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Inhibition of Aldose Reductases from Rat and Bovine Lenses by Hydantoin Derivatives

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The development of potent aldose reductase inhibitors as therapeutic agents for diabetic complications is highly desirable. The inhibitory action of 54 hydantoin derivatives consisting of 25 hydantoins, 21 2-thiohydantoins and 8 2-alkylthiohydantoins was therefore tested on rat and bovine lens aldose reductases *in vitro*. 1-(Phenylsulfonyl)-hydantoin (**18**) and its derivatives, 1-[(substituted phenyl)sulfonyl]hydantoins, were found to be potent inhibitors of the enzymes. 1-[(*p*-Bromophenyl)sulfonyl]hydantoin (**49**) was the most potent among them. It inhibited purified rat and bovine lens aldose reductases by 50% at 7×10^{-7} M and 3.7×10^{-7} M, respectively. Inhibition of rat and bovine lens aldose reductases by this compound (**49**) was due to its non-ionized form, but not the ionized form, and was of a non-competitive type with respect to DL-glyceraldehyde as a substrate.

Keywords——aldose reductase; aldose reductase inhibitor; hydantoin derivatives; rat lens; bovine lens; diabetic complications

Abnormally large amounts of sorbitol and galactitol are known to accumulate in the lenses of diabetic and galactosemic animals, respectively. Elevated levels of sorbitol have also been detected in both the lenses[1] and the peripheral nerves[2] of diabetic patients. Studies in animal models and man suggested that tissue accumulation of excessive sorbitol and galactitol synthesized by the action of aldose reductase (EC 1.1.1.21) on glucose and galactose may trigger sugar cataract formation and peripheral neuropathy in diabetes and galactosemia.[3] In support of this view, inhibition of aldose reductase has been shown to delay the onset of sugar cataract in both diabetic and galactosemic animals, and it has also been shown that aldose reductase is present in tissues in which diabetic complications occur, for example, the lenses and Schwann cells.[3a] These findings suggest that potent aldose reductase inhibitors may be of value in the treatment of some of these complications.

In recent years, Varma and Kinoshita[4] reported that some flavonoids, for example quercitrin, have a striking inhibitory action on lens aldose reductase. More recently, sorbinil (*d*-6-fluoro-spiro[chroman-4,4′-imidazolidine]-2′,5′-dione), which is a hydantoin derivative, was shown to be the most potent inhibitor of the enzyme known so far.[5,6] However, both quercitrin and sorbinil are insufficiently potent inhibitors for the prevention and/or amelioration of complications of diabetic patients.

In this paper, we present the results of a screening test for inhibitory activity of 54 hydantoin derivatives on rat and bovine lens aldose reductases, and describe the effects of structural alterations on the inhibitory activity. We also report the difference in susceptibility to inhibition by hydantoins between rat lens aldose reductase (RLAR) and bovine lens aldose reductase (BLAR), the effect of pH on the inhibition of aldose reductase by hydantoin compounds, and the inhibitory effects of hydantoins on some enzyme activities other than that of aldose reductase.

Experimental

Materials——Lenses were removed from eyes of rats of the Wistar strain weighing 200—250 g. Bovine eyes were obtained from a local abattoir, and the lenses were removed and frozen until used. $NADP^+$, NADPH, lactate dehydrogenase and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast Co., Ltd. (Osaka, Japan). NAD^+, NADH, 2-phosphoenolpyruvate, glucose 6-phosphate, ATP, ADP and glutathione (oxidized form) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydantoin, 2-thiohydantoin, DL-glyceraldehyde and pyridine-3-carbaldehyde were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). 5,5-Diphenylhydantoin was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 3-Ethyl-5-phenylhydantoin (Ethotoin) was a gift from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). DEAE-Sephacel and Sephadex G-75 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Mātrex gel red A was obtained from Amicon Co. (Lexington, MA, U.S.A.). Silica gel plates (Kieselgel 60 F_{254}) were from E. Merck AG (Darmstadt, Germany). All other chemicals were of the highest grade commercially available.

Determination of Infrared Spectra and Melting Points——Infrared (IR) spectra were determined on a Jasco IRA 1 spectrophotometer. Melting points were determined on a Yanaco MP apparatus and are uncorrected.

Preparation of Hydantoin-related Compounds (1—39)——Compounds **3**, **4**, **6**, **10**—**13**, **17**—**23**, **25**—**27**, **29**—**33** and **38** were prepared by the methods reported previously.[7] The preparation of compounds **7**—**9**, **14**—**16**, **24**, **28**, **34**—**37** and **39** will be reported elsewhere.

