p*-aminohippurate and 5 mM $MgCl_2$ with or without 6.7 mM probenecid. $MgCl_2$ was used because we have found it to stimulate probenecid-sensitive uptake of *p*-aminohippurate in our vesicle preparation. The exact conditions of each uptake study are listed in the appropriate figure legends. The probenecid-sensitive uptake was defined as the difference in uptakes measured in the absence and presence of 6.7 mM probenecid. All reagents except alloxan used to study transport were dissolved in 50 mM mannitol and 6.9 mM Tris-HCl buffer (pH 7.0). Alloxan decomposes rapidly in solution above pH 6.0 [7,8]. Therefore, it was freshly prepared daily in 10^{-3} M HCl. The alloxan and β-mercaptoethanol were added to the reaction mixture immediately prior to the uptake reaction which was initiated by the addition of *p*-aminohippurate and Mg^{2+}.

Analytical assays. The total surface bound sulfhydryl groups of basal-lateral membranes were measured using DTNB as described previously [5]. Type I sulfhydryl groups are defined as those measured in the absence of the detergent NP-40. Type II sulfhydryl groups were determined by subtracting the type I sulfhydryl groups from the total number of sulfhydryl groups measured in the presence of NP-40 (0.9% v/v). Type II sulfhydryl groups presumably are located in more hydrophobic domains of the membrane or on the inside surface of the vesicles. The concentration of protein was measured by the method of Bradford [9] using bovine serum albumin as a standard. The assays were linear over the range of concentrations measured.

Chemicals. All chemicals were of reagent grade. *p*-Amino[^{3}H]hippuric acid (374 μCI/mmol) was purchased from Amersham/Searle (Arlington Heights, IL). The other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Results

In the first series of experiments, the effect of 2 mM alloxan on the transport of *p*-aminohippurate into the vesicles was studied. The results (Table IA) show that when alloxan was added at the start of the uptake reaction, both the total uptake and the probenecid-sensitive uptake, measured at one minute, increased by 249% and 216%, respectively. In another series of experiments (Table IB), alloxan was allowed to preincubate with the vesicles for 30 min at 37°C at pH 7.0 before the uptake reaction was started. Under these conditions, the total *p*-aminohippurate uptake was affected minimally while the probenecid-sensitive *p*-aminohippurate uptake tended to be decreased to 45% of its control value (Table IB), although this did not reach statistical significance. The above data show that alloxan had to be added just prior to the start of uptake in order to be stimulatory. In all subsequent experiments, alloxan was used without preincubation.

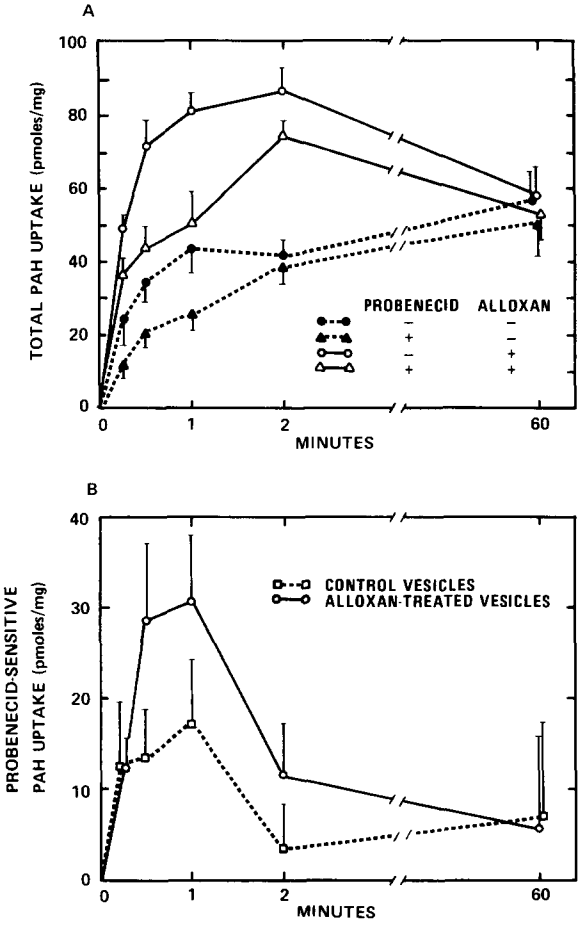
The next objective was to determine how the time-course of *p*-aminohippurate uptake was affected by alloxan. The results of a typical experiment are shown in Fig. 1. The total uptake of *p*-aminohippurate was increased by alloxan at all time points except at 60 min, when all uptakes approached each other (Fig. 1A). At 0.5, 1 and 2

