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Harderian Gland. VI.[1] Effect of Chlorinated Benzenes on Porphyrin Biosynthesis in the Harderian Gland of Rat

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In order to induce chromodacryorrhea observed in pantothenic acid-deficient rats and also to obtain informations on the regulation of porphyrin biosynthetic pathway in the rat Harderian gland, the effect of hepatic porphyria-inducing chemicals, especially chlorinated benzenes, on porphyrin biosynthetic pathway in the Harderian gland of rat was examined and the following results were obtained.

None of the chemicals tested induced the expected chromodacryorrhea. The rat Harderian gland was resistant to the induction of δ-aminolevulinic acid (ALA)-synthetase by hepatic porphyria-inducing chemicals, expected for a few compounds such as lower CB compounds. There was a remarkable difference between the Harderian gland and the liver in the responsiveness of ALA-synthetase and ALA-dehydratase to hepatic porphyria-inducing chemicals.

Keywords——Harderian gland; porphyrin biosynthesis; chlorinated benzenes; δ-aminolevulinate synthetase of Harderian gland; δ-aminolevulinate dehydratase of Harderian gland; cytochrome P-450

Physiological role of free protoporphyrin and regulation of porphyrin biosynthesis in the Harderian gland of rat are still not elucidated.

In the present series of work, we examined the effect of hepatic porphyria-inducing chemicals, especially chlorinated benzenes, on porphyrin biosynthetic pathway in the Harderian gland of rat in order to induce chromodacryorrhea observed in pantothenic acid-deficient rats[1,3] and also to obtain informations on the regulation of porphyrin biosynthetic pathway in the rat Harderian gland.

Material and Method

Chemicals——Phenobarbital was purchased from Fujinaga Chemical Industries, Tokyo, griseofulvin from Schering Co., and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) from Aldrich Chemical Co., U.S.A. Among the chemicals used, monochlorobenzene (MCB), 1,2-, 1,3-, and 1,4-dichlorobenzenes (DCB), 1,2,3-, 1,2,4-, and 1,3,5-trichlorobenzenes (TCB), 1,2,3,4-, 1,2,3,5-, and 1,2,4,5-tetrachlorobenzenes (TECB), pentachlorobenzene (PCB), and hexachlorobenzene (HCB) were purchased from Nakarai Chemical Co., Kyoto, δ-aminolevulinic acid (ALA) from Daiichi Chemical Co., Tokyo, and protoporphyrin-IX from Tokyo Kasei Co. Other chemicals used were usual commercial products. Anion-exchange resin, Dowex 1×8 (200—400 mesh), was purchased from the Dow Chemical Co., Midland, U.S.A.

Animals——Wistar strain female rats, weighing 160—200 g, were given commercial solid diet (Funabashi Farm, Chiba) and water freely. Phenobarbital (100 mg/kg) was suspended in saline and injected subcutaneously every day. Griseofulvin was mixed in a powder diet (5%, w/w) and fed to rat freely after 24 hr fasting. DDC (500 mg/kg) and chlorobenzenes (500 mg/kg) were each dissolved in peanut oil and given to rats by gastric intubation. Animals were sacrificed by decapitation.

Preparation of Tissue Sample——The Harderian gland and the liver were obtained from rats killed by decapitation.

Determination of Porphyrin Content in Harderian Gland——To *ca.* 800 mg of the Harderian gland, 10 ml of a 1:1 buffer-saline solution was added and the mixture was homogenized in a Potter-Elvehjem

1) Part V: K. Eida, N. Kubota, T. Nishigaki, and M. Kikutani, *Chem. Pharm. Bull.* (Tokyo), **23**, 1 (1975).
2) Location: *Bunkyo-cho, Nagasaki, 852, Japan.*
3) *a*) S. Tashiro and H. Stix, *Biol. Bull.*, **64**, 327 (1935); *b*) L.W. McElroy, K. Salomon, F.H.J. Figge, and G.R. Cowgill, *Science*, **94**, 467 (1941).
4) O.H. Lowry, N.J. Rosenbrough, A.L. Forr, and R.T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

glass homogenizer. The homogenate was centrifuged at 2500 rpm for 10 min. An aliquot of the supernatant was placed in a test tube and concentrated HCl was added to make the final concentration of 25% solution. The absorbance of this solution was measured at 412 nm, with a Beckman DB spectrophotometer. The amount of porphyrin was calculated from the calibration curve with protoporphyrin as a standard.

