Pteridine and Riboflavin Patterns During Tail Regeneration in *Triturus* Species and the Effects of Chloramphenicol, Isoxanthopterin and Reserpine

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In *Triturus cristatus* amputation causes the reappearance of larval tetrahydrobiopterin, thus raising the ratios tetrahydrobiopterin/isoxanthopterin and tetrahydrobiopterin/riboflavin from zero to values between 3—5. This increase first occurs in the remaining skin and in the eyes. The increase of both ratios in the regeneration bud, beginning with the 20th day after amputation, coincides with their drop in both other tissues. Chloramphenicol and isoxanthopterin both strongly inhibit the formation of a regeneration bud. They also block the increase of both ratios in the remaining skin and in the rudimental regenerate as well. Reserpine induces regenerative ability in *Triturus vulgaris*, which normally lacks this. It has a strong melanizing effect and, moreover, it causes an increase of both ratios in the regeneration bud and in the remaining skin.

I. Introduction

Prior investigations have shown that in *Triturus alpestris* and *T. cristatus* larval tetrahydrobiopterin disappears with metamorphosis but reappears in the regeneration blastema of the adult newt, whereas isoxanthopterin and riboflavin stay at about the same level. Thus the ratios tetrahydrobiopterin/isoxanthopterin (TH/IX) and tetrahydrobiopterin/riboflavin (TH/RB), starting from zero in the adult animal's skin, rise to a level of 2.5—3 in the regeneration bud; both ratios drop off again with decrease in mitotic activity and proceeding redifferentiation of the regeneration blastema.

To investigate the apparent close relationship between the onset of the regeneration and the high TH/IX and TH/RB ratios further, the time course of the pteridine and riboflavin patterns in the regeneration blastema and in the remaining pteridine containing tissues (skin and eye) were studied in more detail.

Moreover, the action of chloramphenicol, isoxanthopterin and reserpine, which we know to influence protein synthesis, DNA activation or mitotic activity, were studied with respect to bud formation and to the pteridine pattern.

Chloramphenicol is a well known inhibitor of protein synthesis. In bacterial cell free systems it affects the peptide bond synthesis.

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Reserpine is known to interfere with binding of catecholamines in the sympathetic nervous system, thus exposing them to enzymatic degradation. This in turn results in the release of a feed-back inhibition of the tyrosine-hydroxylase (see l.c.\textsuperscript{13}). The coenzyme of this first and rate limiting step of norepinephrine biosynthesis is known to be tetrahydrobiopterin (see l.c.\textsuperscript{14}). Besides the release of endproduct inhibition, the regulation of catecholamine synthesis is also affected by enzyme induction\textsuperscript{15}. This mechanism is initiated by the application of large doses (5 mg/kg) reserpine\textsuperscript{16}. Reserpine, moreover, has been found to act in one more, very different way; it initiates DNA activation in \textit{T. cristatus} and a concomitant increase in the ratio TH/IX\textsuperscript{17}. In contrast to \textit{T. cristatus} and \textit{T. alpestris}, \textit{T. vulgaris} does not form a regeneration bud, nor does it increase its TH/IX and TH/RB ratios\textsuperscript{1}. Moreover, the skin of \textit{T. vulgaris} is characterized by scarce, contracted melanophores in the dermal layer and the lack of melanophores in the epidermis at all. The promoting effect of reserpine on DNA activation and tetrahydrobiopterin formation on one hand and the close relationship between tetrahydrobiopterin and melanin synthesis\textsuperscript{18} (see l.c.\textsuperscript{14}) on the other prompted us to examine its effect on formation of a regeneration bud, the tetrahydrobiopterin level and on the status of the melanophores in this species.

### II. Methods

The raising and amputation of \textit{T. cristatus} and \textit{T. vulgaris} were done as described in l.c.\textsuperscript{1}. The injections were made intraperitoneal. Dosage and timing are given in “Results”. Reserpine (serpasil) was from Ciba, the chloramphenicol used was Kemicetine succinate, Carlo Erba. We thank Dr. PFLEIDERER (Konstanz) and Dr. REMBOLD (München) for the generous gift of isoxanthopterin.