Preparation of 1-(Phenylsulfonyl)-2-thiohydantoin (PTH, 30) and Its Derivatives (40—47)——A suspension containing (substituted phenyl)sulfonyl chloride (10 mmol) and glycine (10 mmol) in 1.1 M K_2CO_3 (5 ml) was heated at 50°C for 10 min and then at 100°C for 30 min. The solution was made alkaline by dropping saturated K_2CO_3 during the reaction. After cooling, the solution was made acid with 10% HCl, and the *N*-[(substituted phenyl)sulfonyl]glycine precipitated was filtered off, washed with water, and recrystallized from hot water (yield 60—80%). A solution containing *N*-[(substituted phenyl)sulfonyl]glycine (2 mmol), NH_4SCN (2.4 mmol), and acetic anhydride (4 mmol) in anhydrous pyridine (1 ml) was heated with stirring at 90°C for 30 min, then cooled. Water (15—20 ml) was added to the solution and the mixture was stirred for 1 h at room temperature. The precipitate was filtered off, washed with water, and recrystallized from H_2O–EtOH. Each of the PTH derivatives prepared gave a single spot on thin layer chromatography with $CHCl_3$–MeOH–HCOOH (40: 10: 1, v/v/v) as a solvent system. Physical and spectral data of these compounds are listed in Table I.

TABLE I. Physical and Spectral Data for 1-(Phenylsulfonyl)-2-thiohydantoin (PTH) and Its Derivatives

Compd. No.	R	Yield (%)	mp (°C)	IR ν_{max}^{KBr} cm^{-1} (C=S)	(C=O)	(SO_2)	Formula	Analysis (%) Calcd (Found) C	H	N
30	(PTH) H	32.0	212—214[a] (210—211)[b]	1220	1760 1780	1160 1360	$C_9H_8N_2O_3S_2$	42.19 (42.18	3.15 3.14	10.94 10.97)
40	*p*-CH_3	36.6	228—232[a]	1230	1745 1775	1160 1360	$C_{10}H_{10}N_2O_3S_2$	44.43 (44.60	3.73 3.64	10.36 10.29)
41	*p*-Br	24.9	248—250.5	1240	1740 1780	1160 1360	$C_9H_7BrN_2O_3S_2$	32.25 (32.30	2.10 2.06	8.36 8.39)
42	*p*-Cl	32.3	231—233	1240	1740 1780	1160 1370	$C_9H_7ClN_2O_3S_2$	37.18 (37.22	2.43 2.34	9.64 9.69)
43	*p*-CH_3O	31.5	205—206	1220	1755 1780	1155 1365	$C_{10}H_{10}N_2O_4S_2$	41.96 (41.94	3.52 3.49	9.79 9.80)
44	*o*-NO_2	53.0	222—224[a]	1230	1730 1770	1170 1365	$C_9H_7N_3O_5S_2$	35.87 (35.89	2.34 2.26	13.95 14.02)
45	*m*-NO_2	23.1	229—231[a]	1240	1740 1780	1170 1380	$C_9H_7N_3O_5S_2$	35.87 (35.87	2.34 2.27	13.95 14.01)
46	*p*-NO_2	30.0	215—216[a]	1240	1745 1780	1160 1340	$C_9H_7N_3O_5S_2$	35.87 (35.88	2.34 2.26	13.95 14.01)
47	*p*-AcNH	31.8	224—225	1260	1650[c] 1740 1800	1150 1360	$C_{11}H_{11}N_3O_4S_2$	42.18 (42.16	3.54 3.54	13.42 13.42)

a) Decomposition.
b) Lit. 7*a*) and T.B. Johnson and W.M. Scott, *J. Am. Chem. Soc.*, **35**, 1130 (1913).
c) (C=O) of acetylamino group.

Preparation of 1-(Phenylsulfonyl)hydantoin (PSH, 18) and Its Derivatives——PSH (**18**) and its derivatives (**48**—**54**) were prepared by oxidation of their 2-thio compounds (**30** and **40**—**46**) with hot nitric acid according to the method of Shirai and Yashiro.[7a] PSH and its derivatives each gave a single spot on thin layer chromatography with $CHCl_3$–MeOH–HCOOH (40: 10: 1, v/v/v) as a solvent system. These compounds are listed in Table II with some of their physical properties.

Table II. Physical and Spectral Data for 1-(Phenylsulfonyl)hydantoin (PSH) and Its Derivatives

