PORPHYRIN METABOLISM

I. EXPERIMENTAL PORPHYRIA IN CHICK EMBRYOS*

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The suggestion of Duesberg (1) that prolonged ingestion of allyliso-
propylacetylcarbamide (Sedormid)1 may have induced acute porphyria in
a human led later workers to produce this disorder in rabbits (2) and rats
(3) by oral administration of the drug. Although this experimental pro-
cEDURE has facilitated the study of acute porphyria, the possibility remains
that some of the porphyrin excreted in this metabolic disturbance originates
from the action of intestinal bacteria (4). This possibility has now been
excluded by use of the embryonated hen’s egg in which injection of Sedor-
mid into the yolk sac, followed by examination of the allantoic fluid for
porphyrins, provides a situation analogous to oral administration of the
drug to animals with subsequent examination of the urine and feces for
porphyrins. Such experiments have demonstrated that the accumulation
of tissue and excretory porphyrins in Sedormid-treated embryos is com-
parable to that occurring in acute porphyria. In addition, congenital de-
formities and retarded growth were noted.

Methods

Preparation of Materials—Pure strain white Leghorn eggs were obtained
from a local hatchery after a week’s incubation, then maintained in a bac-
teriological incubator (37.5°) containing two large pans of water for main-
tenance of humidity. The eggs were turned at least once daily when
 candled for viability. Dead embryos were discarded.

A weighed quantity of Sedormid was “homogenized” in isotonic glycerol
(0.3 M) containing 0.1 per cent Tween 80. The suspension was diluted
with the same solution to a final concentration of 20 mg. per ml. App-
ropriate volumes of the preparation were transferred to vaccine bottles,

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tion Proc., 13, 309 (1954)).

1 Supplied through the courtesy of Hoffmann-La Roche, Inc., Nutley, New Jersey.

663
capped, and sterilized by being heated in a boiling water bath for 1 hour on each of 3 successive days. To prevent caking of the Sedormid, the bottles were shaken frequently by hand during the heating period, and then in a shaking machine until cool.

Experimental Procedure—Sedormid was injected into the yolk sacs of 8 day embryos in doses of 5 and 10 mg. A group of control embryos received 0.50 ml of sterile suspension medium via the same route. At 24 hour intervals for a period of 9 days after injection, three to five living eggs from each group were thoroughly chilled in order to minimize bleeding, the shell and membranes over the air sac were carefully removed, and the allantoic fluid aspirated and collected. After the 9th day following drug treatment, the fluid becomes so scanty and concentrated that samples are not suitable for analysis. Quantitative determinations for coproporphyrin and uroporphyrin and qualitative tests for porphobilinogen were performed daily on pooled allantoic fluids from each group of eggs.

Following aspiration of the allantoic fluid, each embryo was separated from extraembryonic tissues, blotted on absorbent paper, and weighed. Autopsy was performed on many embryos (105 controls, 63 treated with 5 mg., and 116 with 10 mg. of Sedormid) under a dissecting microscope illuminated by ultraviolet light. In every experiment a few eggs from each group were allowed to hatch, and in two experiments the chicks were weighed at hatching and daily thereafter for 11 days, then at convenient intervals until they were 30 days of age. The chicks were fed a commercial starter mash during this period.

Analytical Procedures—Coproporphyrin was determined by the method of Schwartz et al. (5). When the fluid contained more than traces of uroporphyrin, a buffered acetic acid solution made up of equal volumes of glacial acetic acid and saturated aqueous sodium acetate was substituted for the 4:1 buffer originally recommended. After isolation and spectrophotometric identification of coproporphyrin, its isomeric type was established by the fluorescence-quenching technique of Schwartz and associates (6).

Uroporphyrin (unboiled) was estimated in the aqueous phase plus the sodium acetate washes from the coproporphyrin determination by a procedure differing slightly from that of Schwartz et al. (7) in that the adsorption on and elution from alumina were carried out in centrifuge tubes. The term "uroporphyrin" has been used here to indicate ether-insoluble porphyrins.

