The influence of aminopterin on limb regeneration in *Ambystoma mexicanum*

By D. O. E. GEBHARDT & J. FABER

From the National Institute of Public Health, Utrecht, and the Hubrecht Laboratory, Utrecht

During the last twenty-five years a number of authors have studied the influence of chemical substances on limb regeneration in amphibians. Examples of compounds which have been tested so far are: (1) the antimitotic agent, colchicine (Thornton, 1943); (2) the salt, beryllium nitrate (Thornton, 1949, 1950, 1951); (3) the carcinogens, dibenzanthracene and methylcholanthrene (Karczmar & Berg, 1952; Ruben & Balls, 1964); (4) the lathyrus factor, β-aminopropionitrile (Chang, Witschi & Ponseti, 1955); (5) the hormone, thyroxine (Hay, 1956); (6) atropine and other neuropharmacological drugs (Singer, Davis & Scheuing, 1960); (7) the metachromatic dye, toluidine blue (Csaba, Bierbauer & Törö, 1961); and (8) semicarbazide, an inhibitor of histamine formation (Deck & Shapiro, 1963). Most of these substances caused growth retardation as well as malformations of the limb regenerates.

A number of other investigators have studied the effects of chemicals on the ontogenetic development of the amphibian limb. A few examples may suffice here. Bretscher (1949) used colchicine and Tschumi (1954) the radiomimetic chloraethylamine in experiments with *Xenopus* tadpoles. Mustakallio & Telkka (1954) studied the influence of aureomycin, vitamin B₁₂ and aminopterin in *Rana*, whilst Rostand (1955), in his interesting book on limb malformations in frogs, described the effect of trypaflavin on anuran limb development.

It was observed that some of the malformations obtained in amphibians were rather similar to those caused by thalidomide in human embryos. Studies were therefore initiated to determine whether the regenerating amphibian limb would be a suitable system for testing the teratogenic effects of certain drugs (Gebhardt & Faber, 1966). Although no relationship was found between the teratogenic properties of drugs in mammals and their effects on regeneration, some interesting results were obtained with the folic acid analogue aminopterin. In this paper a more detailed report of the work done with this and two other structurally related compounds is presented.

1 Author's address: Rijks Instituut voor de Volksgezondheid, Sterrenbos 1, Utrecht, Netherlands.

2 Author's address: Hubrecht Laboratory, International Embryological Institute, c/o Universiteitscentrum 'De Uithof', Utrecht, Netherlands.
Folic acid or pteroylglutamic acid (PGA) has a number of structural analogues which inhibit enzymic reactions in which folic acid is the substrate. For example, aminopterin (4-amino PGA) is an inhibitor of the enzyme folic reductase, which catalyses the reduction of folic acid to tetrahydrofolic acid (5,6,7,8-tetrahydro PGA). Derivatives of tetrahydrofolic acid act as carbon-donating substances in enzymic carbon transfer reactions. An example of such a process is the synthesis of thymidylate acid from deoxyuridylic acid in the presence of 5,10-methylenetetrahydrofolic acid and the enzyme thymidylate synthetase. As thymidylate acid is a DNA precursor, inhibition of its formation by folic acid analogues will prevent synthesis of DNA.

At the cellular level, the effect of aminopterin on mitosis in tissue culture has been studied by Jacobson (1954, 1964). He showed that cell division in the presence of this substance could not proceed beyond metaphase. After a period of about 24 h, however, the inhibition appeared to be reversible and the cells became insensitive to the folic acid antagonist.

It has been found possible to protect cells and organisms from the action of folic acid analogues by the simultaneous administration of folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid, also known as citrovorum factor or leucovorin). This was demonstrated in chick embryos by Snell & Cravens (1950), in mice by Broquist et al. (1952), in developing Rana by Grant (1960), in bacteria by Woolley (1963), and in dividing cells in tissue culture by Dalen, Oftebro & Engeset (1965). Protection from the effect of aminopterin has also been obtained by the simultaneous administration of thymus DNA or yeast RNA and thymidine. This was shown by O'Dell & McKenzie (1963) in their study on the action of aminopterin on the explanted early chick embryo. Their paper contains a useful review of the literature of the various effects of aminopterin on vertebrate cells and organisms.