Compd. No.	R	Yield (%)	mp (°C)	IR ν_{max}^{KBr} cm^{-1} (C=O)	IR ν_{max}^{KBr} cm^{-1} (SO_2)	Formula	Analysis (%) Calcd (Found) C	H	N
18	(PSH) H	36.4	233—234[a]	1720	1160	$C_9H_8N_2O_4S$	45.01	3.36	11.67
			(236)[b]	1775	1340		(44.92	3.30	11.64)
			(241.5—242.5)[c]						
48	*p*-CH_3	36.3	229—233[a]	1715	1140	$C_{10}H_{10}N_2O_4S$	47.25	3.97	11.02
			(226)[d]	1780	1355		(47.10	3.96	11.00)
49	*p*-Br	29.8	248—250	1720	1150	$C_9H_7BrN_2O_4S$	33.87	2.21	8.78
				1780	1345		(33.82	2.21	8.79)
50	*p*-Cl	32.0	240—242	1720	1150	$C_9H_7ClN_2O_4S$	39.35	2.57	10.20
				1770	1345		(39.30	2.49	10.14)
51	*p*-CH_3O	44.3	228—230.5	1720	1140	$C_{10}H_{10}N_2O_5S$	44.45	3.73	10.37
				1780	1340		(44.31	3.64	10.28)
52	*o*-NO_2	42.5	240—242[a]	1710	1170	$C_9H_7N_3O_6S$	37.90	2.47	14.74
				1780	1350		(37.86	2.35	14.75)
53	*m*-NO_2	30.1	245—246[a]	1720	1160	$C_9H_7N_3O_6S$	37.90	2.47	14.74
				1780	1345		(38.09	2.54	14.34)
54	*p*-NO_2	34.0	248.5—251[a]	1720	1160	$C_9H_7N_3O_6S$	37.90	2.47	14.74
				1780	1340		(37.81	2.34	14.60)

a) Decomposition.
b) Lit. 7*a*).
c) I.S. Bengelsdorf, *J. Am. Chem. Soc.*, **75**, 3138 (1953).
d) G. Holan and E. Samuel, *J. Chem. Soc.*, **1961**, 4660.

Preparation of Inhibitor Solution——Because of the poor water-solubility of many hydantoin derivatives, all compounds were dissolved in propylene glycol. Usually a 10^{-3} M solution was prepared and diluted to the desired concentrations with propylene glycol.

Assay of Aldose Reductase Activity——Aldose reductase assays were conducted according to the procedure of Hayman and Kinoshita[8] except for the addition of ammonium sulfate instead of lithium sulfate to the reaction mixture. Assays were performed at 30°C in 0.1 M sodium phosphate buffer (pH 6.2) containing 0.4 M ammonium sulfate, 10 mM DL-glyceraldehyde, 0.16 mM NADPH and the enzyme (0.010—0.016 unit) in a total volume of 1.0 ml. The effect of enzyme inhibitor was determined by including 10 μl of an inhibitor solution in the reaction mixture. The reference blank to correct for non-specific reduction of NADPH contained all of the above compounds except for the substrate. The reaction was initiated by the addition of substrate, and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm on a Gilford model 250 spectrophotometer.

Purification of Lens Aldose Reductase——RLAR and BLAR were purified according to our method.[9] Briefly, a 40—75% ammonium sulfate fraction was subjected to chromatography on DEAE-Sephacel, following by two column chromatographic steps, *i.e.* affinity chromatography using Mātrex gel red A and gel filtration on Sephadex G-75. By this procedure, RLAR and BLAR were purified over 380-fold and 12000-fold, respectively.

Preparation of Crude Rat Lens Aldose Reductase (Crude RLAR)——The supernatant of the homogenate of rat lenses was prepared according to the method of Kador and Sharpless[10] and used as crude RLAR for determining IC_{50} values (inhibitor concentrations necessary for 50% inhibition of activity) of PSH derivatives to compare with the values obtained with purified RLAR.

Assays of Other Enzyme Activities——All the enzyme assays were performed at 30°C. The assays of hexokinase (EC 2.7.1.1), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate dehydrogenase (EC 1.1.1.47), glutathione reductase (EC 1.6.4.2) and alcohol dehydrogenase (EC 1.1.1.1) were carried out according to the methods of Sharma *et al.*,[11] Staal *et al.*,[12] Stolzenbach,[13]

Cohen and Rosemeyer,[14] Racker[15] and Dalziel,[16] respectively. Aldehyde reductase (EC 1.1.1.2) was assayed by a slight modification of the method of Tulsiani and Touster.[17] The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 6.4), 0.1 mM pyridine-3-carbaldehyde, 0.16 mM NADPH and the enzyme in a total volume of 1.0 ml.

Preparation of crude enzymes was performed at 4°C. Rat lenses were homogenized in 5 mM sodium phosphate buffer, pH 7.4 (0.4 ml/lens) containing 10 mM D-glucose and 1 mM 2-mercaptoethanol, and the homogenate was dialyzed against the same buffer for 3 h and then centrifuged at $20000 \times g$ for 30 min. The supernatant thus obtained was used as a hexokinase preparation. The supernatant of the homogenate of rat lenses for the assays of pyruvate kinase, lactate dehydrogenase and glucose 6-phosphate dehydrogenase was prepared with 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol by the same method as described above. The supernatant containing glutathione reductase was prepared from the homogenate of rat lenses with 5 mM sodium phosphate buffer (pH 7.4) by the same method as used for the preparation of the hexokinase sample. Rat brains were homogenized in 2 vols. of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol. The homogenate was treated by the same method as described for the preparation of the hexokinase sample, and the supernatant obtained was used as an aldehyde reductase preparation. Two major enzymes capable of reducing aldehydes in the presence of NADPH have been found to be present in rat brain.[18] Since these differ in their K_m values for a number of aldehydes, they have been termed the high- and low-K_m aldehyde reductases. In this study we used pyridine-3-carbaldehyde as the substrate instead of *p*-nitrobenzaldehyde, which has been used by many investigators, because it was reported that an NADH-dependent nitro reductase in rat brain is active towards the latter substrate.[19] The Michaelis constants of the high- and low-K_m aldehyde reductases for this substrate are 4.3×10^{-4} M and 2.7×10^{-5} M, respectively.[20] Therefore, the inhibition of the activities of both aldehyde reductases was examined under our assay conditions (0.1 mM substrate). Rat livers were homogenized in 5 vols of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol. The homogenate was then centrifuged at $20000 \times g$ for 20 min, and the supernatant was fractionated with ammonium sulfate. A 50—75% fraction was dissolved in a small volume of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and dialyzed against the same buffer. The solution thus obtained was used as an alcohol dehydrogenase preparation.