TABLE I

EFFECT OF ALLOXAN ON THE *p*-AMINOHIPPURATE UPTAKE IN BASAL-LATERAL MEMBRANOUS VESICLES

The vesicles were incubated at 37°C for 30 min with or without 6.7 mM probenecid before uptakes were carried out. Uptake was measured after one minute. Statistical significance was analyzed by Student's paired *t*-test (two-tailed). n.s., not significant. The results in Expt. A are the averages of nine different experiments. Alloxan was added just prior to the start of *p*-aminohippurate uptake. The reaction occurred at 37°C in the presence of 0.1 mM *p*-aminohippurate, 5 mM $MgCl_2$, with and without 6.7 mM probenecid. The results in Expt. B are the averages of five experiments. Alloxan was incubated with the vesicles for 30 min at 37°C before the uptakes were started.

	PAH uptake (pmol/mg per min, mean ± S.E.)			
	Total uptake		Probenecid-sensitive uptake	
(A) No preincubation				
No alloxan	33.1 ± 2.4	$p < 0.001$	12.8 ± 1.5	$p < 0.02$
Alloxan, 2 mM	82.4 ± 6.6		27.7 ± 4.7	
(B) Preincubation				
No alloxan	43.6 ± 3.8	n.s.	17.7 ± 3.7	n.s.
Alloxan, 2 mM	38.7 ± 10.1		10.8 ± 4.4	



min, the probenecid-sensitive uptake was increased at least 2-fold when alloxan was present (Fig. 1B). In contrast, the probenecid-sensitive *p*-aminohippurate uptake at 15 s was similar for both the control and alloxan-treated vesicles. The lag time of 15 s for alloxan to increase probenecid-sensitive uptake was consistently observed as was the stimulation at 0.5, 1 min, and 2 min. By 60 min the uptakes of *p*-aminohippurate in the presence or absence of alloxan consistently approached each other.

In order to answer the question whether the stimulating effect of alloxan was concentration-dependent, a concentration-response curve was obtained (Fig. 2). Below 1 mM, alloxan had no stimulatory effect on the probenecid-sensitive uptake. However, at concentrations between 1 mM and 5 mM, the effect of alloxan appeared to increase in a linear fashion. Above 5 mM of alloxan, a maximal stimulation of *p*-aminohip-

Fig. 1. The effect of alloxan on the time-course of *p*-aminohippurate (PAH) uptake. Each time point represents the mean of quadruplicate determinations ± S.E. (A) The total uptake of 0.1 mM PAH was measured in the presence (+) and absence (−) of both 2 mM alloxan and 6.7 mM probenecid. (B) Probenecid-sensitive uptake in the presence and absence (control) of alloxan was determined by subtracting the PAH uptake in the presence of probenecid from the uptake in the absence of probenecid.

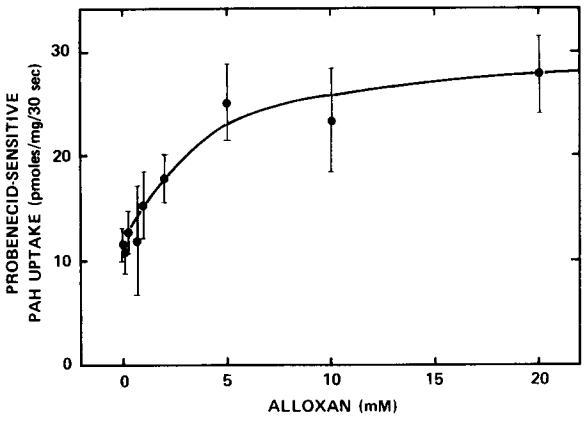


Fig. 2. Concentration-response curve for the stimulatory effect on probenecid-sensitive uptake. The concentration of *p*-aminohippurate (PAH) was 0.1 mM and that of probenecid was 6.7 mM. Each point is the average of the results from four experiments ± S.E.

purate uptake of about 25 pmol/mg per 30 s was attained.