In the light of the above information, experiments were designed in an attempt to solve the following questions:

1. At which stage of development of the blastema does administration of aminopterin disturb regeneration, and at what dosage?
2. Which kinds of morphological abnormalities are produced by the drug?
3. Does the level of amputation of the limb affect the mode of regeneration in the treated animal?
4. Do compounds structurally related to aminopterin such as methotrexate (4-amino-\(N^{10}\)-methyl PGA) and \(N^{10}\)-methyl PGA have similar effects on regeneration?
5. Is it possible to counteract the effect of aminopterin by the administration of substances such as folinic acid or thymidine whose synthesis is inhibited by the folic acid analogue?
Aminopterin and limb regeneration

METHODS

The axolotl larvae used in these experiments were approximately 9 cm long and weighed about 10 g. The forelimbs were amputated either through the middle of the upper arm (operation A, Text-fig. 1) or through the distal part of the lower arm (operation B, Text-fig. 1), according to the method described by Hamburger (1960); in some cases the hindlimbs were also amputated. The substances to be tested were administered orally in gelatin capsules (no. 5, Eli Lilly & Co.) together with a small piece of beef heart to fill the capsule. Each animal received 1 mg of the drug, corresponding to a dose of 100 mg/kg. In two instances in which either the dose-response relationship of aminopterin or the protective action of folinic acid was studied, other doses were also used. The animals were maintained in separate containers in quarters where the temperature was approximately 20°C.

Text-fig. 1. Diagram of the forelimb showing the two levels of amputation (operations A and B).

The aminopterin used in the experiments was obtained from Koch-Light Laboratories, Colnbrook, England. Methotrexate and \(N^{10}\)-methyl PGA were supplied by Dr A. Heltai of Cyanamid International, Pearl River, N.Y. Thymidine was obtained from Sigma Chemical Co., St Louis, Mo., and folinic acid from Serva Entwicklungslabor, Heidelberg.

Two main sets of experiments were carried out in which aminopterin was given to animals undergoing forelimb amputation either by the method of operation A or operation B (see Text-fig. 1). The A series consisted of 95, and the B series of 75 operated animals. The series were subdivided into smaller groups and each of these received aminopterin at different times before or after amputation.

The hindlimbs were also amputated in a limited number of animals by either operation A or operation B. Sometimes both operations A and B were performed on the same animal, using the fore- and hindlimbs. In 40 control animals which received no aminopterin, forelimb regeneration after either operation A or B always resulted in limbs with the normal number of four digits.

The cartilaginous skeleton of the regenerated limbs was examined following \textit{in toto} staining with methyl green and clearing in benzyl benzoate according to Tschumi (1954).
Determination of the stages of regeneration

The staging of forelimb regenerates after operation A for 9 cm axolotls at approximately 20° is given in Table 1. A more detailed description of the stages is given by Faber (1960).

Table 1. Staging of forelimb regeneration (operation A) at ± 20°

<table>
<thead>
<tr>
<th>Post-amputation age (days)</th>
<th>Ratio length/breadth of blastema</th>
<th>Stage according to Faber (1960)</th>
</tr>
</thead>
<tbody>
<tr>
<td>± 8</td>
<td>0.50</td>
<td>I. Middle cone</td>
</tr>
<tr>
<td>± 10</td>
<td>0.75</td>
<td>II. Late cone</td>
</tr>
<tr>
<td>± 14</td>
<td>1.00</td>
<td>III. Early paddle</td>
</tr>
<tr>
<td>± 19</td>
<td>1.20</td>
<td>IV. Paddle, 1 digital rudiment</td>
</tr>
<tr>
<td>± 22</td>
<td>1.30</td>
<td>V. Paddle, 2 digital rudiments</td>
</tr>
</tbody>
</table>

After operation B the blastema appears at the same time, but does not grow as fast as after operation A. Nevertheless, since the amount of limb material to be replaced after distal amputation is much smaller than after proximal amputation, the first digit appears several days earlier, viz. when the length/breadth ratio of the blastema is still smaller than 1.0. The other digits also appear several days earlier than after operation A.