For the determination of uroporphyrin (unboiled), an aliquot of allantoic fluid was adjusted to pH 5.0 with acetic acid-saturated sodium acetate buffer (1:1) and heated for 1⁄4 hour in a boiling water bath. Uroporphyrin was then estimated as outlined above. After the isolation of uroporphyrin

by repeated adsorption on and elution from alumina, its isomeric type was established by the decarboxylation technique of Edmondson and Schwartz (8).

Porphobilinogen was identified as described by Watson and Schwartz (9).

**Results**

**Mortality**—The mortality rates in the first 24 hours after treatment were 6 per cent for controls and 12 per cent for treated eggs (10 mg. of Sedormid). Many of these deaths resulted from injury to the embryo during inoculation. On the 7th day after treatment, the rates were 3 and 6 per cent, respectively. At all other times the mortality rates were low (<2 per cent).

**Qualitative Observations on Allantoic Fluid**—The red fluorescence characteristic of porphyrins in ultraviolet light became visible in the native allantoic fluids of Sedormid-treated eggs within 24 hours and increased progressively. In the latter part of these experiments, the fluids from eggs treated with 10 mg. of Sedormid exhibited a tan or brownish color in contrast to the yellow or orange-yellow color of normal fluids. Porphobilinogen usually accompanied the tan color.

**Porphyrins in Allantoic Fluid**—Quantitative data on porphyrin concentrations in allantoic fluid are summarized in Table I. Coproporphyrin

### Table I

**Porphyrin Concentrations in Pooled Allantoic Fluids from Embryonated Eggs following Injection of Sedormid into Yolk Sacs**

<table>
<thead>
<tr>
<th>Age of embryo, days</th>
<th>Days after treatment</th>
<th>Coproporphyrin, y per cent</th>
<th>Uroporphyrin (unboiled), y per cent</th>
<th>Uroporphyrin (boiled), y per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 5 mg. Sedormid</td>
<td>10 mg. Sedormid</td>
<td>Control 5 mg. Sedormid</td>
<td>10 mg. Sedormid</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>2.75 4.23 5.86</td>
<td>0.20 2.30 2.30</td>
<td>0.13 0 0.74</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>3.52 14.5 18.1</td>
<td>0.23 6.55 5.88</td>
<td>0.19 0.04 1.73</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>3.70 26.0 35.7</td>
<td>0.81 14.1 17.8</td>
<td>0.30 0.40 2.55</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>4.46 45.8 50.7</td>
<td>1.32 21.3 31.1</td>
<td>0.33 6.00 11.4</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>4.59 51.2 77.0</td>
<td>2.43 43.5 47.4</td>
<td>1.07 6.55 12.6</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>6.41 33.6 197</td>
<td>2.20 27.3 140</td>
<td>2.22 4.60 28.6</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>8.24 72.5 208</td>
<td>2.12 24.2 86.5</td>
<td>1.07 5.16 21.1</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>4.72 44.1 261</td>
<td>4.78 29.3 164</td>
<td>2.82 3.62 23.0</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
<td>4.12 55.8 295</td>
<td>6.27 42.5 195</td>
<td>5.44 6.30 30.0</td>
</tr>
</tbody>
</table>

*Average values derived from five separate experiments for control and 10 mg. dose and two separate experiments for 5 mg. dose. Allantoic fluids from three to five eggs pooled daily in each experiment.

† Expressed in terms of a coproporphyrin standard.
concentrations in the fluids of eggs treated with 10 mg. of Sedormid showed a progressive increase throughout the period studied. Unboiled uroporphyrin in the same samples increased similarly except for the 7th day after treatment. In all other groups there was a progressive rise in por-

\[ \text{CALCULATIONS BASED UPON DATA FROM 445 EGGS} \]

![Graph](http://www.jbc.org/)  

**Fig. 1.** Total coproporphyrin content per egg of allantoic fluid following Sedormid.

porphyrin concentrations through the 5th day following treatment, with an irregular pattern thereafter. It is clear that porphyrin levels in allantoic fluid are markedly increased after treatment with Sedormid, and that boiling at pH 5.0 results in the disappearance of a large proportion of the ether-insoluble porphyrin. Direct interpretation of these concentration figures may be misleading since the volume of allantoic fluid increases through the 13th day of embryonic development and then decreases. For this reason, the allantoic fluid volumes of a large number of eggs were de-
terminated from which the absolute quantities of porphyrins present in the allantoic fluid of a single egg were estimated. The results of these calculations are shown graphically in Figs. 1, 2, and 3. Fig. 1 illustrates the presence of a small amount of coproporphyrin in the allantoic fluids of control eggs. Single 5 mg. doses of Sedormid resulted in an increase in the quantity of coproporphyrin with the maximal drug effect appearing 4 to 5 days after treatment. Coproporphyrin excretion following a 10 mg. dose was higher than that following a 5 mg. dose during the first 4 days, and con-