Determination of Protein Concentration——Protein concentration was determined by the modified Folin method of Lowry, *et al.*[4]

Enzyme Assay——Activity of ALA-synthetase [succinyl-CoA: glycine C-succinyl transferase (decarboxylating) EC 2.3.1.37] was assayed by the method of Marver, *et al.*[5] and by the method of Narisawa and Kikuchi.[6] The reaction was carried out in the reaction system of Marver, *et al.* and the ALA formed was determined by the method of Narisawa and Kikuchi. The activity of ALA-synthetase was expressed as nmole/g tissue/hr.

The activity of ALA-dehydratase [5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing) EC 4.2.1.24] was assayed by the method of Gibson, *et al.*[7]

Measurements of Cytochrome P-450 and b_5——The cytochrome P-450 and b_5 were determined by the method of Omura and Sato,[8] with a Shimadzu multipurpose MPS-50 recording spectrophotometer.

Result

Effect of Various Porphyria-Inducing Chemicals on Porphyrin Content, ALA-synthetase and ALA-dehydratase in Harderian Gland

Rats treated with porphyria-inducing chemicals did not show any symptoms such as chromodacryorrhea observed in pantothenic acid-deficient rats. Table I summarizes the porphyrin content in the Harderian gland of rat treated with various porphyria-inducing chemicals. Porphyrin content increased considerably at 1 day after administration of MCB, 1,2- or 1,3-DCB, but it markedly decreased at 5 days after consecutive administration of MCB and 1,4-DCB. Strong hepatic porphyria-inducing chemicals such as DDC, phenobarbital and griseofulvin, had no effect on the porphyrin content.

Table I. Effect of Chlorinated Benzenes on Porphyrin Content in Rat Harderian Gland

Treatment	Porphyrin content in days after administration (ng/mg Harderian gland)		
	1	3	5
Control	457± 12	442± 19	449± 25
MCB	955±176	296± 41	198± 53
1,2-DCB	950± 87	481± 13	453± 50
1,3-DCB	940± 83	459± 38	450± 23
1,4-DCB	411± 42	192± 33	113± 11
1,2,3-TCB	556± 82	497± 15	570± 88
1,3,5-TCB	630± 98	616± 27	559± 32
1,2,4-TCB	606± 40	610± 37	538±110
1,2,3,4-TECB	534± 19	650± 56	624± 58
1,2,3,5-TECB	462± 50	536± 66	600±115
1,2,4,5-TECB	430± 99	480± 85	498± 69
PCB	550± 40	660±103	566±208
HCB	607±120	550± 30	654± 47
DDC	455± 41	449± 54	455± 20
Griseofulvin	547± 40	503± 51	610± 48
Phenobarbital	410± 47	596± 27	603± 36

Animals were treated as described in "Text". All values represent the mean ± SD of the results obtained from six rats.

5) H.S. Marver, D.P. Tschudy, M.G. Perlroth, and A. Collins, *J. Biol. Chem.*, **241**, 2803 (1966).
6) K. Narisawa and G. Kikuchi, *Biochim. Biophys. Acta*, **123**, 596 (1966).
7) K.D. Gibson, A. Neuberger, and J.J. Scott, *Biochem. J.*, **61**, 618 (1956).
8) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2379 (1964).

The Harderian gland of rats treated with 1,4-DCB was extremely different from that treated with other dichlorobenzenes, in that the former showed a remarkable decline in deep fluorescence of porphyrin under ultraviolet light (Manaslu light. wavelength 3650 Å, Manaslu Chemical, Inc.) as shown in Fig. 1.

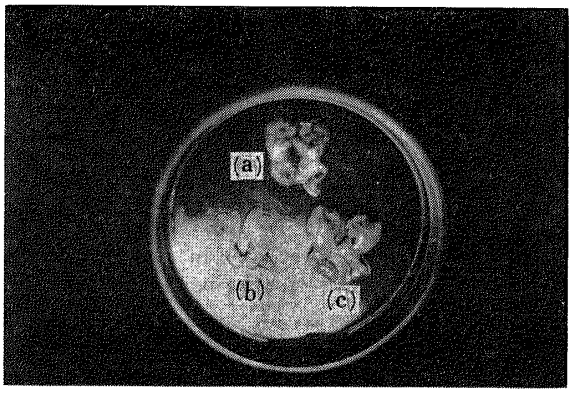

Fig. 1. Harderian Gland of Rats treated with Chemicals Under Ultraviolet Light

(a) Harderian gland of normal rat (a red-fluorescent color)
(b) Harderian gland of a rat treated with 1,4-DCB (a faded color)
(c) Harderian gland of a rat treated with 1,3-DCB (a red-fluorescent color)