For extraction, the samples were ground in the Potter-Elvehjem with cold methanol, to which mercaptoethanol (0.25\% final conc.) and NH\textsubscript{4}OH (up to pH 10) were added. The homogenate was centrifuged at 8000 \(g\) for 15 min and the supernatant was dried in the presence of P\textsubscript{2}O\textsubscript{5}. All samples were adjusted to 0.5 ml with a 0.25\% solution of mercaptoethanol. Aliquots (between 50—200 \(\mu\)l, varying with the material) were chromatographed on Whatman I with butanol/acetic acid/water (4 : 1 : 5), to which 0.25\% mercaptoethanol was added. All operations were done in red safe light; the chromatograms were run in the dark.

The identification of the different pteridines as well as of tetrahydrobiopterin is described in l.c.\textsuperscript{1}. The quantitative determination of riboflavin was done by the growth test with \textit{Lactobacillus casei}\textsuperscript{1, 19}.

### III. Results

The compounds identified in the skin of \textit{T. cristatus} and \textit{T. vulgaris} were isoxanthopterin, pterincarbonic acid(6), 2-amino-4-hydroxypteridine, biopterin, and riboflavin. In addition to these tetrahydrobiopterin appeared under given experimental conditions. As pterincarbonic acid and 2-amino-4-hydroxypteridine were present at a very minor, but constant level, only isoxanthopterin, tetrahydrobiopterin and riboflavin are to be consiered here.

Even though the absolute amounts of pteridines vary in the normal adult skin of the different animals, the ratios TH/IX and TH/RB are constant and denote the stable ratios of the adult, non proliferating tissue, used as control in the following experiments.

#### A. The ratios TH/IX and TH/RB during tail regeneration in \textit{Triturus cristatus}

Figs. 1 a and 1 b show that the increase in the ratios TH/IX and TH/RB not only takes place in the regeneration bud, but in all tissues with unconjugated pteridines. Furthermore it is clearly indicated that it first occurs in the remaining skin and that it then follows in the eyes. In the regeneration bud it does not start before the 20\textsuperscript{th} day after amputation. The increase here immediately is followed by a marked drop of both ratios in the remaining skin and in the eyes.

#### B. The action of drugs on regeneration and on the ratios TH/IX and TH/RB

**a) Chloramphenicol**

The injections were made at a dose of 0.2 mg/g body weight at intervals of 6 hours. For the experiments the animals were divided into 3 groups, as follows: In group I the animals were amputated, chloramphenicol was simultaneously administered, and the treatment was maintained until the end of the experimental period (20 days after amputation). The other 2 groups (II and III) of the amputated newts also received chloramphenicol, however...
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longitudinal growth of the regenerates (regeneration time 20 days). Apart from the numerical data about the length of the regenerates, the general appearance of the regeneration tails clearly indicated (without any statistical evaluation) the considerable difference between the tails under normal conditions and those of animals treated with chloramphenicol at various times after amputation. There was no gross or microscopic evidence of blastema formation in the drug-treated animals, when chloramphenicol was administered immediately after amputation (time 0, group I), while the regenerates obtained when the application of chloramphenicol was started 3 days (group II) or 10 days (group III) after amputation were definitely smaller than those of the control.

ß) The effect of chloramphenicol on pteridine and riboflavin pattern of tail regenerates and skin

After a regeneration time of 20 days, quantitative examinations showed significant variations in the ratios TH/IX and TH/RB as chloramphenicol was applied at different times after amputation. These variations were found not only in the regeneration bud itself (Table 1) but also in the remaining skin of the animals (Table 2).