In order to determine if the time-dependent and concentration-dependent stimulation of *p*-aminohippurate uptake by alloxan was due to an effect on something other than the *p*-aminohippurate transporter, we determined whether alloxan simply affected the intravesicular volume, as determined by the glucose space of the vesicles [10]. Thus, the uptake of glucose measured at 60 min was determined at various concentrations of alloxan (Table II). No effect on the glucose space was observed in the presence of 2 mM and 5 mM alloxan. There was a trend for 10 mM and 20 mM alloxan to diminish the glucose space by 30% and 43%, respectively; the latter reached statistical significance. Since 2 mM alloxan had no effect on the glucose space, the remainder of the experiments were performed with 2 mM alloxan.

TABLE II

EFFECT OF ALLOXAN ON 60-min GLUCOSE UPTAKE IN BASAL-LATERAL MEMBRANOUS VESICLES

Number of experiments in parentheses. The data were analyzed by Student's paired *t*-test (two-tailed) before converting to percent. 100% = 1440 ± 213 pmol/mg per 60 min. The concentration of glucose in the reaction mixture was 0.47 mM.

	% of control		
Control	100		
2 mM alloxan	91 ± 27		(6)
5 mM alloxan	85 ± 7		(6)
10 mM alloxan	70 ± 79		(4)
20 mM alloxan	57 ± 10	$p < 0.05$	(4)

In order to test whether alloxan enhanced *p*-aminohippurate uptake by changing the net charge of the membrane, which could then facilitate the movement of anions in a nonspecific manner, the Na^+-dependent, and the Na^+-independent uptake of glutamate which probably represents diffusion into the vesicles, was studied (Table III). 2 mM alloxan had no significant effect on the Na^+-independent uptake of glutamate measured in the presence of an initial 100 mM gradient of K^+. The only change noted in the uptake of glutamate was a decreased uptake in the presence of Na^+ occurring at 0.5 min. In contrast to affects on *p*-aminohippurate, alloxan had either no effect or a slightly inhibitory effect on the uptake of glutamate.

TABLE III

EFFECT OF ALLOXAN ON GLUTAMATE UPTAKE IN BASAL-LATERAL MEMBRANOUS VESICLES

The results are the average of four experiments (mean ± S.E.). The uptakes for each experiment were standardized using the average 1-min uptake in the presence of 100 mM Na^+ as 100% (51.2 pmol/mg). The concentration of glutamate in the reaction mixture was 0.025 mM.

	Glutamate uptake (%) of control uptake measured at 1 min)					
	100 mM Na^+			100 mM K^+		
	15 s	30 s	1 min	15 s	30 s	1 min
Control	49 ± 10	92 ± 14	100	9 ± 7	28 ± 7	58 ± 15
2 mM alloxan	45 ± 9	69 ± 8	81 ± 9	12 ± 4	22 ± 8	57 ± 13

The above results suggested that the effect of alloxan on the probenecid-sensitive *p*-aminohippurate uptake is due to an interaction with the transporter rather than due to some general effect on the membrane. However, the mechanism of this stimulation remained unknown. The following series of experiments were designed to elucidate this mechanism. It has been known that alloxan is a sulfhydryl reagent [7]. Therefore, the stimulatory effect of alloxan could result from this property. The interaction of alloxan with sulfhydryl groups in the basal-lateral membranes was measured. The vesicles were incubated for 30 min with 2 mM alloxan at 37°C, and then washed two times with 6 ml of buffer by centrifugation at 13000 rpm for 30 min in an SS34 rotor (Sorvall). Sulfhydryl groups on the vesicles were then determined with DTNB. 2 mM alloxan decreased the measurable type I sulfhydryl groups from an average control value of 48.8 ± 15.6 ($\pm$S.D., $n = 5$) to 22.5 ± 6 nmol/mg protein ($\pm$S.D., $n = 4$), indicating that about 26 nmol/mg or 54% of the type I sulfhydryl groups reacted with alloxan. However, only 5% of the type II sulfhydryl groups (average control value of 35.4 ± 12.3 nmol/mg protein) reacted with alloxan.