EXPERIMENTS AND RESULTS

Effect of oral administration of 100 mg/kg aminopterin at various times before or after amputation on the number of regenerating digits

In the evaluation of the results of the two main experimental series, A and B, the left and right malformed forelimbs of each treated animal were considered separately, because they were only rarely mirror images of each other. The results of these two series are summarized in Tables 2 and 3. The numbers of normal, oligodactylous and polydactylous forelimbs are given as percentages of the total number of regenerating limbs. All data are graphically represented in Text-fig. 2.

As shown in the tables and graphs, polydactylism never occurred in the B series. Oligodactylism, on the other hand, occurred in both series and at all times of treatment up to day 19 for the A series, and up to day 16 for the B series. An illustration of polydactylism and oligodactylism in the A series is given in the Plate, figs. A, B.

Following operation A polydactylism never occurred when the drug was given later than 10 days after amputation. The highest numbers of polydactylous limbs were found after administration within the first week following amputation.

In series A there was a distinct optimal period for the induction of oligodactylism around the fourteenth day after amputation. At this time of treatment
All animals are seen in ventral view.

Fig. A. Aminopterin given 2 days after amputation of both forelimbs by operation A. The left forelimb regenerate is polydactylous. The right forelimb regenerate was also polydactylous but was re-amputated 6 weeks later. It has yielded a normal second regenerate.

Fig. B. Aminopterin given 14 days after amputation of both forelimbs by operation A. The right forelimb regenerate is oligodactylous. The left forelimb regenerate was also oligodactylous but was re-amputated 6 weeks later. It has yielded a normal second regenerate.

Fig. C. Aminopterin given 6 days after amputation of both forelimbs by operation B and of the left hindlimb by operation A. The left forelimb has yielded an oligodactylous and the left hindlimb a polydactylous regenerate. The right forelimb regenerate was also oligodactylous but was re-amputated 6 weeks later. It has yielded a normal second regenerate. The right hindlimb was not amputated.

Fig. D. Aminopterin given 6 days after amputation of the left hindlimb by operation A and of the right hindlimb by operation B. The left regenerate is polydactylous, the right regenerate oligodactylous.

D. O. E. GEBHARDT & J. FABER
Aminopterin and limb regeneration

the degree of oligodactylism induced was most pronounced, and all regenerates were affected. In the B series the incidence of oligodactylism increased up to day 6 of treatment, when about three-quarters of the resulting regenerates were malformed, and declined thereafter.

Table 2. Operation A (both forelimbs amputated)
Effect of a single oral dose of 1mg aminopterin.

<table>
<thead>
<tr>
<th>Day of administration*</th>
<th>No. of limbs amputated</th>
<th>Regenerates with normal no. of digits (%):</th>
<th>Oligodactylous regenerates (%)</th>
<th>Polydactylous regenerates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>−7</td>
<td>10</td>
<td>90</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>−2</td>
<td>10</td>
<td>60</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>+2</td>
<td>10</td>
<td>30</td>
<td>40†</td>
<td>30</td>
</tr>
<tr>
<td>+6</td>
<td>58</td>
<td>47</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>+10</td>
<td>14</td>
<td>50</td>
<td>43</td>
<td>7</td>
</tr>
<tr>
<td>+14</td>
<td>32</td>
<td>—</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>+19</td>
<td>46</td>
<td>39</td>
<td>61</td>
<td>—</td>
</tr>
<tr>
<td>+23</td>
<td>10</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* − = days prior to amputation; + = days after amputation.
† The apparent peak in sensitivity on day +2 is uncertain because of the small number of observations.

Table 3. Operation B (both forelimbs amputated)
Effect of a single oral dose of 1mg aminopterin.

<table>
<thead>
<tr>
<th>Day of administration*</th>
<th>No. of limbs amputated</th>
<th>Regenerates with normal no. of digits (%):</th>
<th>Oligodactylous regenerates (%)</th>
<th>Polydactylous regenerates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>−7</td>
<td>10</td>
<td>90</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>−4</td>
<td>20</td>
<td>70</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>+2</td>
<td>10</td>
<td>40</td>
<td>60</td>
<td>—</td>
</tr>
<tr>
<td>+6</td>
<td>46</td>
<td>24</td>
<td>76</td>
<td>—</td>
</tr>
<tr>
<td>+11</td>
<td>20</td>
<td>30</td>
<td>70</td>
<td>—</td>
</tr>
<tr>
<td>+16</td>
<td>10</td>
<td>70</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>+19</td>
<td>34</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* − = days prior to amputation; + = days after amputation.