Determination of IC_{50}——The concentration of inhibitor needed to elicit 50% inhibition (IC_{50}) was determined by the method described in ref. 21.

Results

Inhibition of Lens Aldose Reductases by Hydantoin Derivatives

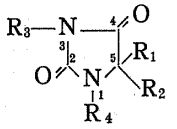
Homogeneously purified RLAR and BLAR were employed in the screening test for inhibitory activity unless otherwise stated. The percentages of inhibition of RLAR and BLAR by hydantoins, 2-thiohydantoins and 2-alkylthiohydantoins at 10^{-5} M are summarized in Tables III—V, respectively. The compounds listed in these tables were randomly selected from our stock of hydantoin derivatives which had been synthesized for the purpose of finding potent antituberculotics and analgesics.

Hydantoin (**1**) and 2-thiohydantoin (**19**) themselves did not inhibit RLAR and BLAR at 10^{-5} M (Tables III and IV). As shown in Tables III and IV, nearly all of the hydantoins (**2**—**12**) and 2-thiohydantoins (**20**—**25**) substituted at the C-5 position were ineffective or only slightly effective at this level of concentration, as far as we tested. Hydantoin and 2-thiohydantoin derivatives (**13**—**16** and **26**—**28**) substituted at the N-3 position also appeared to be ineffective. Some (**18**, **29** and **30**) of the compounds substituted only at the N-1 position were found to be highly potent. Of these three compounds, 1-(phenylsulfonyl)hydantoin (PSH, **18**) was the most potent, and it inhibited RLAR and BLAR by 81 and 85%, respectively, at 10^{-5} M. It is interesting that PSH is more potent than its 2-thio derivative, 1-(phenyl-sulfonyl)-2-thiohydantoin (PTH, **30**), while 1,3-dibenzoylhydantoin (**16**) and 1-benzoylhydantoin (**17**) are less potent than their 2-thio compounds (**28** and **19**, respectively).

Some (**37**, **38** and **39**) of the 2-alkylthiohydantoins (**32**—**39**) were inhibitory towards lens aldose reductases (Table V). Even the most potent one (**39**) of these compounds, however, inhibited RLAR and BLAR by less than 50% at 10^{-5} M.

Since PTH (**30**) and PSH (**18**) were the two best inhibitors among the 2-thiohydantoin and hydantoin derivatives tested and they had a phenylsulfonyl group, we tried to synthesize

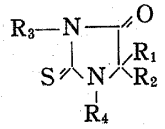
TABLE III. Inhibition of Lens Aldose Reductases by Hydantoin Derivatives



Compd. No.	R_1	R_2	R_3	R_4	Inhibition (%) RLAR	Inhibition (%) BLAR
					10^{-5} M	10^{-5} M
1	H	H	H	H	0	0
2	-Ph	-Ph	H	H	22	10
3	=CBr-Ph		H	H	11	0
4	=CCl-Ph		H	H	6	0
5	H	-Ph	$-CH_2CH_3$	H	0	0
6	H	$-(CH_2)_3CH_3$	-Ph	H	8	0
7	=CH-Ph		-CO-Ph	H	0	0
8	=CH-Ph		$-SO_2$-Ph	H	22	9
9	=CH-Ph		-CO-Ph	-CO-Ph	8	12
10	$=CH-(CH_2)_2CH_3$		-Ph	-Ph	9	6
11	H	$-(CH_2)_3CH_3$	-Ph	-Ph	20	9
12	H	$-(CH_2)_3CH_3$	-Ph	$-CH_2$-Ph	13	11
13	H	H	-Ph	-Ph	9	0
14	H	H	-Ph	$-CH_2$-Ph	0	0
15	H	H	$-CH_2$-Ph	-N=CH-Ph	9	0
16	H	H	-CO-Ph	-CO-Ph	17	10
17	H	H	H	-CO-Ph	13	0
18	H	H	H	$-SO_2$-Ph	81	85

Assays were carried out as described in "Experimental." Each compound was tested three to five times and the standard deviation of each value listed was less than 5 percent.

