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# Harderian Gland. IV.[1] Porphyrin Formation from δ-Aminolevulinic Acid by the Harderian Gland of Rats

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Enzymic formation of porphyrins in the extract of Harderian gland of a rat was examined. Porphyrins formed from δ-aminolevulinic acid by the extract of Harderian gland were uroporphyrin, coproporphyrin-III, a small quantity of protoporphyrin-IX, and unidentified porphyrins. According to the quantitative study of porphyrins at specified incubation time, the main part of porphyrins formed were uroporphyrin and coproporphyrin-III, and protoporhyrin-IX was formed only in a small amount.

Accumulation of a red material on the nose and whiskers of rats has been attributed to the deficiency of pantothenic acid and riboflavin in these animals.[3] This peculiar symptom had been erroneously described as "nose-bleed" or "blood-caked whiskers,"[4] because this red material contained a large amount of porphyrin.[5,6] It was demonstrated that the Harderian gland is the source of this porphyrin secretion, and a large proportion of this porphyrin was found to be a coproporphyrin.[6] On the other hand, it is known that the Harderian gland of normal rats contains a relatively large quantities of protoporphyrin.[7]

These past reports suggest that pantothenic acid and riboflavin derivatives participate in decarboxylation and oxidation in the biosynthesis of protoporphyrin from coproporphyrinogen. The mechanism of protoporphyrin biosynthesis from δ-aminolevulinic acid has been almost elucidated, but there is still some missing links in the last step of protoporphyrin biosynthesis.

The problem of immediate interest is that, since pantothenic acid and riboflavin derivatives take part in biosynthesis of protoporphyrin from coproporphyrinogen, further studies on this enzymatic mechanism might throw additional light on the inhibition of protoporphyrin biosynthesis by the Harderian gland in pantothenic acid- and riboflavin-deficient rats.

We first investigated what kinds of porphyrin are formed in Harderian gland of rats, using δ-aminolevulinic acid as substrate and Harderian gland extract as the enzyme preparation. The result of this investigation showed that a large amount of uroporphyrin and coproporphyrin-III are formed, while formation of protoporphyrin increased only slightly.

## Experimental

**Materials and Methods**——δ-Aminolevulinic acid was purchased from the Daiichi Pure Chemicals Co., Ltd. Protoporphyrin-IX was prepared from hemin by the method of Grinstein[8] and coproporphyrin-III

1) Part III: M. Kikutani and K. Hirose *Chem. Pharm. Bull.* (Tokyo), **12**, 760 (1964).
2) Location: *Bunkyo-cho, Nagasaki.*
3) S.G. Smith, *Proc. Soc. Exptl. Biol. Med.*, **49**, 691 (1942).
4) K. Unna, *J. Nutrition*, **20**, 565 (1940).
5) H. Chick, T.F. Macrea, and A.N. Worden, *Biochem. J.*, **34**, 580 (1940).
6) L.W. McElroy, K. Salomon, F.H.J. Figge, and G.R. Cowgill, *Science*, **94**, 467 (1941).
7) E. Derrien and J. Turchini, *Compt. Rend. Soc. Biol.*, **91**, 637 (1924); J. Thomas, *Bull. Soc. Chim. Biol.*, **20**, 471 (1938); E.J. Towbin, P.E. Eauta, and H.C. Hodge, *Proc. Soc. Exptl. Biol. Med.*, **60**, 228 (1945); C. Rimington, *Endeavour*, XIV, **55**, 126 (1955).
8) M. Grinstein, *J. Biol. Chem.*, **167**, 515 (1947).

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was isolated from diphtheria toxin.[9] Male Wistar rats weighing 200 to 250 g were used. The animals were killed with ether and the Harderian glands were dissected out. After removal of any excess blood, 800 mg of Harderian glands were homogenized with 10 ml of 1:1 buffer–saline solution in a Potter–Elvehjem homogenizer. This buffer–saline solution consisted of an equal amount of isotonic NaCl solution and buffer solution (of various pH of Kolthoff's buffer solution). After the tissues were homogenized, the homogenate was centrifuged at 2500 rpm for 10 min and the supernatant solution was used as the crude enzyme extract (2.10 mg protein/ml. Protein concentration was determined by the modified Folin method of Lowry, *et al.*[10]). All these treatments were carried out at a temperature below 4°.