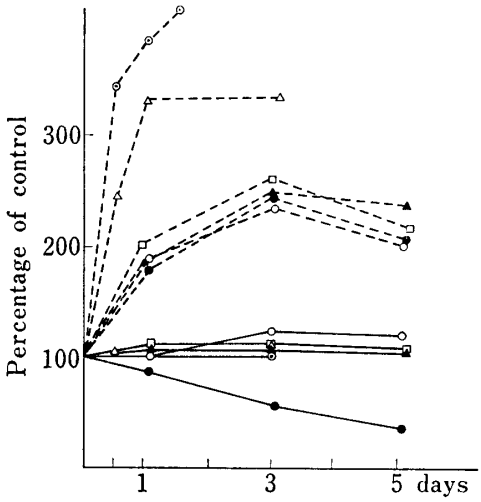

Fig. 2. Change of ALA-synthetase Activities in the Harderian Gland and the Liver of Rats treated with Porphyria-inducing Chemicals

Each value plotted represents an average of ALA-synthetase activities per gram tissue of 6 experimental animals as compared with those of 6 control rats.

--○--: liver, phenobarbital
--△--: liver, griseofulvin
--⊙--: liver, DDC
--□--: liver, 1,2-DCB
--▲--: liver, 1,3-DCB
--●--: liver, 1,4-DCB
—○—: Harderian gland, phenobarbital
—△—: Harderian gland, griseofulvin
—⊙—: Harderian gland, DDC
—□—: Harderian gland, 1,2-DCB
—▲—: Harderian gland, 1,3-DCB
—●—: Harderian gland, 1,4-DCB

In order to elucidate the effect of these chemicals on porphyrin content of rat Harderian gland, the activity of ALA-synthetase was examined. Figure 2 illustrates the response of Harderian gland ALA-synthetase and hepatic ALA-synthetase to the chemicals. All the chemicals used had no effect on the activity of Harderian gland ALA-synthetase, with an exception of 1,4-DCB, while hepatic ALA-synthetase activity increased 2—3 times the normal level. When 1,4-DCB was administered, the ALA-synthetase level of Harderian gland continued to decrease without showing a phase of increase.

To examine further the relationship between ALA-synthetase activity and porphyrin content in the Harderian gland, periodical changes in ALA-synthetase activity and porphyrin content of the rat Harderian gland after a single administration of two chlorobenzenes, 1,3-DCB for increasing the porphyrin content and 1,4-DCB for decreasing it, were determined. The ALA-synthetase activity reached a maximum at 6 hr and 12 hr after administration of 1,4- and 1,3-DCB ,respectively (Fig. 3(a)), and the porphyrin content reached a maximum at about 6 hr after the maximum induction of ALA-synthetase (Fig. 3(b)). The Harderian gland ALA-synthetase activity in rat treated with 1,3-DCB was significantly higher than that in rat treated with 1,4-DCB. This may explain an increase of porphyrin content in the Harderian gland at 1 day after administration of MCB, 1,2- and 1,3-DCB. However, the increase of Harderian gland ALA-synthetase activity was transient and 1,3-DCB did not give a subsequent increase of Harderian gland ALA-synthetase activity after 1st day in the enzyme assay at 12 hr intervals, despite continued daily administration for 5 days.

Then we examined the ALA-dehydratase, the second enzyme of the porphyrin biosynthetic pathway, whose activity is markedly inhibited in erythropoietic porphyria due to lead poisoning. Figure 4 shows the periodical changes in ALA-dehydratase activities after a single

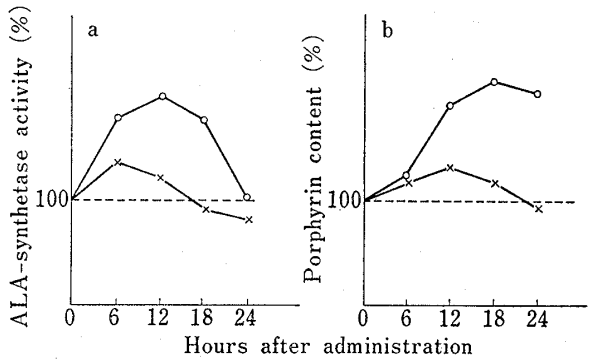

Fig. 3. Time Course of Changes of ALA-synthetase Activity (a) and Porphyrin Content (b) in the Harderian Gland of Rats treated with 1,3-DCB and 1,4-DCB

