IN VIVO DEPRESSION OF HEPATIC SUCCINOXIDASE BY SULFHYDRYL COMPOUNDS

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SUMMARY:

Thioglycolate injected i.v. in dosages of 50 mg/kg, significantly depressed hepatic succinoxidase in immature rats of either sex and adult males whereas, about a five-fold excess was required to elicit a significant response in adult females. Ovariectomy increased and estradiol pretreatment, decreased the sensitivity to thioglycolate. Of a number of sulfur compounds screened on hepatic succinoxidase in males, only 2-mercaptoethanol shared this action with BAL and thioglycolate. The latter two agents were without effect on cytochrome oxidase and diaphorase but both depressed NADH cytochrome c reductase. It is suggested that the mechanism of succinoxidase inhibition involves a combination with Slater's factor, thereby causing a disruption in the normal flow of electrons.

BAL has been shown to inhibit hepatic succinoxidase (SO) at lower dosages in male rats and rabbits as compared to adult females (1). The present report extends such studies to other sulfhydryl compounds in an attempt to relate structure to SO depressive activity and to further explore the sex difference in regard to the sensitivity.

MATERIALS AND METHODS:

The compounds and reagents were synthesized or purchased from Eastman Kodak and other chemical sources. BAL, $CH_2(SH)CH(SH)CH_2OH$, originated from Hynson, Westcott and Company and Evans Chemetics Company supplied thioglycerol, $CH_2(SH)CH(OH)CH_2OH$, as a 50% aqueous solution, 2-mercaptoethylamine hydrochloride and thioglycolic acid, $CH_2(SH)COOH$ (TC). The latter was purified further by two distillations (b. 105-108° at 20 mm; collected in water); the oxidation product, dithioglycolic acid, melted at 107-108° after several recrystallizations from water. Except for cysteine hydrochloride and reduced glutathione, concentrated stock solutions containing the acids in amounts of

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up to 500 mg/ml were brought to a pH of 7.2-7.4 with cold aqueous sodium hydroxide; hydrogen sulfide was also prepared in alkaline medium. In all instances, the dosages are given in terms of the free acids. SO was assayed manometrically in the Warburg apparatus (2). For the in vitro evaluation of cytochrome oxidase and NADH cytochrome c reductase in rat liver mitochondria in the presence of sulfhydryl agents, the decrease in absorption at 550 mμ was ascertained (3). Diaphorase was determined by the 2,6-dichlorophenolindophenol procedure (4).

Holtzman rats were maintained on Purina chow and water ad lib. The agent was injected i.v., the controls receiving an equivalent volume of saline. The rats were sacrificed 30 min later by swift decapitation, the abdomen incised and the liver removed rapidly and placed in chilled Sorensen buffer of pH 7.4. Homogenates were prepared with this buffer and 0.5 ml of the final mixture removed for SO assay. In one series, the animals were sacrificed at other specified periods following the injections. In yet another group of experiments, immature males as well as bilaterally gonadectomized and sham-operated females were treated with estradiol benzoate in sesame oil, the controls receiving the oil alone, prior to the administration of TG or saline.

RESULTS:

Effect of TG and Other Compounds on Hepatic SO Levels. The action of intravenously administered TG and a variety of other sulfur compounds on hepatic SO together with the respective Fisher t-values in the comparisons with the controls is shown in Table 1. In all cases, the animals survived and the tissues were essentially normal on gross examination. Preliminary screening indicated that dosages of TG at 10 and 30 mg/kg did not significantly alter hepatic TG in males but inhibition was definite at a level of 50 mg/kg. The decrease is quite persistent, the effect continuing beyond the third day after injection of 75 mg/kg in males with a return to the control range on the seventh day. In marked contrast, when similar experiments were conducted with adult females, dosages of about 250 mg/kg and up were necessary to produce a

Table 1

Hepatic SO of Rats Injected I.V. with Various Sulfur Compounds on a Sex Basis[a]