In order to test whether alloxan stimulated the *p*-aminohippurate uptake by reacting with membrane sulfhydryl groups, we tested whether pre-alkylation of membrane sulfhydryl groups by *N*-ethylmaleimide would abort the stimulatory effect of alloxan. We have shown that 2 mM *N*-ethylmaleimide reacts with 83% of type I and 25% of type II membrane sulfhydryl groups without affecting *p*-aminohippurate uptake [5]. The result (Table IV) showed that a 30 min preincubation with 2 mM *N*-ethylmaleimide prevented the stimulation of probenecid-sensitive *p*-aminohippurate uptake by alloxan. The result suggests that the stimulatory effect of alloxan is the result of a reaction with a membrane sulfhydryl group.

In order to further test the hypothesis that alloxan affects transport of *p*-aminohippurate by interacting with sulfhydryl groups, we tested the effect of β-mercaptoethanol, which contains a sulfhydryl group and which could act as a scavenger for alloxan. β-Mercaptoethanol, in concentration of 2 mM, 5 mM and 10 mM, was added to the reaction mix just prior to the addition of alloxan. We also examined the effect of β-mercaptoethanol alone on *p*-aminohippurate uptake. Both alloxan and β-mercaptoethanol were freshly prepared. The uptake of *p*-aminohippurate was measured at 37°C for 30 s after the vesicles were preincubated for 30 min in the presence or absence of 6.7 mM probenecid. The results of these experiments are listed in Table V. As before, 2 mM alloxan approximately doubled the proben-

TABLE IV

EFFECT OF *N*-ETHYLMALEIMIDE (NEM) ON THE STIMULATORY EFFECT OF ALLOXAN

Reagent I was incubated with the vesicles at 37°C for 30 min before the uptake reaction was started. Reagent II was added immediately before the uptakes were started. Uptakes were measured at 1 min. The data were analyzed by Student's paired *t*-test (two-tailed). n.s., not significant. PAH, *p*-aminohippurate.

Reagent I (2 mM)	Reagent II (2 mM)	Probenecid-sensitive PAH uptake		
		pmol/mg/min (mean ± S.E.)		% of control
None [a]	None	12.7 ± 1.2	$p < 0.01$	100
None [a]	Alloxan	26.3 ± 4.0		207
NEM [b]	None	10.6 ± 2.4	n.s.	82
NEM [b]	Alloxan	11.1 ± 1.7		90

[a] The results are the average of 11 experiments.
[b] The results are the average of four experiments.

TABLE V

EFFECTS OF β-MERCAPTOETHANOL ON THE STIMULATORY EFFECT OF ALLOXAN

The results are expressed as the means ± S.E. Number of experiments in parentheses. Uptakes were measured at 30 s. PAH, *p*-aminohippurate.

	Probenecid-sensitive PAH uptake (pmol/mg per 30 s)
Control	23.0 ± 3.1 (4)
2 mM alloxan	52.7 ± 7.3 (3)
5 mM β-mercaptoethanol	23.6 ± 4.0 (4)
2 mM β-mercaptoethanol + 2 mM alloxan	26.7 ± 3.9 (4)
5 mM β-mercaptoethanol + 2 mM alloxan	24.0 ± 6.6 (4)
10 mM β-mercaptoethanol + 2 mM alloxan	25.5 ± 7.1 (3)

TABLE VI

EFFECTS OF SULFHYDRYL REAGENTS ON THE PROBENECID-SENSITIVE *p*-AMINOHIPPURATE UPTAKE INTO BASAL-LATERAL MEMBRANOUS VESICLES