The most striking effect occurred in group I. No blastema formation had taken place, consequently no tetrahydrobiopterin was to be detected. Also no tetrahydrobiopterin was found in the remaining skin of the animals. The amount of tetrahydrobiopterin in the regeneration bud and in the skin increases as the application of chloramphenicol after amputation is deferred. The opposite ef-

<table>
<thead>
<tr>
<th>Group</th>
<th>Starting time of chloramphenicol application</th>
<th>TH</th>
<th>IX</th>
<th>RB</th>
<th>TH/IX</th>
<th>TH/RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>--a</td>
<td>--a</td>
<td>--a</td>
<td>--a</td>
<td>--</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>1.15 ± .06</td>
<td>402 ± 18</td>
<td>382 ± 17</td>
<td>806 ± 31</td>
<td>1.31 ± .07</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>3.05 ± .09</td>
<td>585 ± 23</td>
<td>312 ± 15</td>
<td>497 ± 20</td>
<td>1.87 ± .12</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>4.20 ± .05</td>
<td>-b</td>
<td>-b</td>
<td>2.87b</td>
<td>3.00b</td>
</tr>
</tbody>
</table>

Table 1. Effect of chloramphenicol on longitudinal growth and on pteridine and riboflavin of tail regenerate of Triturus cristatus. Each group: 10 animals.

a There was no blastema formation at all in this group; consequently no determinations of pteridines and riboflavin were made.
b Results from another serie of experiments, where attention only was drawn to the relative changes. See l. c. 1.
c Time after amputation in days, after which the animals were treated with chloramphenicol until the end of the experiments (20 days). For explanations see also text. d Fluorometer units in the same weight of regenerates. The deviations here are given in absolute values. The deviation in TH/IX and TH/RB represent the square deviations. e The difference in length between the controls and the drug treated animals was clear without need of any statistical evaluation. Abbreviation: TH = tetrahydrobiopterin; IX = isoxanthopterin; RB = riboflavin.

Fig. 1 a. Ratio tetrahydrobiopterin/isoxanthopterin after tail amputation in T. cristatus. square deviations.

Fig. 1 b. Ratio tetrahydrobiopterin/riboflavin after tail amputation in T. cristatus. square deviations. regeneration bud, — — — remaining skin, ⋅ ⋅ ⋅ eye.

the injections did not start with the day of amputation, but rather 3 and 10 days respectively after it.

a) The effect of chloramphenicol on the growth rate of the regeneration bud

Apart from its effect on the formation of the regeneration bud, chloramphenicol in the concentrations used showed no apparent toxic effect on the animals. Table 1 summarizes the data concerning
fect is seen in the case of isoxanthopterin and riboflavin. In general their amount increases in the regeneration bud and in the skin the sooner the application of chloramphenicol is started after amputation. Only the level of riboflavin in the skin shows no differences between group II and III.

Thus, as seen in Tables 1 and 2, the ratios TH/IX and TH/RB increase with the deferment of the starting time of chloramphenicol application, exhibiting their maximum value in group III. Both ratios strictly parallel the lengths of the regeneration.

b) Isoxanthopterin

The injections were made at a dose of 3 μg/g body weight at intervals of 12 hours. The difference between groups I, II, and III with respect to the timing of injection corresponds to the experiments done with chloramphenicol, with the exception that the injections in group III started with the 7th day after amputation and all animals were killed after 17 days.

a) Effect of isoxanthopterin on the growth rates of the regeneration bud

As does chloramphenicol, isoxanthopterin also exerts a strong inhibitory effect on the formation of the regeneration bud (Table 3). The inhibition increases the earlier the injections were started after amputation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Starting time of isoxanthopterin application [day]</th>
<th>TH/IX</th>
<th>TH/RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>0.5 ± .01</td>
<td>0.37 ± .029</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>0.6 ± .01</td>
<td>0.67 ± .050</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>0.8 ± .01</td>
<td>0.73 ± .062</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>3.5 ± .05</td>
<td>1.14 ± .071</td>
</tr>
</tbody>
</table>

Table 3. Changes of pteridines and riboflavin of the regenerates and of the remaining skin, during regeneration of *T. cristatus* under the influence of isoxanthopterin. Each group: 10 animals. For explanations (a—e) see Table 1.