δ-Aminolevulinic acid (16 μmoles) was added to 5 ml of Harderian gland extract and 5 ml of buffer–saline solution to make a final volume of 10 ml. The reaction mixture was incubated at 37° in a colored flask plugged loosely with cotton. After the specified incubation time, concentrated HCl was added to the flask to give the final concentration of 25% HCl, which stopped the enzyme reaction and cleared the solution. The total porphyrin formed was determined by measuring the absorbancy at 411 mμ with a Shimadzu spectrophotometer, QR-50. Protoporphyrin-IX in 25% HCl was taken as a standard solution.

Chromatographic analysis and quantitative determination of porphyrins were carried out as follows. At the end of the reaction, porphyrins formed were extracted from the reaction mixture with AcOEt–AcOH (3:1, v/v) and dried in vacuum. The dried residue was dissolved in a minimum volume of dilute $NH_4OH$ and subjected to paper chromatography. Modified Kehle's method[11] was used in this procedure. The development was performed by the ascending method in a sample jar saturated with $NH_3$ vapor at 27—29° in a dark room. The chromatographic solvent system used was a mixture of 2,6-lutidine and water (2:1, v/v),[11,12] and the paper used was Tōyō Roshi No. 51 (10×18 cm). The porphyrins on the chromatogram were detected by their fluorescence under ultraviolet ray (Manaslu–light, longwave 3650 Å), cut off, and each piece was eluted with 1N HCl in a dark room. Porphyrins eluted were identified by the *Rf* values and by measuring the Soret maxima with a Hitachi Recording Spectrophotometer, EPS-2. Porphyrin contents were calculated from the calibration curve prepared at the respective Soret muximum in 1N HCl solution to determine the amount of porphyrin.

A few ml of the reaction mixture was taken out at certain intervals and Soret muximum of porphyrin in 25% HCl solution was also checked by the Recording Spectrophotometer.

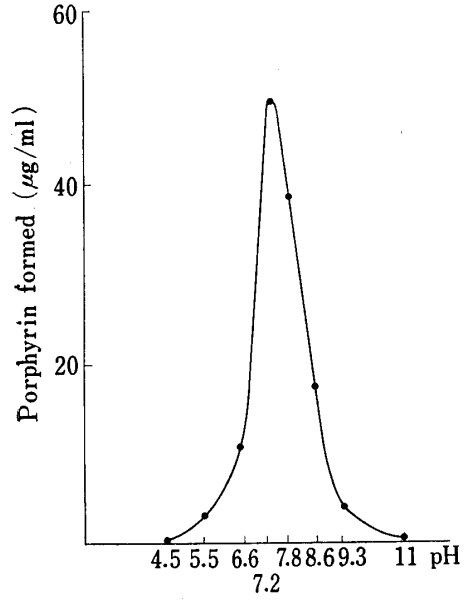


Fig. 1. pH-Activity Curve of Harderian Gland Extract

δ-Aminolevulinic acid (16 μmoles) was added to 5 ml of Harderian gland extract and 5 ml of buffer–saline solution consisting of equal amounts of isotonic NaCl solution and Kolthoff's buffer solution at various pH to make a final volume of 10 ml. The reaction mixture was incubated at 37° for 24 hours. After incubation, total porphyrin formed was determined in 25% HCl solution by a spectrophotometer.

## Results

Porphyrin formation from δ-aminolevulinic acid by the enzyme extract of Harderian gland: Fig. 1 shows the amount of total porphyrins formed at various pH after 24 hours of incubation. Optimum pH for the porphyrin formation by the enzyme extract of Harderian gland was 7.2.

Fig. 2 shows the rates of porphyrin formation at the specified incubation time. Total porphyrin formed by the Harderian gland extract[13] increased with lapse of time during 24 hours of incubation, the total amount formed being as much as approximately 98 times the amount at 15–minute incubation, and 20 times that formed at one–hour incubation.

Effect of blood stain on the surface of the gland to porphyrin formation was also examined. As shown in Fig. 2, the amount of porphyrin formed by

9) C. Rimington, *Acta Med. Scand.*, **143**, 161 (1952).
10) O.H. Lowry, N.J. Rosenbrough, A.L. Forr, and R.T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) R. Kehl and W. Stich, *Z. Physiol. Chem.*, **289**, 6 (1951).
12) L. Eriksen, *Scand. J. Clin. Lab. Invest.*, **5**, 155 (1953).
13) The amount of porphyrin originally present in this gland was deducted.

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blood was less than 2% of that formed by the Harderian gland extract. Therefore, the effect of blood remaining on the Harderian gland can be neglected, especially when Harderian gland had been rinsed thoroughly.