![Graph showing the increase in uroporphyrin content after Sedormid treatment.](http://www.jbc.org/)

**Fig. 2.** Total "uroporphyrin" content per egg of allantoic fluid following Sedormid.

tinued at about the same rate through the 5th day with a maximal drug effect on the 6th day after treatment. After a direct relationship between dosage and response had been established by two experiments, the 5 mg. dose was discontinued. The decrease in allantoic fluid coproporphyrin noted on the 7th day after a 10 mg. dose was a consistent finding. Since allantoic fluid volume decreased approximately 20 per cent in the 24 hour period between the 6th and 7th days following treatment, some porphyrin may have been reabsorbed with this large volume of water and then re-excreted.

Fig. 2 shows that the actual quantities of uroporphyrin present were

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*Unpublished data obtained by an isotopic dilution technique.*
somewhat smaller but the over-all pattern of excretion resembled that of coproporphyrin. The reduction in uroporphyrin after the boiling at pH 5.0, even in the presence of porphobilinogen, is illustrated by Fig. 3. The positive test for porphobilinogen was strongest when the rate of porphyrin excretion was greatest.

Identification of Porphyrins—The absorption spectrum of coproporphyrin (1.5 N HCl) isolated from control and Sedormid-treated eggs corresponded to that of pure coproporphyrin. Coproporphyrin from treated eggs proved to be the type III isomer (>90 per cent) while that from control eggs was approximately 80 per cent type III and 20 per cent type I.

The ether-insoluble porphyrin (uroporphyrin) isolated from the allantoic fluids of treated eggs showed an absorption maximum at 405 μm compared to 406 μm for pure uroporphyrin in 1.5 N HCl. Decarboxylation (8) of the ether-insoluble porphyrin from treated eggs yielded coproporphyrin type III (>90 per cent). Uroporphyrin from control eggs exhibited an absorption maximum corresponding exactly to that of a pure sample. Its isomeric configuration has not been established.

A porphyrin having the solubility characteristics and HCl number of protoporphyrin was detected in the allantoic fluids of treated eggs. Maxi-
mal absorption appeared at 408 m\(\mu\), which corresponds exactly to the maximum of known protoporphyrin.

![Graph](http://www.jbc.org/)

**Fig. 4.** Growth curves of control and Sedormid-treated chick embryos. Each point represents an average weight derived from sixteen to nineteen embryos.

*Embryo Size and External Appearance*—Growth curves for control and treated embryos are compared in Fig. 4. Treated embryos were significantly smaller than the controls from the 5th through the 9th days following treatment (95 per cent confidence level on the 5th day, 99 per cent from the 6th through the 9th days). Beginning about the 15th day of embryonic life, many treated embryos exhibited gross external abnormali-
ties, including maldevelopment of down (clubbed down), cysts over the caudal region, and a flaccid condition of the legs and feet with the toes distorted.

Observations on Internal Organs—In 8 day embryos the mesonephros is the functional excretory organ (10). 24 hours after the injection of 10 mg. of Sedormid, red fluorescence was noted in the mesonephri from more than half of the embryos when first exposed to ultraviolet light. This fluorescence increased in intensity after 5 minutes irradiation, and many initially non-fluorescent mesonephri became fluorescent afterwards. About the 13th day of age, the metanephri showed fluorescence, which increased greatly upon exposure to ultraviolet light. The fluorescence of both mesonephri and metanephri was localized in small intracellular granules which coalesced as they increased in number. The frequency of occurrence of fluorescence in these organs before and after irradiation is summarized in Table II.