Agent (mg/kg)	Average Body Weight, g	SO Activity, Q_{O_2} ± SE Control (No. of Rats)	Treatment (No. of Rats)	t
MALES				
TG (50)	172	104 ± 8.7 (8)	82 ± 4.9 (8)	2.20*
TG (75)	175	93 ± 4.1 (13)	71 ± 4.3 (13)	3.63**
TG (75; 18 hr)[b]	323	106 ± 5.6 (8)	72 ± 5.0 (8)	4.30**
TG (75; 3 days)		104 ± 4.8 (8)	85 ± 4.4 (8)	2.90
TG (75; 7 days)		109 ± 5.7 (12)	100 ± 4.6 (11)	1.34
Methyl thioglycolate (173)	120	66 ± 1.8 (11)	40 ± 2.5 (11)	8.38**
2-(2-Methoxyethoxy)ethyl thioglycolate (323)			35 ± 0.3 (11)	15.10**
2-Mercaptoethanol (125)	116	65 ± 6.9 (11)	38 ± 4.3 (10)	3.20**
Thiodiglycolic Acid (500)			69 ± 3.4 (12)	0.64
Dithioglycolic Acid (300)	111	106 ± 4.2 (8)	91 ± 6.1 (10)	1.65
α-Mercaptopropionic acid (175)	90	89 ± 7.1 (10)	87 ± 8.1 (11)	0.19
β-Mercaptopropionic acid (175)	112	106 ± 4.2 (8)	97 ± 6.4 (10)	1.02
2-Mercaptoethanesulfonic acid (220)	105	73 ± 4.4 (9)	74 ± 3.4 (11)	0.18
2-Mercaptoethylamine·HCl (175)			73 ± 3.5 (11)	
Thiomalic acid (500)	297	95 ± 4.5 (9)	94 ± 4.0 (12)	0.12
α,α'-Dimercaptoadipic acid (500)			94 ± 5.2 (12)	0.11
Thiourea (125)	275	73 ± 4.9 (10)	74 ± 4.1 (12)	0.14
Thioglycerol (300)			79 ± 3.0 (12)	0.96
Hydrogen Sulfide (5.0)[c]	143	64 ± 6.2 (10)	68 ± 5.9 (11)	0.49
Glutathione, reduced (500)	110	80 ± 1.5 (10)	87 ± 6.4 (12)	0.88
Cysteine·HCl (400)	243	94 ± 7.6 (8)	87 ± 3.2 (7)	0.77
FEMALES				
TG (75)	63	90 ± 7.4 (10)	65 ± 4.0 (11)	3.09**
TG (150)	165	81 ± 5.4 (10)	71 ± 3.0 (11)	1.74
TG (250)	189	104 ± 7.0 (14)	69 ± 3.4 (16)	4.68**

[a]Except for the one series as specified, the animals were sacrificed 30 min after injection and the individual liver homogenates prepared.

[b]Rats in this series were sacrificed 18 hr, 3 days and 7 days after i.v. TG, a saline-injected or control group being employed at each interval.

[c]Adjusted to pH 7.8 with 1.26 N NaOH.

*$P < 0.05$.

**$P < 0.01$.

significant depression. However, as with the males, a level of 75 mg/kg led to a decrease in activity in immature females. Of a number of compounds

tested in males, aside from BAL and TG and its esters, only 2-mercaptoethanol

Table 2

Influence of Ovariectomy and Estrogen Administration on the Hepatic Response of Rats to TG

Treatment	No. of Rats	Body Weight, g ± SE Initial	Body Weight, g ± SE At Necropsy	SO Activity Q_{O_2} ± SE	t
FEMALES[a,b]					
A. Sham-operated; 8 injections of oil, then saline	11	197 ± 9.7	206 ± 9.9	46 ± 4.2	
B. Sham-operated; 8 injections of oil; TG i.v. at 100 mg/kg	12	201 ±10.1	204 ± 9.5	54 ± 5.1	1.13
C. Ovariectomized; as per Group A	11	221 ±11.8	221 ±10.7	53 ± 5.0	
D. Ovariectomized; as per Group B	12	234 ±13.0	229 ±11.1	36 ± 5.0	2.40*
E. Ovariectomized; 5-0.05 mg injections of estradiol benzoate, then 3-0.25 mg injections of the hormone; i.v. saline	12	218 ±12.3	200 ±10.2	56 ± 4.4	
F. Ovariectomized; as per Group E except that TG at 100 mg/kg was given i.v.	12	214 ±12.1	191 ± 9.6	49 ± 4.9	1.80
MALES[b]					
AA. 4 injections of oil followed by i.v. saline	13	84 ± 1.8	94 ± 2.2	78 ± 5.2	
BB. As per Group AA except for TG at 100 mg/kg i.v.	13	78 ± 1.4	92 ± 2.3	55 ± 3.5	3.53**
CC. 4-0.25 mg estradiol benzoate injections prior to i.v. saline	13	84 ± 2.1	91 ± 1.9	67 ± 4.4	
DD. As per Group CC but with i.v. TG at 100 mg/kg	13	88 ± 1.5	67 ± 1.0	64 ± 3.0	0.45

[a]The animals comprised a single shipment and averaged 80 g in weight at surgery. They were ovariectomized or sham-operated 59 days prior to injection with estradiol benzoate in sesame oil or the oil as such. The initial weights are the means at the start of such treatment.

[b]Estrogen solution or sesame oil alone in amount of 0.25 ml was injected s.c. on alternate days. Saline or TG was administered i.v. 48 hr following the last oil injection, the rats being sacrificed 30 min later.

*$P < 0.05$. **$P < 0.01$.

(125 mg/kg) proved inhibitory. In addition to those cited in Table 1, the following were also ineffective at the levels specified: 3-mercapto-2-hydroxypropane-1-sulfonic acid (500 mg/kg), thiosorbitol (3,000 mg/kg), isethionic acid (200 mg/kg) and acetate (5,000 mg/kg).