The normal hindlimb has five digits. After amputation through the middle of the upper leg (operation A) the number of regenerating digits was again dependent on the day of administration of the drug. Just as in the forelimb, the sensitive phase for the induction of polydactyly ended earlier than that for oligodactylism, but here both phases extended longer. In the hindlimbs it was still possible to induce polydactylism by administration on day 14, and oligo-
dactylism by administration on day 22. After amputation through the distal end of the lower leg (operation B) abnormalities were again restricted to oligodactylism. Here, too, the sensitive phase was somewhat longer than in the forelimb. Poly- and oligodactylism in the hindlimbs are illustrated in the Plate, figs. C, D.

Text-fig. 2. Graphical representation of the data of Table 2 (operation A) and Table 3 (operation B). The total height of each column represents 100 %. The two graphs are on the same time-scale. □ Regenerates with normal number of digits; □ oligodactylous regenerates; □ polydactylous regenerates.

Two contralateral fore- or hindlimb regenerates of one animal, undergoing the same operation, were seldom affected to the same degree by aminopterin. Sometimes one was poly- or oligodactylous, whereas the other had the normal number of digits, However, poly- and oligodactylism never occurred together in contralateral limb pairs of the same animal if only operation A was performed. If, on the other hand, one limb had undergone operation A and the contralateral (or ipsilateral) one operation B, and if aminopterin was administered
Aminopterin and limb regeneration

before the end of the sensitive phase for polydactylysm, the former limb often became polydactylysm and the latter oligodactylysm (Plate, figs. C, D).

Limb malformations were sometimes induced by aminopterin when it was given prior to amputation. This was an indication that the rate of metabolism of the drug was low in these animals and that the analogue was present in an active state for a number of days. On the other hand, re-amputation of malformed limbs 6 weeks after administration of aminopterin always yielded normal second regenerates (Plate, figs. A, B, C).

Aminopterin generally had a growth-retarding effect leading to a delay in the appearance of the digits of about 2 weeks. This was observed both in oligodactylysm and in polydactylysm limbs, as well as in cases where the normal number of digits regenerated. After administration during the optimal phase for the induction of oligodactylysm in the forelimb, particularly on day 14 after operation A, this delay amounted to as much as 3 weeks.

Morphological analysis of malformed limbs

The axolotl forelimb normally has four digits. The structure of the hand skeleton has been described by Faber (1960). The phalangeal formula is 2–2–3–2. There are four metacarpals and eight carpal elements. The hindlimb has five digits with the phalangeal formula 2–2–3–4–2. There are five metatarsals and ten tarsal elements. In the forelimb, polydactylysm involved the formation of one additional digit. Although in general hindlimb polydactylysm was likewise restricted to the formation of one extra digit, a small number of seven-digit limbs were also encountered (Plate, fig. C).

An analysis of the type of polydactylysm showed, with one exception, that it was postaxial, i.e. it did not involve the anterior two digits in the forelimb and the anterior three in the hindlimb. Often the third digit of the forelimb was reduplicated (Plate, fig. A); there were two metacarpalia 3 and also two carpalia distalia 3. The phalangeal formula became 2–2–3–3–2. Sometimes we found a reduplication of the fourth digit instead of the third. This was frequently accompanied by a reduplication of the carpale distale 4. In a number of cases the centrale was also reduplicated. The phalangeal formula for the polydactylysm hindlimbs was 2–2–3–4–3–2 or 2–2–3–3–3–2 (i.e. a reduplication of the fourth digit). In the latter case polydactylysm involved a reduction in number of phalanges of both the fourth and the extra digit.

Oligodactylysm forelimbs had three, two or one digits and usually less than the normal number of phalanges. As there was also a strong reduction in the number of carpal elements, it became impossible to identify individual digits. One-digit limbs (‘spikes’) only occurred after operation A if aminopterin was administered on day 14. After operation B a common type of oligodactylysm limb was one with two short digits. An additional abnormality of oligodactylysm limbs was the tendency of the praepollex and the radiale to fuse. Likewise, fusion of the carpalia distalia 3 and 4 was often found. More interesting from a
morphogenetic point of view was the frequent occurrence of a strongly reduced radius and ulna, associated with a normal or only slightly reduced upper arm. This happened most often in oligodactylyous limbs, but occurred also in limbs with four digits and in polydactylyous limbs. In extreme cases the skeleton of the lower arm was altogether absent or only represented by ill-defined cartilaginous nodules.