The presence of porphyrin precursors in these tissues is indicated by the increased red fluorescence resulting from ultraviolet irradiation. Porphyrin isolated from these organs was ether-insoluble and the absorption spectrum of its methyl ester in chloroform exhibited maxima at 405, 502, 536, 571, and 626 mp. Once recrystallized uroporphyrin methyl ester isolated from the urine of an acute porphyric showed maxima at 406, 503, 536, 572, and 626 mp.

Embryos with strongly fluorescent kidneys also displayed striking red fluorescence in the calcified portions of all bones. The stomachs, intestines, and cloacae of embryos 17 days of age and older contained large quantities of red fluorescent material when the metanephri were brightly fluorescent. The tissues of control embryos never exhibited fluorescence typical of the porphyrins.

### Table II

| Incidence of Red Fluorescence under Ultraviolet Light in Mesonephri and Metanephri |
|---|---|---|---|---|
| No. of embryos | Mesonephri per cent | Metanephri per cent |
|  | 5 min. ultraviolet irradiation | 5 min. ultraviolet irradiation |
|  | Before | After | Before | After |
| Controls | 105 | 0 | 0 | 0 | 0 |
| 5 mg. Sedormid | 63 | 31 | 66 | 0 | 10 |
| 10 " " | 116 | 85 | 91 | 19 | 46 |
Red fluorescence was seen in the liver in only four embryos in which most other tissues also fluoresced. Alterations in the gross appearance of livers from Sedormid-treated embryos included variations in color from olive-drab to dark green, white necrotic areas, and extreme friability. Normal livers are pink or yellow in color, depending upon the stage of development. Studies in progress have demonstrated a prompt and marked decline in the liver catalase activity of Sedormid-treated embryos. Enzyme activity was significantly reduced 24 hours after drug injection and had reached a minimum of 5 to 10 per cent of normal within 72 hours.

Observations on Hatched Chicks—In hatching, at least three-fourths of the Sedormid-treated chicks were not able to leave the shell unassisted. More than a third of these chicks had seriously deformed feet and poor con-
control of the legs. Neurological involvement was suggested by difficulty in maintaining equilibrium and by the appearance of tremors, especially in the wings.

The growth curves in Fig. 5 show that a single 10 mg. dose of Sedormid given during embryonic development results in definite retardation of growth for at least 30 days after hatching. Since the embryo draws the yolk sac into its abdominal cavity during its last 24 to 48 hours in the egg, average weights of the two groups at hatching did not differ markedly. Gains in weight did not begin until the 3rd and 6th days after hatching for the control and treated groups, respectively. Henceforth, treated chicks grew at a slower rate than the controls. Feather growth was poor in many of the treated chicks.

**DISCUSSION**

Earlier workers have demonstrated the presence of porphyrins in several parts of the egg. Protoporphyrin has been found in the shell (11), yolk (12), albumin (13), and egg-shell membranes (14). Coproporphyrin has been isolated from normal embryos, crystallized, and identified as the type I isomer (15). A search of the literature revealed no previous reports of the occurrence of porphyrins in allantoic fluid.