β) Effect of isoxanthopterin on pteridine and riboflavin pattern in tail regenerate and remaining skin

As seen in Table 3, the ratios TH/IX and TH/RB are strongly reduced both in the regeneration bud and in the remaining skin. This decrease is also here increasingly expressed the earlier the injections started after amputation. Seemingly the percentage of reduction in both ratios, compared with the control is expressed even more in the remaining skin than it is in the regeneration bud itself.

c) Reserpine

The injections were made at a dose of 10 μg/g body weight at intervals of 24 hours. This is double the amount needed for induction of the hydroxylating enzyme system (see introduction). Even though the regeneration time (17 days) was the same for all animals, the timing of injections was different in the groups I—VII. In group I the injections were started 12 days, in group II 7 days preceding the amputation and were stopped 24 hours before it. In groups III and IV, injections were started at the time of amputation and were continued for 16 days (group III) or 7 days (group IV) again. In groups V and VI injection of reserpine was started 5 days and 10 days respectively after amputation; thus the animals of group V received 12, those of group VI 7 injections. Group VII was run as a control.
The most striking effect is that in all drug treated animals tetrahydrobiopterin is found in the regeneration bud and in the remaining skin. Therefore the ratios TH/IX and TH/RB in the regeneration bud increase with the deferment of starting time of the drug application after amputation; they reach their highest values in group VI, where the application started 10 days after amputation. This does not account for the remaining skin, where the drug application preceding the amputation causes the highest levels of tetrahydrobiopterin (Table 4).

Besides tetrahydrobiopterin a yellow fluorescing substance appeared to a minor extent in the skin of the drug treated animals. It seems to be a degradation product, caused by acidic chromatography. It is neither riboflavin (or any of its derivatives like FMN or FAD) nor it is identical with 7,8-dihydro-6-lactoylpteridine (sepiapterin), isosepiapterin or 7,8-dihydro-6-lactoyllumazine. It is not a growth factor for *Crithidia* (see l. c. \(^9\)). Its chemical nature has still to be identified.

**Discussion**

The time course of tetrahydrobiopterin synthesis shows that the initiating event, the amputation, first affects the pteridine metabolism of the remaining skin. The subsequent appearance of tetrahydrobiopterin in the regeneration bud coincides with its decline in the remaining skin. Therefore one tends to assume that it is synthesized in the remaining skin and then transported into the regeneration bud. This view is supported by the fact that no increase in degradation products, e.g. isoxanthopterin, goes along with the decline of tetrahydrobiopterin and that changes in the ratio RB/IX do not take place. Furthermore, the action of reserpine, administered before amputation, causes the highest levels of tetrahydrobiopterin in the remaining skin. This may be due to the fact that the drug causes tetrahydrobiopterin synthesis immediately after amputation, however, it cannot be trans-
ported quickly. In contrast, later application of reserpine may cause the newly formed tetrahydrobipterin to be immediately transported into the growing bud.

The period of intensive tetrahydrobipterin synthesis in the remaining skin in turn coincides with the time of DNA synthesis, which starts after 5 days in the limbs of amputated newts and in the liver after partial hepatectomy.

Histochemical and biochemical analyses have shown that intensive DNA and RNA synthesis as well as ribosomal protein synthesis are important metabolic activities in dedifferentiating tissues. This agrees with the above results: both drugs which interfere with these processes also inhibit the formation of a regeneration bud. In parallel, the level of tetrahydrobipterin is kept low. In contrast, reserpine, which activates DNA and increases the regenerative ability, also does improve tetrahydrobipterin accumulation.

Thus two main problems arise. The first is the question of whether the drugs affect the synthesis of tetrahydrobipterin from its purin precursor (see l.c. 8, 14), or whether they mainly influence its further metabolism. The second question is, whether the basic events of blastema formation, which are DNA, RNA and ribosomal protein synthesis on the one hand, and, a high value of TH/IX and TH/RB ratios on the other, have any causative connection, or whether they are parallel phenomena initiated by one common event.