**Dose-response relationship of aminopterin treatment**

A small additional series of experiments was carried out in order to obtain some information on the dose-response relationship of aminopterin with special regard to its morphogenetic effects. Twenty animals of approximately 12 cm in length were subjected to bilateral forelimb amputation of type A. All animals received a single oral dose of aminopterin on the fourteenth day after amputation, i.e. at a time of known high sensitivity for the induction of oligodactylism. The results are given in Table 4. The lowest dose produced neither morphogenetic effects nor growth retardation. The two higher doses resulted in essentially similar numbers of malformed limbs, and in considerable growth retardation. It should be noted that the effect of a dose of 100 mg/kg in this series was less than 100 %, in contrast to the series of Table 2. It is possible that this is due to the greater size of the animals used in this experiment.

**Table 4. Dose-response relationship of aminopterin**

Effect of a single oral dose given at 14 days after operation A.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of animals</th>
<th>Regenerates with normal no. of digits</th>
<th>Oligodactylous regenerates</th>
<th>Growth retardation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>20</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>Strong</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>Strong</td>
</tr>
</tbody>
</table>

**Effects of two other folic acid analogues on regeneration**

The effects of methotrexate (4 amino-$N^{10}$-methyl PGA) and $N^{10}$-methyl PGA on limb regeneration were studied after amputation of all four limbs according to operation A. The results of this study are given in Table 5. The structural relationships of these analogues with aminopterin and with folic acid are shown in Text-fig. 3.

As shown in Table 5 the effects of methotrexate on regeneration were very similar to those of aminopterin. On the other hand, $N^{10}$-methyl PGA had neither morphogenetic nor growth-retarding effects on the regenerating limb. Thus, of the three analogues tested, only the two having a substituted amino group at position 4 were capable of influencing the regeneration process.
Table 5. Effects of other folic acid analogues on regeneration

Administration of a single oral dose of 1 mg after operation A.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>6</td>
<td>6</td>
<td>24</td>
<td>7</td>
<td>6</td>
<td>—</td>
<td>Strong</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>14</td>
<td>6</td>
<td>24</td>
<td>3</td>
<td>1</td>
<td>—</td>
<td>Strong</td>
</tr>
<tr>
<td>$N^{10}$-methyl PGA</td>
<td>6</td>
<td>6</td>
<td>24</td>
<td>12</td>
<td>12</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>$N^{10}$-methyl PGA</td>
<td>14</td>
<td>6</td>
<td>24</td>
<td>12</td>
<td>12</td>
<td>—</td>
<td>None</td>
</tr>
</tbody>
</table>

Text-fig. 3. Structural relationships of the folic acid (pteroylglutamic acid) analogues used.

Protective action of folinic acid

In these experiments an attempt was made to counteract the morphogenetic effect of aminopterin by the simultaneous administration of folinic acid (citrovorum factor, leucovorin) or thymidine, or of a combination of both. In bacteria either substance is known to counteract the action of aminopterin (Woolley, 1963), whilst in the explanted chick embryo thymidine gives no protection (O'Dell & McKenzie, 1963).

In three groups of animals the forelimbs alone or both the fore- and hindlimbs were amputated according to method A. The substances were given in one gelatin capsule on the fourteenth day after amputation. Because Grant (1960) had shown in his study on embryos of *Rana* that only low doses of aminopterin could be counteracted by folinic acid, the lowest aminopterin dose known to have an effect on regeneration was used, viz. 20 mg/kg (cf. Table 4).

The results presented in Table 6 clearly demonstrated that folinic acid was able to protect regenerating limbs from the deleterious action of aminopterin. The differences between groups I and Ia and between groups III and IIIa are
highly significant. Thymidine, on the other hand, had no protective action, and the combination of 1 mg folinic acid and 2 mg thymidine gave no better protection than 0.4 mg folinic acid alone.