In contrast to the quantitative relationships usually observed in the urine in acute porphyria, allantoic fluids from Sedormid-treated eggs contain larger amounts of coproporphyrin than uroporphyrin. However, in the chick embryo, the nephric ducts and the gut all terminate in the cloaca, which in turn opens into the allantoic cavity so that the allantoic fluid may contain materials excreted by the liver as well as those excreted by the kidneys. The fact that the porphyrin isolated from kidney tissue proved to be ether-insoluble suggests that the major part of the coproporphyrin is excreted by some other route. Therefore, the porphyrin picture in allantoic fluid is probably more nearly comparable to that of the combined urinary and fecal porphyrin output of mammals than to urinary excretion alone. Wells and Rimington (16) have recently demonstrated the presence of large quantities of endogenous copro- and protoporphyrins in the stools of a patient suffering from porphyria cutanea tarda. Thus, if fecal porphyrin is taken into account, the coproporphyrin output of porphyrin animals also probably exceeds that of uroporphyrin. The reduction in uroporphyrin in allantoic fluid after being heated at pH 5.0, even in the presence of porphobilinogen, is not in agreement with the earlier observations on rabbits (2), rats (3), and humans (17), and remains to be explained. Conversion of porphyrin precursors to uroporphyrin by heating at pH 5.0 is usually required to demonstrate the preponderance of uro- over coproporphyrin in porphyria urines. Thus, failure to note such an increase in
allantoic fluid provides an additional explanation for the larger quantities of coproporphyrin as compared to uroporphyrin. Despite these relatively minor differences in the uroporphyrin picture, the appearance of porphobilinogen and large amounts of type III porphyrins in the allantoic fluids of Sedormid-treated eggs parallels the excretory pattern observed in rabbits (2) and rats (3) suffering from experimental porphyria and in human acute porphyria sufficiently closely to allow this disorder of chick embryos to be labeled acute porphyria. Other similarities between acute porphyria and the condition induced in chick embryos include the marked reduction in liver catalase activity (2), the appearance of material giving red fluorescence under ultraviolet light in several tissues (2, 3, 18), and the probable conversion of porphobilinogen to porphobilin as evidenced by the tan color of the allantoic fluids in the latter stages of an experiment. In this organism, acute porphyria has been demonstrated in the absence of bacteria and therefore is undoubtedly the result of an alteration in porphyrin metabolism induced solely by allylisopropylacetylecarbamide.

Several reports of the production of abnormalities in chick embryos as a result of the injection of a number of compounds into the embryonated egg (19–23) have appeared in the literature. However, the effects of Sedormid treatment did not closely resemble the effects of these other substances. Embryonic abnormalities resulting from Sedormid treatment are very similar to those described by Romanoff and Bauerfeind (24) in embryos developing in eggs from riboflavin-deficient hens. This similarity is especially noteworthy in view of the findings of Stich and Eisgruber (25) that riboflavin promotes hemin synthesis and inhibits production of coproporphyrin I by a yeast grown under conditions ordinarily leading to the formation of increased amounts of coproporphyrin I. However, the anomalies resulting from Sedormid treatment also resembled in certain respects those observed in embryos developing in eggs from vitamin B₁₂-deficient hens (26). Poultry experts, consulted regarding the condition of chicks hatched from Sedormid-treated eggs, expressed the opinion that certain features of several vitamin deficiencies were apparent but that the symptoms were not characteristic of any one nutritional deficiency syndrome.⁴

The recent work of Shemin and Russell (27), demonstrating that porphyrins and purines arise from a common precursor, suggests that disorders of purine metabolism may be associated with disorders in porphyrin metabolism. Inhibition of purine synthesis with its resultant decrease in nucleic acid formation offers a logical explanation for the retarded growth of por-

⁴ The authors wish to acknowledge the generous advice and cooperation of Dr. G. H. Arscott, Dr. J. E. Parker, and Dr. P. E. Bernier of the Department of Poultry Husbandry, Oregon State College, Corvallis, Oregon.
PORPHYRIN METABOLISM. I

Phyric embryos and chicks. Moreover, symptoms suggesting avitaminosis might appear as a consequence of purine deficiency, since many vitamins function as a moiety of purine-containing prosthetic groups. Studies in progress in this laboratory indicate that there may indeed be an alteration in purine synthesis in experimental porphyria. Another factor which may contribute to retarded growth is the suppression of normal liver function implied by a marked reduction in liver catalase activity. Whatever the mechanism involved, it is clear that a single 10 mg. dose of Sedormid given to an 8 day embryo produces a metabolic defect lasting for at least 30 days after hatching.

SUMMARY

Injection of Sedormid into the yolk sacs of 8 day chick embryos results in the accumulation of large quantities of type III porphyrins in their allantoic fluids. When the rate of porphyrin excretion is high, porphobilinogen also appears in these fluids.

At autopsy, many of the embryonic tissues display brilliant red fluorescence under ultraviolet light. The livers are frequently olive-drab or green in color with white necrotic areas. Liver catalase activity is markedly reduced.

Significant retardation of embryonic growth becomes apparent by the 5th day after drug treatment. Congenital anomalies appear in many chicks hatched from treated eggs and, in addition, the growth of these chicks is retarded for at least 30 days after hatching.

The possibility of an associated derangement in purine metabolism as a factor in experimental porphyria is considered.

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BIBLIOGRAPHY

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