Reagents [a]	PAH uptake (pmol/mg per 30 s, mean ± S.E.)		PAH uptake (% of control)
Control (none)	13.1 ± 1.2 (12)		100
2 mM alloxan	25 ± 1.8 (12)	$p < 0.001$	208
2 mM iodoacetamide	15.6 ± 4.4 (3)		109
2 mM NEM	9.1 ± 1.7 (6)		82
0.2% H_2O_2 (0.11 M)	16.1 ± 2.3 (7)	$p < 0.1$	128
0.02% H_2O_2	12.7 ± 2.9 (6)		116

[a] The reagents were added just prior to the start of uptakes which were carried out at 37°C for 30 s in the presence of 5 mM Mg^{2+} and 0.1 mM *p*-aminohippurate (PAH). Uptakes were measured at 30 s. Number of experiments in parentheses. The data were analyzed by Student's paired *t*-test (two-tailed). Statistical differences refer to differences from control. NEM, *N*-ethylmaleimide.

ecid-sensitive uptake measured at 30 s. 5 mM β-mercaptoethanol alone had no effect on probenecid-sensitive *p*-aminohippurate uptake. In addition, 2 mM, 5 mM and 10 mM β-mercaptoethanol completely abolished the stimulatory effect of alloxan. Thus in the presence of a sulfhydryl containing scavenger for alloxan, all the effects of alloxan were blocked.

The effects of other sulfhydryl reagents are listed in Table VI. The alkylating sulfhydryl reagent, 2 mM iodoacetamide, had very little stimulatory effect. However, the oxidizing agent, 0.2% H_2O_2 (0.11 M) seemed to have a small stimulatory effect, albeit much less than that of 2 mM alloxan. The result suggests that the effect of alloxan is fairly unique because no other sulfhydryl reagent duplicated alloxan's action. Furthermore, ninhydrin, a compound with chemical properties similar to that of alloxan [11], and glucose, a structural analogue of alloxan, did not mimic the stimulatory effect of alloxan.

Discussion

Several conclusions can be drawn from the present studies.

(1) Alloxan, but not its breakdown product, alloxanic acid, enhanced the probenecid-sensitive *p*-aminohippurate uptake into basal-lateral membranous vesicles.

(2) This stimulatory effect was dependent on the concentration of alloxan.

(3) Alloxan did not affect either the transport or diffusion of another anion, glutamate.

(4) Alloxan did not increase the apparent intravesicular volume as measured by the glucose space. Since preincubating the vesicles with *N*-ethylmaleimide abolished the stimulatory effect of alloxan it seems that alloxan may exert its effect by a reaction with specific sulfhydryl groups. This finding, coupled to previously reported data on the importance of sulfhydryl groups to the integrity of the *p*-aminohippurate transporter [5,6], indicates that sulfhydryl groups play an integral role in the function of a probenecid-sensitive *p*-aminohippurate transporter of renal basal-lateral membranes.

The stimulation of probenecid-sensitive *p*-aminohippurate uptake by alloxan only occurred more than 15 s after the addition of alloxan, and the stimulation reached its maximal value within the next 15 to 45 s. When alloxan was allowed to incubate with the vesicles for 30 min, not only was the stimulatory effect not observed, but the probenecid-sensitive *p*-aminohippurate uptake seemed to be inhibited (Table I). Furthermore, when alloxan and the vesicles preincubated together for a period of 30 s or less, stimulation of the probenecid-sensitive *p*-aminohippurate uptake 30 s into the uptake reaction was still observable (data not shown). It has been known that alloxan is unstable in solution when the pH is above 6.0 [7]. Under this condition, alloxan decomposes to form alloxanic acid [12]. The half-life of alloxan at 37°C and pH 7.4 is about 1.38 min [8]. Thus, the stimulation by alloxan required a short activation time and the effect was evanescent, possibly due to the rapid decomposition of alloxan.