Table 6. Protection experiments
Oral administration at 14 days after operation A.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>No. of limbs amputated</th>
<th>Dosage (mg) am.  folin.ac. thym.</th>
<th>Regenerates with normal no. of digits</th>
<th>Oligodact. regenerates</th>
<th>$\chi^2$ (Yates correction applied)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>22</td>
<td>0.2 0.2 0.4 0.2 0.2 0.2 0.2 0.2 0.2</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>11.4†</td>
</tr>
<tr>
<td>Ia</td>
<td>11</td>
<td>22</td>
<td>0.2 0.2 0.4 0 0 0 0 0 0</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>11.4†</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>16</td>
<td>0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>0.5</td>
</tr>
<tr>
<td>IIa</td>
<td>6</td>
<td>24</td>
<td>0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>11.4†</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>20</td>
<td>0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>11.4†</td>
</tr>
<tr>
<td>IIIa</td>
<td>6</td>
<td>24</td>
<td>0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2</td>
<td>7 (32%) 19 (86%) 3 (19%) 4 (17%) 4 (20%) 18 (75%)</td>
<td>15 (68%) 3 (14%) 13 (81%) 20 (83%) 16 (80%) 6 (25%)</td>
<td>11.4†</td>
</tr>
</tbody>
</table>

* am. = aminopterin; folin.ac. = folinic acid; thym. = thymidine.
† Significant at the 0.1% level. $\chi^2 = 10.83$ for one degree of freedom.

Administration by injection

The method of oral administration is very suitable, but has the disadvantage that it is not known how fast the drug is resorbed from the gut, particularly since aminopterin as such is insoluble. Therefore, in a series of preliminary experiments, the soluble sodium salt of aminopterin was injected intraperitoneally in a dose of 100 mg/kg. Surprisingly, this treatment had hardly any effect on limb regeneration. It is possible that the soluble salt was rapidly excreted and therefore failed to reach the site of regeneration. When insoluble aminopterin, suspended in 1% carboxymethyl cellulose, was injected intraperitoneally in a dose of 100 mg/kg, it occasionally led to malformed regenerates, but the incidence of malformations was never as high as after oral administration.

DISCUSSION

The results of this study show that following amputation through the upper arm (operation A) maximal sensitivity of the regenerate for the induction of polydactylism occurs earlier than that for the induction of oligodactylism. If the data of Tables 1 and 2 are compared, it is seen that the period of sensitivity for polydactylism extends through the 'cone' stages of blastemic development and ends before the appearance of the digital plate. The sensitivity for oligodactylism, on the other hand, is at a maximum at the early 'paddle' stage and persists until the first digital rudiment appears. In other words, the phase of sensitivity for polydactylism coincides with the period of rapid growth of the blastema, whereas oligodactylism is induced most frequently at the time when the digital plate starts to expand, but the overall growth of the blastema is slowing down. It is interesting to note that essentially similar sensitive phases for
the induction of poly- and oligodactyly exist in the embryonic urodele limb with respect to the action of ultraviolet light. This was shown in developing forelimbs of *Amblystoma maculatum* by Rieck (1954), who found that polydactyly (digit reduplication) was induced predominantly in late limb bud and early paddle stages, whereas oligodactyly (digit suppression) was induced most frequently in the later stages of digit formation.

A similar sequence of sensitive phases has also been shown to exist during the ontogenetic development of the mammalian limb. In a study on X-ray induced abnormalities in the mouse embryo, Russell (1950) (see Russell & Russell, 1954, fig. 4) demonstrated that the sensitive period for limb ‘overgrowth’ and polydactyly lies in the stages of the growing limb bud (9½, 10½ days), and to some extent in even earlier stages before the actual appearance of the limb bud. Oligodactyly, on the other hand, is predominantly induced by irradiation in the stages of digital plate development (11½, 12½ days). Russell’s work has recently been confirmed by Murakami, Kameyama & Nogami (1963). The existence of a difference in sensitive phases for poly- and oligodactyly follows also from work done on the action of chemical teratogens on the developing mammal. Dagg (1960) induced polydactyly prior to oligodactyly in mice with 5-fluorouracil, and similar results were obtained by Kageyama (1961) in mice with triethylene melamine, and by von Kreybig (1965) in rats with cyclophosphamide.