Endocrine Factors Influencing the Response of Hepatic SO to TG. In view of the sex differences noted above, immature rats previously spayed or sham-operated (controls) were treated with estrogen or the oil as such as detailed in Table 2. The animals were subdivided and injected i.v. 30 min before necropsy with (a) saline and (b) TG at 100 mg/kg. The latter dosage, previously shown to be too low for a response from the adult female, was likewise ineffective in the sham-operated controls and ovariectomized rats given estradiol, but proved inhibitory to spayed animals injected with oil. Further confirmation was obtained with intact immature males, estrogen blocking the effect of TG. Body weights were well maintained except for about 10% losses for females of Groups E and F and a 20% decrease for males of Group DD (Table 2).

Effect of Sulfur Compounds on Other Enzyme Levels (In vitro). Diaphorase and NADH cytochrome c reductase data for rat mitochondrial preparations in the presence of about 0.1 mg each of TG and BAL are presented in Table 3. Both

Table 3

Liver Mitochondrial Diaphorase and NADH Cytochrome c Reductase and Homogenate Cytochrome Oxidase Levels as Influenced by BAL and TG[a]

Agent (mg)	Diaphorase ΔA_{600}/5 min		NADH Cytochrome c Reductase ΔA_{550}/5 min	Cytochrome Oxidase ΔA_{550}/5 min
Control (Saline)	0.710[b]	0.620[c]	0.300	0.300
TG (0.10)	0.650[b]	0.540[c]	0.130	0.290
BAL (0.08)	0.660[b]	0.560[c]	0.135	0.270

[a]Different rat liver mitochondrial preparations were used for each enzyme.

[b]NAD added to the system.

[c]NADP-specific.

compounds depressed the level of the reductase whereas, dithioglycolate and α,α'-dimercaptoadipate, each at 0.1 mg, were without action. The four agents did not alter the control diaphorase and cytochrome c values to any appreciable extent.

DISCUSSION:

The structural similarity is quite evident between mercaptoethanol and TG, a primary alcohol and the corresponding carboxylic acid, which in addition to BAL, caused in vivo depression of hepatic SO. The structure of TG is unique in this regard when it is considered that other α-mercapto-acids as well as β-mercapto types proved ineffective. Yet BAL which could be converted to α,β-dimercaptopropionic acid, a derivative containing a mercaptomethyl group adjacent to the "TG residue" and unlike thiolactic or α-mercaptopropionic acid, may cause an inhibitive effect. However, no experiment of this type was instituted. Demercaptomethylation of this compound to TG is not too likely but the anticipated action may be predicated on differences in electronegative properties, among others, as compared to thiolactate. The presence of a free carboxyl group as such is not necessary for an effect since esters and the alcohol are also active but the presence of a sulfhydryl group is a prime requisite. Aside from certain structural relationships, the mechanism of SO inhibition by TG is similar, if not identical, to BAL, since both inhibit SO without affecting cytochrome oxidase and they depress NADH cytochrome c reductase and have no action on diaphorase.

According to the classical work of Slater employing heart muscle preparations, the aerobic oxidation of succinate requires an additional factor operating between succinic dehydrogenase and cytochrome c. The addition of BAL causes complete inactivation of SO without affecting succinic dehydrogenase or the cytochrome oxidase portion because of the oxidation of a factor essential for the transfer of electrons from cytochrome b to cytochrome c (5,6). As a result, the normal flow of electrons is interrupted and inhibition ensues. The depression of hepatic SO by BAL has also been demonstrated in vivo (1) and presumably, the mechanism also applies to TG. Destruction of the BAL-labile

factor does not influence the oxidation of succinate when methylene blue is employed as the electron carrier, nor the oxidation of various reducing agents through cytochrome c and cytochrome oxidase. This would explain the failure of both TG and BAL to inhibit diaphorase which in the analysis utilizes 2,6-dichlorophenolindophenol as an electron acceptor and by-passes the pathway in which the labile factor is an integral component. Slater also demonstrated that BAL inactivates the NADH oxidase system. In this connection, the addition of cytochrome c in the presence of SO does not alter the inhibitory effect of TG and BAL on the NADH oxidase system. This finding lends further support to the contention that the BAL-sensitive factor operates as a link between the succinic and NADH oxidase systems.

In regard to the sex differences noted in the present study, Yielding and Tomkins (7) found that low levels of progesterone and other steroid hormones inhibit the oxidation of NADH in rat skeletal muscle without impairment of either NADPH or succinate oxidation. The participation of NADP and the transhydrogenation reaction was very unlikely. It might be mentioned that as one of the components of the NADH cytochrome c reductase system is a flavoprotein, higher levels of steroids inhibit various flavoproteins (8). It is shown in the current report that ovariectomized rats displayed a greater susceptibility to TG than rats which had been pretreated with estradiol (Table 2). This observation is in complete agreement with those of Hollander and Stephens (9) who showed an estrogen-induced oxidation of NADH. Consequently the flow of electrons could be maintained and the effect of TG negated.

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