TABLE IV. Inhibition of Lens Aldose Reductases by 2-Thiohydantoin Derivatives



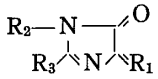
Compd. No.	R_1	R_2	R_3	R_4	Inhibition (%) RLAR	Inhibition (%) BLAR
					10^{-5} M	10^{-5} M
19	H	H	H	H	0	0
20	=CH-Ph-*p*-Cl		H	H	0	0
21	=CH-Ph-*p*-OCH_3		H	H	0	15
22	=CH-Ph		-Ph	H	0	0
23	=CH-Ph		-Ph	-Ph	19	0
24	=CH-Ph		-CO-Ph	-CO-Ph	16	12
25	H	$-(CH_2)_3CH_3$	-Ph	H	6	0
26	H	H	-Ph	H	0	0
27	H	H	-Ph	-Ph	8	0
28	H	H	-CO-Ph	-CO-Ph	34	28
29	H	H	H	-CO-Ph	50	52
30	H	H	H	$-SO_2$-Ph	71	57
31	H	H	H	$-COCH_3$	0	11

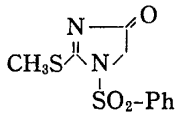
The details of the experiments were the same as described in the legend to Table III.

derivatives (**40**—**47** and **48**—**54**) which were substituted on the phenyl ring (Tables VI and VII). The inhibitory potencies of the derivatives of PTH and PSH were similar to or higher than those of the parent compounds (PTH and PSH). All of the PSH derivatives (**48**—**54**) were more potent than the corresponding PTH derivatives (**40**—**47**). Among the PSH derivatives, 1-[(*p*-bromophenyl)sulfonyl]hydantoin (*p*-Br–PSH, **49**) was the most potent inhibitor. It inhibited both RLAR and BLAR by 60% even at 10^{-6} M.

The IC_{50} values (inhibitor concentrations necessary for 50% inhibition of activity) of PSH (**18**) and its derivatives (**48**—**54**) were estimated and are listed in Table VIII. A crude preparation of RLAR as well as purified RLAR was used as a test enzyme, because almost all the previous papers of other investigators have dealt with the IC_{50} values of crude (or partially

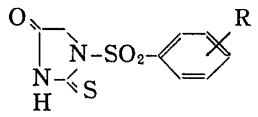
TABLE V. Inhibition of Lens Aldose Reductases by 2-Alkylthiohydantoin Derivatives



Compd. No.	R_1	R_2	R_3	Inhibition (%) RLAR 10^{-5} M	Inhibition (%) BLAR 10^{-5} M
32	=CH-Ph-*p*-Cl	H	$-SCH_3$	9	0
33	=CH-Ph-*p*-OCH_3	$-CH_3$	$-SCH_3$	0	0
34	=CH-Ph	$-SO_2$-Ph	$-SCH_3$	8	7
35	=CH-Ph	-CO-Ph	$-SCH_2CH_3$	0	0
36	=CH-Ph	$-SO_2$-Ph	$-SCH_2CH_3$	11	0
37	=CH-Ph	-Ph	$-SCH_2COCH_2CH_3$	31	21
38	=CH-Ph	H	$-SCHCH_2CH_3$ (CH$_3$ above)	24	24
39				44	47

The details of the experiments were the same as described in the legend to Table III.

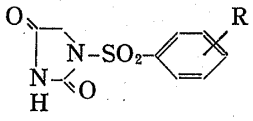
TABLE VI. Inhibition of Lens Aldose Reductases by PTH and Its Derivatives



Compd. No.	R	Inhibition (%) RLAR 10^{-5} M	RLAR 10^{-6} M	RLAR 10^{-7} M	BLAR 10^{-5} M	BLAR 10^{-6} M	BLAR 10^{-7} M
30	H	71	20	0	57	7	0
40	*p*-CH_3	58	19	0	59	7	0
41	*p*-Br	63	18	0	69	6	0
42	*p*-Cl	63	11	0	70	0	0
43	*p*-CH_3O	53	13	0	56	0	0
44	*o*-NO_2	83	40	8	87	27	0
45	*m*-NO_2	73	28	0	62	0	0
46	*p*-NO_2	56	20	0	42	0	0
47	*p*-AcNH	65	22	0	59	0	0

The details of the experiments were the same as described in the legend to Table III.

Table VII. Inhibition of Lens Aldose Reductases by PSH and Its Derivatives



Compd. No.	R	Inhibition (%) RLAR 10^{-5} M	RLAR 10^{-6} M	RLAR 10^{-7} M	BLAR 10^{-5} M	BLAR 10^{-6} M	BLAR 10^{-7} M
18	H	81	50	14	85	44	12
48	*p*-CH_3	82	52	20	88	54	14
49	*p*-Br	88	59	20	91	60	36
50	*p*-Cl	84	54	18	88	53	18
51	*p*-CH_3O	77	49	7	81	42	9
52	*o*-NO_2	85	57	22	86	35	7
53	*m*-NO_2	79	47	16	85	32	10
54	*p*-NO_2	83	52	12	88	48	15

The details of the experiments were the same as described in the legend to Table III.