An exact explanation of the differential effect of aminopterin on successive stages of limb regeneration cannot be given at present. Oligodactyly may be attributed to the antimitotic effect of the drug. If aminopterin is given during the phase of formation of the digital plate, inhibition of mitosis may last until differentiation of the digits begins. In that case inhibition will lead to a decrease in the size of the digital plate and a reduction in the number of digits.

Polydactyly, on the other hand, cannot be explained on the basis of an antimitotic effect alone. If the drug is given during an earlier phase of regeneration, the antimitotic effect may wear off before digital differentiation starts. The blastema may then resume its growth. It is possible that the phase of renewed growth is characterized by ‘overshoot’, a phenomenon observed in certain growth processes after experimental interference (cf. Nowinski, 1960). In the blastema, ‘overshoot’ may ultimately lead to an increased width of the digital plate such as has been described in certain polydactylous chick and mouse mutants (see Grüneberg, 1963).

The polydactyly observed in our treated animals usually consists of a reduplication of the third digit of the forelimb. In histological sections of normal axolotl forelimb regenerates (Faber, unpublished observations), a small extra digital rudiment is found which branches off from the postaxial side of the third digit. Shortly after its appearance this rudiment regresses completely. It is possible that a greater width of the digital plate in treated animals permits this rudiment to develop into the extra digit; whether a similar situation exists in the hindlimb is not known at present.
The longer duration of the phases of sensitivity for the induction of poly- and oligodactyly in the hindlimb is in accordance with the fact that here the main growth phase of the blastema lasts longer and the digital plate appears later than in the forelimbs.

In a number of animals undergoing operation A (see Table 2) polydactyly is induced by administration of aminopterin on or prior to the tenth day after amputation. Following the same treatment oligodactyly is observed in other animals of this group. This difference of response may be the result of a difference in rate of metabolism of aminopterin. The drug is probably still present in the system of some of the animals after the sensitive phase for polydactyly has ended and that for oligodactyly has begun. The effect of the drug in the latter phase would then override any effect it may have had in the former, i.e. the continuous presence of aminopterin in the body would not allow polydactyly to express itself. Evidence for such a hypothesis comes from an additional series of experiments in which doses of 1 mg aminopterin were administered three times a week during 3 weeks following operation A. Only oligodactylous limbs regenerated after such treatment. It is unknown at present whether a suppression of postaxial polydactyly can also be achieved in mammals by the continuous administration of a teratogenic agent during the sensitive period of gestation. Our hypothesis does not seem to be valid for all cases of polydactyly, as Nishimura, Kageyama & Hayashi (1962) found that continuous administration of methionine sulfoximine to pregnant mice from the ninth to fifteenth day of gestation resulted in fetuses with preaxial polydactyly only.

The selective inhibitory effect of aminopterin on the growth of the radius and ulna suggests a parallel with similar phenomena observed in certain avian and mammalian mutants (Grüneberg, 1963), and also in mammals following the administration of teratogenic agents during pregnancy (von Kreybig, 1965; Wilson & Warkany, 1965).

The results obtained after amputation through the distal end of the lower arm (operation B) show interesting differences from those obtained after operation A. The earlier maximal sensitivity for the induction of oligodactyly and the shorter duration of this phase of sensitivity are in accordance with the fact that the digital plate appears several days earlier than after operation A. The complete absence of polydactyly in this series, even when aminopterin was given prior to amputation, cannot be explained at present. It would be of interest to know whether it is in any way related to the fact that the main growth phase of the blastema after operation B is of shorter duration than after operation A.

An answer to this and other questions raised in the discussion cannot in our opinion be given until a histological analysis has been made of the sequence of events leading to polydactyly on the one hand and oligodactyly on the other. Also, the biochemical changes taking place in the cells of the blastema
Aminopterin and limb regeneration

after aminopterin treatment need to be examined carefully. It is to be hoped that such studies will provide a deeper insight in the processes of regeneration under normal and abnormal conditions.

SUMMARY

1. A study was made of the effect of the folic acid analogue aminopterin (4-aminopteroylglutamic acid) on limb regeneration (particularly of the forelimbs) in *Ambystoma mexicanum* larvae. The limbs were amputated either through the upper arm (operation A), or through the distal end of the lower arm (operation B).