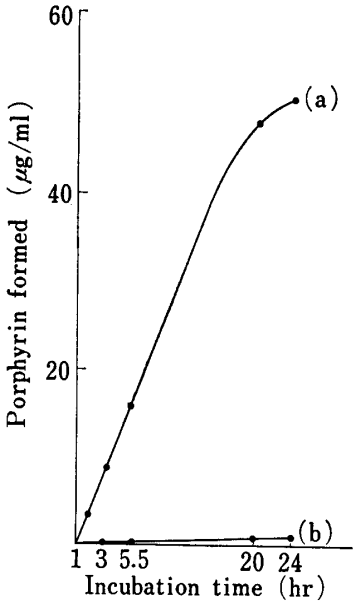


Fig. 2. Rate of Porphyrin Formation by Harderian Gland Extract and by Blood Stain around Harderian Gland Tissues

Five ml of Harderian gland extract (a) and 5 ml of a solution after washing blood around Harderian gland tissues (b) were incubated at 37° with 16 μmoles of δ-aminolevulinic acid in 5 ml of buffer–saline solution (pH 7.2). Total porphyrin was determined in 25% HCl.

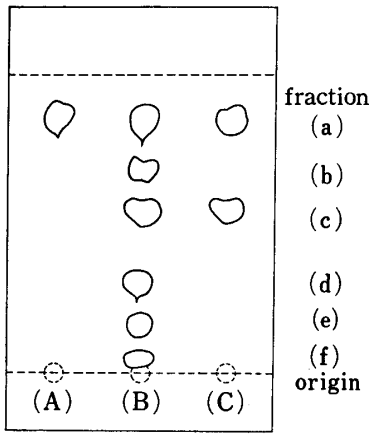


Fig. 3. Paper Chromatogram of Porphyrin produced

Solvent system of 2,6-lutidine–water (2:1, v/v), 29°, ascending, saturated $NH_3$ vapor. (A), (B), and (C) indicate application of porphyrins before incubation, after 24 hours of incubation, and authentic protoporphyrin-IX and coproporphyrin-III, respectively.

The paper chromatograms of porphyrins before incubation, after 24 hours of incubation at 37°, pH 7.2, and authentic porphyrins are shown in Fig. 3,(A), (B), and (C), respectively.

Only one spot (a) was found in 3 (A), while there were many distinct spots, (a), (b), (c), (d), (e), and (f) in 3 (B).

Table I shows *Rf* values and Soret maxima of porphyrins formed, with the values found in the references. Protoporphyrin-IX, coproporphyrin-III, uroporphyrin, and a few spots

Table I. *Rf* Values[a] and Soret Maxima[b] of Porphyrins formed in 24 hours of Incubation

| Fraction | *Rf* | Soret maxima (mμ) | Porphyrins |
|---|---|---|---|
| (a) | 0.78—0.82 | 408 | protoporphyrin-IX[c] |
| (b) | 0.60—0.68 | 401 | unidentified |
| (c) | 0.48—0.56 | 401 | coproporphyrin-III[c] |
| (d) | 0.23—0.24 | 401 | unidentified |
| (e) | 0.11—0.16 | 406 | unidentified |
| (f) | 0.02—0.06 | 406 | uroporphyrin-III (?)[d] |

*a*) 2,6-lutidine–water (2:1, v/v), Tōyō Roshi No. 51, 27—29°, ascending, saturated $NH_3$ vapor, by Eriksen's method.
*b*) Measured in 1N HCl solution.
*c*) Identified with pure substance.
*d*) D. Mauzerall, *J. Am. Chem. Soc.*, 62, 2601 (1960)

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of unidentified porphyrins were formed after 24 hours of incubation. Protoporphyrin-IX and coproporphyrin-III formed were respectively identified with authentic protoporphyrin-IX and coproporphyrin-III.

Variation of Soret Maximum during the incubation was examined by spectrophotometry. As shown in Fig. 4, Soret maximum moved from the longer to shorter wavelength region during incubation. The maximum at 412 mμ[14] before incubation shifted to 408 mμ[14] after a 24-hour incubation. Periodical changes in the amount of each fraction of porphyrins formed were then examined. As shown in Fig. 5, the amount of uroporphyrin and coproporphyrin-III tended to increase with lapse of incubation time, but that of protoporphyrin stopped increasing after about 6 hours.