Table VIII. IC_{50} of PSH and Its Derivatives for Lens Aldose Reductases

Compd. No.	IC_{50} (10^{-6} M) Crude RLAR	RLAR	BLAR
18 (PSH)	0.40	1.06	1.72
48	0.30	1.00	0.88
49	0.20	0.70	0.37
50	0.27	0.92	0.86
51	0.40	1.70	1.48
52	0.25	0.68	1.95
53	0.43	1.20	2.20
54	0.29	1.12	1.00

The assay of crude RLAR was carried out according to the method of Kador *et al.*[15] The IC_{50} values were determined as described in "Experimental."

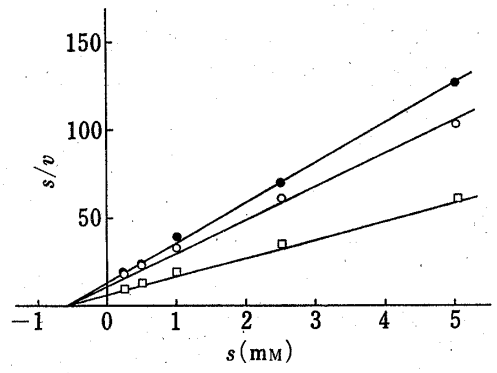


Fig. 1. Hanes–Woolf Plots of [DL-Glyceraldehyde]/v against [DL-Glyceraldehyde] with RLAR

(□——□) control, (○——○) in the presence of 10^{-6} M PSH (**18**), and (●——●) in the presence of 10^{-6} M *p*-Br–PSH (**49**). The velocity of the aldose reductase reaction was measured at each substrate concentration, in the presence and absence of inhibitors. The reactions were carried out as described in "Experimental" except that the DL-glyceraldehyde concentration was varied.

purified) RLAR, not purified RLAR. Crude RLAR was 2.5- to 4-fold nore sensitive to inhibition by the compounds tested than purified RLAR. The susceptibility of purified BLAR to inhibition was different from that of purified RLAR. No definite trends, however, could be deduced.

Kinetic studies were performed with PSH (**18**), *p*-Br–PSH (**49**) and 1-[(*o*-nitrophenyl)-sulfonyl]hydantoin (*o*-NO_2–PSH, **52**) to determine their inhibitor constants (K_i) and the type of inhibition by PSH derivatives. The Hanes–Woolf plots of [DL-glyceraldehyde]/v *vs*, [DL-glyceraldehyde] with the two compounds (10^{-6} M) as inhibitors are shown in Fig. 1. These compounds were found to be non-competitive inhibitors of both RLAR and BLAR. Their K_i values are summarized in Table IX.

Table IX. Inhibitor Constants of PSH and Its Derivatives

Compd. No.	Type of inhibition	K_i (M) RLAR	K_i (M) BLAR
18 (PSH)	Non-competitive	1.32×10^{-6}	9.16×10^{-7}
49	Non-competitive	9.50×10^{-7}	4.44×10^{-7}
52	Non-competitive	9.80×10^{-7}	1.35×10^{-6}

K_i values were obtained from the Hanes–Woolf plots of [DL-glyceraldehyde]/v against [DL-glyceraldehyde] as described in the legend to Fig. 1.

Inhibition of Some Enzymes other than Aldose Reductase by PSH and Its Derivatives

It has recently been reported that 5,5-diphenylhydantoin (**2**) is a potent inhibitor of bovine brain aldehyde reductase, which has many properties similar to those of lens aldose reductase.[22] To study the specificity of the inhibition of aldose reductase by PSH and its derivatives (**18**, **49** and **52**), the inhibitory effects of these compounds on a number of adenine nucleotide-requiring enzymes, hexokinase, pyruvate kinase, lactate dehydrogenase, alcohol dehydrogenase, glucose 6-phosphate dehydrogenase, glutathione reductase and aldehyde reductase, were investigated. The results obtained are summarized in Table X. The enzymes, except for aldehyde reductase, were unaffected or only slightly inhibited by PSH and its derivatives at 2×10^{-5} M. On the other hand, aldehyde reductase was significantly inhibited. The IC_{50} values of PSH (**18**), *p*-Br–PSH (**49**) and *o*-NO_2–PSH (**52**) for aldehyde reductase were 1.9×10^{-6} M, 3.5×10^{-6} M and 3.7×10^{-6} M, respectively.