Rats were killed 6, 12, 18, and 24 hr after a single administration of 1,3- and 1,4-DCB (500 mg/kg body wt.). Each value plotted represents an average of 6 experiments.
—○—: 1,3-DCB, —×—: 1,4-DCB

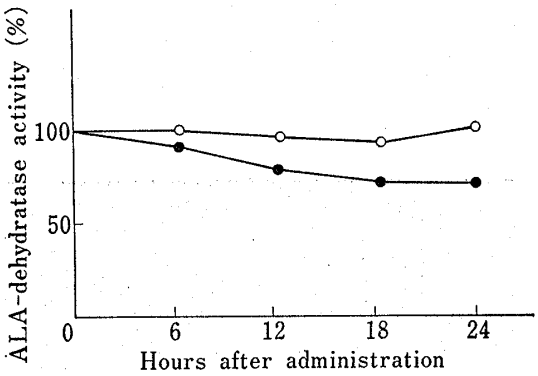

Fig. 4. Time Course of Change of ALA-dehydratase Activity in the Harderian Gland of Rats treated with 1,3- and 1,4-DCB

Each value plotted represents an average of ALA-dehydratase activities per gram tissue of 6 experimental animals as compared with those of 6 control rats.
—○—: 1,3-DCB, —●—: 1,4-DCB

administration of 1,3- and 1,4-DCB. 1,3-DCB had no effect on the ALA-dehydratase activity, but 1,4-DCB decreased the ALA-dehydratase activity gradually.

Changes in the ALA-dehydratase activity in the Harderian gland and the liver of rats treated with daily administration of chemicals were further examined. As shown in Table II, there was no change in the ALA-dehydratase activity in the liver, but the enzyme activity in the Harderian gland markedly decreased by the administration of MCB and 1,4-DCB, which markedly reduced the porphyrin content at 5 days after administration. In the Harderian gland, there seems to be a proportional relationship between the lowering of porphyrin content and that of ALA-dehydratase activity.

Table II. Change of ALA-Dehydratase Activity in the Harderian Gland and the Liver of Rats treated with 1,2-, 1,3-, 1,4-DCB and MCB

Tissues	Experimental No.	Treatment[a)]	ALA-dehydratase activity (μmole/g/hr)		
			Day 1 (%)	Day 3 (%)	Day 5 (%)
Harderian gland	6	control	0.158(100)	0.154(100)	0.155(100)
	4	MCB	0.131(83)	0.126(82)	0.086(55)
	6	1,2-DCB	0.148(94)	0.148(93)	0.146(94)
	6	1,3-DCB	0.160(101)	0.175(110)	0.157(101)
	5	1,4-DCB	0.143(90)	0.103(67)	0.054(35)
Liver	6	control	0.297(100)	0.297(100)	0.281(100)
	4	MCB	0.300(101)	0.318(107)	0.247(88)
	6	1,2-DCB	0.286(96)	0.290(99)	0.293(104)
	6	1,3-DCB	0.296(100)	0.330(111)	0.309(110)
	5	1,4-DCB	0.304(102)	0.337(113)	0.281(100)

a) Female rats were treated orally for 1,-3, or 5 days with each chemical (500 mg/kg) or peanut oil and sacrificed 24 hr after the last dose. All values represent the means of the results obtained.

Hemoprotein of the Harderian Gland Microsome

The decrease of ALA-synthetase activity and ALA-dehydratase activity in the Harderian gland during consecutive daily administration of MCB and 1,4-DCB seems to be closely related to the physiological role of this tissue. Therefore, the presence of cytochrome P-450, closely related to drug metabolism, was examined in the microsome of the rat Harderian gland. As

shown in Fig. 5, cytochrome P-450 which shows a positive peak at 450 nm, was not detected and only cytochrome b_5 was detected, showing a γ-band at 424 nm and α-band at 556 nm in the oxido-reduction difference spectrum. The content of cytochrome b_5 was about one-third of that of the liver.