The stimulatory effect of alloxan on probenecid-sensitive uptake of *p*-aminohippurate appeared to be due to an action on the transporter. This conclusion is based on the following observations. First, alloxan did not increase the apparent intravesicular volume of the membranous vesicles as evidenced by the experiments showing that up to 10 mM alloxan did not significantly alter the

60-min glucose uptake, a common indicator of the intravesicular volume [10]. The same conclusion can also be drawn from the result of turbidimetric determinations [13]. A 1-min or a 30-min incubation of vesicles with 2 mM alloxan did not change the percent transmission of the vesicles at 700 nm. This suggests that there is no fragmentation of the vesicles. Also, when compared to control, alloxan did not stimulate the uptake of glutamate, another anion, suggesting that the enhanced uptake of *p*-aminohippurate was not due to a change in the net charge or charge density of the vesicles.

The mechanism by which alloxan stimulates *p*-aminohippurate uptake is not certain. However, the present data suggest that the stimulatory effect of alloxan is probably related to its reactivity with sulfhydryl groups. Alloxan reacted with 54% of the type I sulfhydryl groups and 5% of the type II sulfhydryl groups in the basal-lateral membrane. Preincubation of the vesicles with *N*-ethylmaleimide, which reacts with 83% of the Type I membrane sulfhydryl groups [5], abolished the stimulatory effect of alloxan. Furthermore, when alloxan and β-mercaptoethanol were added simultaneously to the reaction solution, no stimulation was seen. β-Mercaptoethanol contains a sulfhydryl group which would react with alloxan, thus blocking alloxan's ability to react with a sulfhydryl group of the transporter. Preliminary work with iodoacetamide, which reacts with 22% of type I sulfhydryl groups but no type II sulfhydryl groups [5], has shown that a 30-min preincubation with 2 mM iodoacetamide also eliminates the stimulatory effect of alloxan. The reaction with type I sulfhydryl groups suggests that alloxan reacts primarily with those sulfhydryl groups on the surface of the membrane since type I sulfhydryl groups are defined as those measured in the absence of detergent.

The reactions of alloxan with protein sulfhydryl groups are complex and are not well understood [7]. It probably oxidizes sulfhydryl groups to disulfide as well as forming addition products [14]. Both reactions are possible when alloxan reacts with the membrane proteins. It remains to be seen which of these two reactions contribute to alloxan's stimulatory effect. However, H_2O_2, which oxidizes sulfhydryl groups to disulfide, had a tendency to stimulate probenecid-sensitive *p*-aminohippurate uptake, albeit at a much higher concentration (110 mM) than alloxan (2 mM). This suggests that oxidation of sulfhydryl groups to disulfide by alloxan may be a factor in its mechanism of action.

It is intriguing that whereas mercurial sulfhydryl reagents inhibit *p*-aminohippurate uptake in both proximal tubules [15,7] and in basal-lateral membranous vesicles [5,6] alloxan stimulates the probenecid-sensitive *p*-aminohippurate uptake. Interestingly, a stimulation of *p*-aminohippurate uptake has also been observed in proximal tubules by very low concentrations of mercurials [15]. It is possible that alloxan and the higher concentrations of the mercurials are affecting different organic anion uptake systems, whereas the low concentration of mercurials and alloxan are affecting the same system. There is ample evidence to support the concept of multiple transport systems for organic anions in the kidney [16–18].

It has been reported that disulfide bonds are important to the function of a D-glucose transporter in brush-border membranes [19]. Robillard and Konings [20] have also proposed that thiol-disulfide exchanges may play a role in transport processes.

It is also possible that the stimulation of alloxan may involve the perturbation of the structure of the membrane's bilayer as has been suggested for a stimulatory effect of *N*-ethylmaleimide on the transport of leucine in the Chang liver cell [21]. Finally, it must be pointed out that alloxan, *N*-ethylmaleimide and iodoacetamide all have the ability to react with protein amino groups at a rate much lower than the rate of reaction with sulfhydryl groups. The possibility that the stimulatory effect of alloxan is linked to its reactivity with protein amino groups cannot be totally ruled out. Future experiments are required to elucidate mechanisms of action of alloxan.

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