Table X. Inhibitory Effects of PSH and Its Derivatives on Adenine Nucleotide-requiring Enzymes

Enzyme	Inhibition (%) 18 (PSH)	49	52
Hexokinase	0	0	0
Pyruvate kinase	0	0	0
Lactate dehydrogenase	0	0	0
Glucose 6-phosphate dehydrogenase	0	0	8
Glutathione reductase	9	9	0
Alcohol dehydrogenase	0	0	0
Aldehyde reductase	75	78	65

The supernatants of the rat lens homogenate were used as the crude enzyme preparations of hexokinase, pyruvate kinase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase and glutathione reductase as described in "Experimental." The supernatant of the rat brain homogenate and the 50—75% ammonium sulfate fraction of the rat liver homogenate were used as the crude enzyme preparations of aldehyde reductase and alcohol dehydrogenase, respectively, as described in "Experimental." Glucose 6-phosphate dehydrogenase was contaminated by an appreciable amount of 6-phosphogluconate dehydrogenase. Assays were carried out in a reaction system with or without an inhibitor at 2×10^{-5} M. Each value is the mean of two experiments.

Effect of pH on Inhibition of Lens Aldose Reductases by Hydantoin Derivatives

It was previously reported that inhibition of brain aldehyde reductase activity by 5,5-diphenylhydantoin (**2**), phenobarbital, dimethadione and ethoximide was due to the ionized forms of these compounds.[22a,23] We were also interested in determining whether or not inhibition of lens aldose reductase by various hydantoin derivatives is due to their ionized forms. When the pH of the reaction mixture was increased from 6.0 to 7.5, the percent inhibition of crude RLAR by 5-substituted hydantoins, *i.e.* 5,5-diphenylhydantoin (**2**) and 5-(α-bromobenzylidene)hydantoin (**3**), was increased (Fig. 2a), while that by 1-substituted hydantoins,

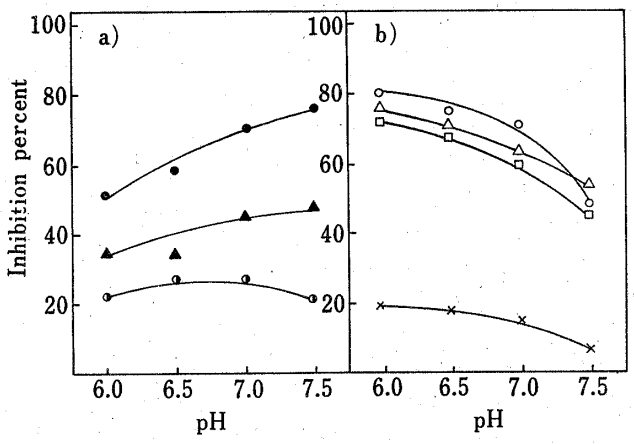


Fig. 2. Effect of pH on Inhibition of Aldose Reductase Activity by Hydantoin Derivatives

a) 5-substituted hydantoins and 3,5-disubstituted hydantoin. b) 1-substituted hydantoins. The reaction mixture contained 0.016 unit of crude RLAR preparation, 0.16 mM NADPH and 10 mM DL-glyceraldehyde in 0.1 M sodium phosphate, at the pH values indicated, in a final volume of 1 ml. The concentrations of various inhibitors were as follows: *p*-Br–PSH (**49**) (○) 2×10^{-6} M; *p*-CH_3–PSH (**48**) (△) 2×10^{-6} M; PSH (**18**) (□) 2×10^{-6} M; 1-benzoylhydantoin (**17**) (×) 2×10^{-4} M; 5,5-diphenylhydantoin (**2**) (●) 5×10^{-5} M; 5-(α-bromobenzylidene)hydantoin (**3**) (▲) 10^{-4} M; 3-(phenylsulfonyl)-5-benzylidenehydantoin (**8**) (◑) 2×10^{-4} M. Values of inhibition are expressed as percentages of the rate of enzyme activity at various pH values in the absence of inhibitor. Points are the mean for 3 experiments. Curves were drawn by eye.

i.e. *p*-Br–PSH (**49**), 1-(*p*-tolylsulfonyl)-hydantoin (*p*-CH_3–PSH, **48**), PSH (**18**) and 1-benzoylhydantoin (**17**), was decreased (Fig. 2b). Inhibition by 3-(phenylsulfonyl)-5-benzylidenehydantoin (**8**), which is not ionizable within the pH range of 6.0 to 7.5, was not altered by varying the pH (Fig. 2a). These data show that inhibition of lens aldose reductase by 5-substituted hydantoins is due to their ionized forms, while that by 1-substituted hydantoins is due to their non-ionized forms. The concentrations of the ionized form of 5,5-diphenylhydantoin (**2**) and the non-ionized form of *p*-Br–PSH (**49**) were determined from the Henderson-Hasselbach equation by the use of p*K* values (8.02 for the former and 6.04 for the latter), which were estimated from the change in absorption of aqueous solutions at 220 nm as a function of pH values. The p*K* value of 5,5-diphenylhydantoin (**2**) was in good agreement with the value (8.05) which was reported by other investigators.[22a] The IC_{50} values for RLAR based on the concentrations of the ionized form of 5,5-diphenylhydantoin (**2**) and of the non-ionized form of *p*-Br–PSH (**49**) were 5.2×10^{-7} M and 8.2×10^{-8} M, respectively.