Effect of Chemicals on Tissue Weight of Harderian Gland

Table III shows changes in the weights of Harderian gland and the liver after administration of chemicals. In case of the liver the tissue weight generally increased with any of the chemicals. In contrast, the Harderian gland showed a characteristic periodical decrease in the tissue weight after the administration of MCB and 1,4-DCB, but hardly showed any change with other chemicals. It is presumed that administration of the two chemicals caused the tissue damage of Harderian gland.

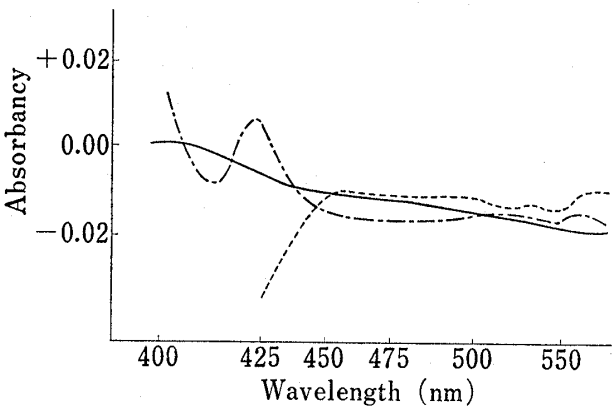

Fig. 5. Difference Spectra of Cytochrome P-450 and Cytochrome b_5 in the Harderian Gland Microsome

The amounts of cytochrome P-450 and b_5 in microsomes of the Hardreian gland were determined by the method of Omura and Sato.

--------: cytochrome P-450,
−·−·−: cytochrome b_5,
———: base line

	Cytochrome P-450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)
Liver	0.73	0.53
Harderian gland	not detected	0.19

TABLE III. Changes in the Harderian Gland and the Liver Weight after the Administration of 1,2-, 1,3-, 1,4-DCB and MCB

Tissue	Treatment[a)]	Tissue wt./body wt. × factor[b)]		
		Day 1	Day 3	Day 5
Harderian gland	control	1.32±0.07	1.39±0.05	1.36±0.04
	MCB	1.26±0.13	1.18±0.09	1.09±0.11
	1,2-DCB	1.40±0.05	1.39±0.10	1.45±0.06
	1,3-DCB	1.40±0.06	1.40±0.09	1.50±0.05
	1,4-DCB	1.26±0.04	1.05±0.03	0.94±0.02
Liver	control	4.20±0.11	4.16±0.19	4.18±0.15
	MCB	4.20±0.28	6.34±0.58	5.72±0.12
	1,2-DCB	4.30±0.14	5.96±0.17	6.73±0.35
	1,3-DCB	4.73±0.21	6.38±0.35	6.49±0.38
	1,4-DCB	4.66±0.18	5.29±0.30	5.40±0.23

a) Experimental conditions are as given in Table II. All values are represented as the mean ± SD of 6 rats.
b) Harderian gland wt./body wt. × 1000
Liver wt./body wt. × 100

Discussion

The present study shows that there is a considerable difference between the Harderian gland and the liver in their drug responsiveness. The rat Harderian gland was resistant to the induction of ALA-synthetase by hepatic porphyria-inducing chemicals, except for a few lower CB derivatives. This fact suggests that the regulation of porphyrin formation in rat Harderian gland considerably differs from that in the liver.

However, lack of responsiveness to the induction of ALA-synthetase is observed in the liver of newborn animals. The neonatal animal is resistant to the induction of ALA-synthetase by AIA or DDC,[9] an extremely potent porphyria-inducing chemicals known to induce readily this enzyme in adult animals. This resistance is related to a deficiency in the levels of microsomal cytochrome P-450.[10]

Since cytochrome P-450, which plays an important role in the liver for drug metabolism, is not detected in the Harderian gland of rat, a lack of responsiveness to induction of ALA-synthetase by chemicals may be related to a deficiency of cytochrome P-450 in this gland. Therefore, Harderian gland is affected directly by chemicals, and administration of chemicals may cause the tissue damage of the Harderian gland and nonspecific depression of enzyme activities. Considering the deficiency of cytochrome P-450 and the higher level of ALA-synthetase activity in the rat Harderian gland, it is presumed that the physiological significant of the porphyrin formation in this gland differs apparently from that in the liver.

9) C.S. Song, J.W. Singer, R.D. Levere, D.F. Harris, and A. Kappas, *J. Lab. Clin. Med.*, **72**, 1019 (1968).
10) C.R. Short and L.E. Davis, *The Pharmacologist*, **10**, 191 (1968).