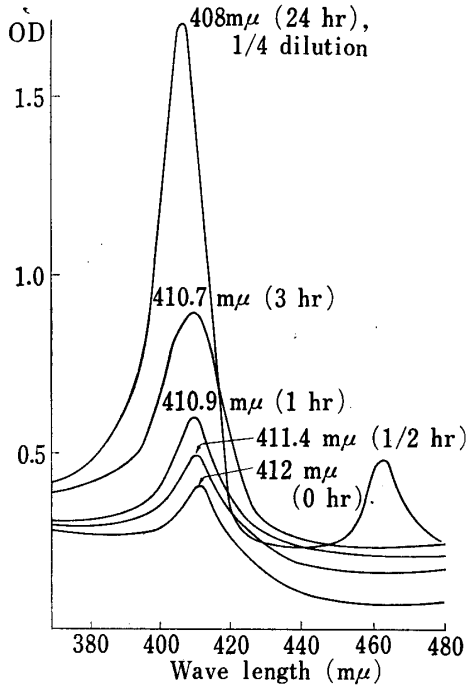


Fig. 4. Variation in Soret Maxima during Incubation

δ-Aminolevulinic acid (32 μmoles) was added to 10 ml of Harderian gland extract and 10 ml of Kolthoff's buffer–saline solution (pH 7.2) to make a final volume of 20 ml. The reaction mixture was incubated at 37°. A few ml of the reaction mixture was taken out at certain intervals and Soret maximum in 25% HCl solution was checked with a recording spectrophotometer.

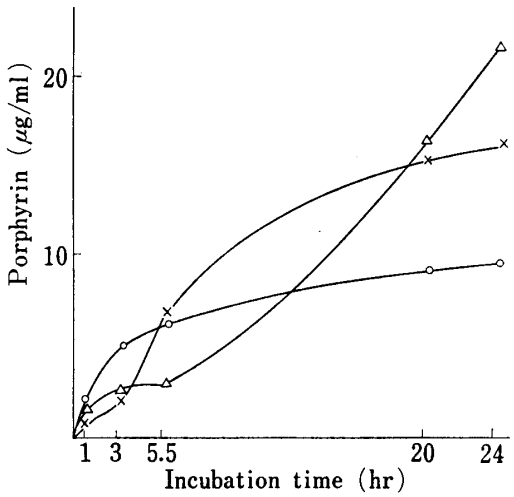


Fig. 5. Time Course of Changes in Some Porphyrin Concentration and Rate of Porphyrin Formation *in Vitro*

δ-Aminolevulinic acid (96 μmoles) was added to 30 ml of Harderian gland extract and 30 ml of Kolthoff's buffer–saline solution (pH 7.2) to make a final volume of 60 ml. The reaction mixture was incubated at 37°. Eight ml of the reaction mixture was taken out to quantitative determination (as described in "Experimental Procedure") of porphyrins at certain intervals. The amount of porphyrin was determined in 1N HCl solution respectively.

—○— : protoporphyrin-IX
—×— : coproporphyrin-III
—△— : uroporphyrin

## Discussion

It was found that uroporphyrin and coproporphyrin-III are formed mainly, with a small amount of protoporphyrin-IX, from δ-aminolevulinic acid by the extract of Harderian gland of rats.

The shift of the Soret maximum to shorter wavelength seems to indicate that the maximum at 412 mμ before incubation is that of protoporphyrin-IX and that at 408 mμ is due to coproporphyrin-III and uroporphyrin formed during the incubation. The rate of porphyrin

14) Soret maxima of each porhyrin in 25% HCl solution are as follows: Protoporphyrin-IX, 411.2 mμ; uroporphyrin-III, 410.5 mμ; coproporphyrin-III, 405.0 mμ.

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formation at specified incubation time indicates that the formation of uroporphyrin and coproporphyrin-III is stimulated, whereas that of protoporphyrin-IX is inhibited.

It is well known that the precursors of protoporphyrin in the biosynthesis pathway are uroporphyrinogen-III and coproporphyrinogen-III, and the compounds like uroporphyrin-III and coproporphyrin-III are by-products. Considering such a fact, it seems that the increase of uroporphyrin and coproporphyrin-III is a consequence of the oxidation of the intermediate, which accumulated from the inhibition of protoporphyrin formation, by light and oxygen in the air.

As the Harderian gland of a rat originally contains a large amount of protoporphyrin, it is assumed that all the enzymes in the pathway of protoporphyrin formation are contained in the Harderian gland of normal rats and, therefore, the Harderian gland extract also contains all the enzymes of protoporphyrin formation from $\delta$-aminolevulinic acid. In spite of that, inhibition of protoporphyrin formation has been observed in porphyrin synthesis by the Harderian gland extract, as was seen in the case of pantothenic acid- and riboflavin-deficient rats, and this is presumed to be due to inactivation of the enzymes or some co-factors necessary for this reaction system during the incubation.

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