Discussion

Currently an active search for aldose reductase inhibitors is being conducted. While known inhibitors of aldose reductase have been used to establish a role for the enzyme in the development of cataract and peripheral neuropathy in diabetic and galactosemic animals, their value in the clinical setting may be limited since they are effective only at relatively high oral dose.[24] The present work was conducted as part of a search for potent aldose reductase inhibitors which may possibly be useful for the prevention of cataract formation in diabetic patients.

Sorbinil, a hydantoin derivative, is one of the most potent aldose reductase inhibitors both *in vitro* and *in vivo* reported to date.[5,6] The IC_{50} values of this compound against crude RLAR and partially purified BLAR have been reported to be 7×10^{-8} M and 5×10^{-7} M, respectively. *p*-Br–PSH (**49**), which is the most potent inhibitor among the hydantoin derivatives tested in this study, was estimated to be almost equipotent to sorbinil. Sorbinil has been shown to uncompetitively inhibit partially purified BLAR,[5a] while *p*-Br–PSH (**49**) is an inhibitor of non-competitive type, as shown in Fig. 1.

Kador *et al.*[21] have reported significant differences in the susceptibility of aldose reductases from rat lens, human placenta and human lens to inhibition by various aldose reductase inhibitors. They have pointed out that the evaluation of aldose reductase inhibitors for potential clinical use may require the use of human aldose reductase from the appropriate target tissue. Because of the difficulty in obtaining human lenses, however, earlier workers have used aldose

reductases from animal lenses in surveys of potent inhibitors against human lens aldose reductase. For the same reason, we also used aldose reductases from animal lenses in the present study. We compared the susceptibility of purified RLAR to inhibition by PSH derivatives with that of crude RLAR and purified BLAR (Table VIII). Purified RLAR was found to be less susceptible to inhibition by all PSH derivatives tested than crude RLAR. This difference in susceptibility between crude RLAR and purified RLAR may be due to the contamination of purified RLAR with an inactive enzyme which is capable of interacting with inhibitors or due to the existence of unknown factors, which enhance the susceptibility to inhibition, in crude RLAR preparation.

Some hydantoin derivatives and their congeners have been reported to inhibit adenine nucleotide-requiring enzymes other than aldose reductase. These enzymes include aldehyde reductase,[22] ATPase,[25] glutathione reductase[26] and diaphorase.[27] No systematic study on inhibition of these enzymes by hydantoin derivatives, however, has yet been reported. We measured, in the present study, the inhibitory activities of PSH (**18**) and its derivatives (**49** and **52**) against seven adenine nucleotide-requiring enzymes (hexokinase, glucose 6-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, glutathione reductase, alcohol dehydrogenase and aldehyde reductase). As shown in Table X, only aldehyde reductase was inhibited to a major extent (about 70%) by all three compounds at 2×10^{-5} M. The inhibitory activity of each of these compounds against aldehyde reductase, however, was one order of magnitude lower than that against aldose reductase as judged from the IC_{50} values for the two enzymes, indicating that the PSH (**18**) and its derivatives may fairly selectively inhibit only aldose reductase. This feature probably makes PSH derivatives promising as clinically useful drugs. Aldehyde reductase has recently been suggested to be an important site of action of anticonvulsant drugs, because the enzyme is potently inhibited by almost all anticonvulsant drugs, with K_i values in the range of 10^{-4} M to 10^{-5} M.[22,23] PSH derivatives, which have K_i values of less than 10^{-5} M for aldehyde reductase, may also be useful as anticonvulsant drugs.

It has recently been proposed that aldehyde reductase, especially low-K_m form, very closely resembles aldose reductase in terms of substrate and cofactor specificity, sensitivity to inhibitors, particularly flavonoids and 3,3-tetramethyleneglutaric acid, and response to sulfate ion.[28–31] That both aldose reductase and aldehyde reductase were potently inhibited by PSH derivatives (**18**, **49** and **52**) suggests that these two enzymes may be identical. However, the relationship of aldose reductase to aldehyde reductase is unclear at present.

We found that the inhibition of aldose reductase by 1-substituted hydantoins was due to their non-ionized forms possessing the –CO–NH–CO– group, while the inhibition by 5-substituted hydantoins was due to their ionized forms (Fig. 2). It may be concluded that the extent of inhibition of aldose reductase by these ionizable hydantoin compounds is governed rather by the concentrations of either ionized forms or non-ionized forms at physiological pH than by total concentrations, and that the binding site of 1-substituted hydantoin to the enzyme is different from the binding site of 5-substituted hydantoin. An *in vivo* study of inhibition of aldose reductase by PSH derivatives is now in progress. Further search may reveal even more potent hydantoin derivatives which could ultimately be useful in diabetic patients.

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