As to the first question, a possible inhibition of tetrahydrobipterin synthesis by chloramphenicol and isoxanthopterin and its enhancement by reserpine respectively, has to be examined by application of guanosine-14C-phosphate (see l.c. 8, 14). By an additional effect, chloramphenicol may prevent the formation of folic acid reductase, which is to some degree active in the reduction of 7,8-dihydrobipterin, to form the tetrahydrocompound; induction of folic acid reductase has already been shown to be blocked in S. faecium by chloramphenicol. As we know, not only isoxanthopterin but also lumazines, including the lumazine derivative riboflavin, are endproducts of pteridine metabolism (see l.c. 8): tetrahydrobipterin is converted by the action of a pterin deaminase and by xanthin-oxidase into lumazines. Table 1 and 2 show that chloramphenicol, for instance, causes a marked increase in isoxanthopterin as well as in riboflavin, indicating an enhanced catabolism of tetrahydrobipterin under the influence of this drug. Haldar and Freeman showed that the NADH oxidizing activity is reduced by chloramphenicol, and it was found that two different sites are attached to the enzyme molecule, one for pterin and another for NADH. Thus it seems not unlikely that an inhibition of NADH oxidation capacity enhances its pteridine metabolizing activity.

As to the second question, the action of tetrahydrobipterin as a cofactor in hydroxylation reactions (see l.c. 8) does not seem to be involved here. It should be emphasized that in tissues with high protein synthesis levels (and a high rate of phenylalanine hydroxylation) the amount of tetrahydrobipterin is high indeed, but the ratios TH/IX and TH/RB remain low due to the high levels of both products of pteridine metabolism. Therefore tetrahydrobipterin and, respectively, the ratios TH/IX and TH/RB may act in some additional way, controlling mitotic activity and blastema formation. The only indication up to now we have for a direct causal connection between DNA and RNA activation and pterins is the observation that isoxanthopterin inhibits DNA and ribosomal RNA synthesis and forms an unstable association with DNA. The strong inhibition of blastema formation by isoxanthopterin found here and its suppressing effect on tumor growth favors the idea that neoplastic growth and low content in metabolites of tetrahydrobipterin may be directly connected. The suppression of a high TH/IX ratio by application of isoxanthopterin in turn may exert an amplifying action.

The lack of regenerative ability in the scarcely melanized T. vulgaris, in contrast to the well established regeneration ability in both melanized species T. cristatus and T. alpestris on one hand and the coupled enhancement of increase in melanization and regeneration by reserpine on the other emphasize the close connection of rapid cell proliferation and melanization. The failure of Biber and Hitching to find an inhibitory action of chloramphenicol on regeneration in tadpoles may be connected with this, as at this stage tetrahydrobipterin is still present in large amounts and melanization is going on. The action of reserpine on melanization observed here may be explained to some degree by its dispersing action on melanin granules, rendering them more accessible to the...
N. Kokolis, N. Mylonas, and I. Ziegler, *Pteridine and Riboflavin Patterns During Tail Regeneration in Triturus Species and the Effects of Chloramphenicol, Isoxanthopterin and Reserpine* (page 285)

Fig. 2 a. Melanization of the wound healing zone in normal *Triturus vulgaris*.

Fig. 2 b. Melanization of the regeneration bud in *Triturus vulgaris*, under the influence of reserpine (Group VI). Magnified: x 132.
tyrosine hydroxylation cofactor tetrahydrobiopterin and thus activating melanin synthesis. However, melanization seems to be more generally connected with cell proliferation; the melanomas, especially those caused by hybridization of Xiphophorus species (see l.c. 30), seem to be a most suitable tool for further investigations of the connection between tetrahydrobiopterin metabolism, melanin synthesis and cell proliferation.

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6 B. M. Burnet u. R. A. Liversage, Amer. Zoologist 4, 427 [1964].
8 S. Biebel u. G. H. Hitchings, Cancer Res. 19, 112 [1959].