2. Following operation A it was found that a single oral dose of 100 mg/kg could induce polydactylism or oligodactylism, depending on the stage of regeneration at which the drug was administered. Polydactylism, which was of the postaxial type, occurred in up to one-third of the regenerating forelimbs after aminopterin administration within the first 10 days of regeneration, i.e. during the ‘cone’ stages of blastemic development. Oligodactylism could be induced by administration at all stages of regeneration, up to the appearance of the first digital rudiment. However, its incidence was maximal (100 %) after administration at 14 days after amputation, i.e. at the early ‘paddle’ stage of blastemic development. These data are compared with the results of other authors, who have studied the effects of teratogenic agents on embryonic limb development in amphibians and mammals; here, too, the sensitive phase for the induction of polydactylism usually precedes that for the induction of oligodactylism.

3. Following operation B the sensitive phase for the induction of oligodactylism was of shorter duration and the optimal response was observed earlier than after operation A. Polydactylism did not occur after operation B.

4. Aminopterin had a growth-retarding effect on the regenerating limb. Occasionally it had the additional effect of reducing the length of the lower arm.

5. Besides aminopterin, two other folic acid analogues were studied with respect to their effects on limb regeneration, 4-amino-<sup>N</sup>10-methylpteroylglutamic acid (methotrexate), and <sup>N</sup>10-methylpteroylglutamic acid. Of the three compounds studied only the two having a substituted amino group at position 4 (aminopterin and methotrexate) had an effect on the regeneration process.

6. The morphogenetic effects of aminopterin could be counteracted by the simultaneous administration of folinic acid (citrovorum factor). This is in agreement with the results obtained by other authors using various other biological systems for testing the activities of folic acid analogues.
L'influence de l'aminoptérine sur la régénération des membres chez Ambystoma mexicanum

1. On a étudié les effets d'un analogue de l'acide folique, l'aminoptérine (acide 4-aminoptéroylglutamique), sur la régénération des membres (antérieurs en particulier) de larves d'Ambystoma mexicanum. Les membres ont été amputés soit à travers le bras (opération A), soit à travers l'extrémité distale de l'avant-bras (opération B).

2. Après l'opération A, on a trouvé qu'une dose orale unique de 100 mg/kg pouvait induire la polydactylie, ou bien l'oligodactylie, selon le stade de régénération auquel le produit était administré. La polydactylie, qui était de type post-axial, est survenue dans un tiers maximum des membres antérieurs en de la régénération quand le traitement était appliqué pendant les dix premiers jours de rénovation, c'est-à-dire pendant les stades 'cône' de la formation du blastème. L'oligodactylie a pu être induite par administration de l'aminoptérine à tous les stades de la régénération, jusqu'à l'apparition du premier rudiment de doigt. Néanmoins, sa fréquence était maximale (100%) lors de l'administration au 14ème jour après l'amputation, c'est-à-dire au début du stade 'palette' du développement du blastème. On compare ces résultats avec ceux d'autres auteurs qui ont étudié les effets d'agents tératogènes sur le développement embryonnaire des membres d'Amphibiens et de Mammifères; là aussi, la phase sensible pour l'induction de la polydactylie précède habituellement celle de l'induction de l'oligodactylie.

3. Après l'opération B, la phase sensible pour l'induction de l'oligodactylie était de durée plus courte et la réponse optimale a été observée plus tôt qu'après l'opération A. La polydactylie n'est pas survenue après l'opération B.


5. Outre l'aminoptérine, deux autres analogues de l'acide folique ont été étudiés sous la rapport de leurs effets sur la régénération des membres, l'acide 4-amino-N10-méthylptéroglutamique (méthotrexate) et l'acide N10-méthylptéroglutamique. Des trois composés étudiés, seuls les deux possédant un groupe aminé substitué en position 4 (aminoptérine et méthotrexate) ont eu un effet sur le processus de régénération.

6. Les effets morphogénétiques de l'aminoptérine ont pu être contrariés par l'administration simultanée d'acide folinique. Ceci s'accorde avec les résultats obtenus par d'autres auteurs utilisant divers autres systèmes biologiques pour éprouver l'activité d'analogues de l'acide folique.
This project was suggested to us by Professor W. Lammers, whom we wish to thank for his continuing interest and stimulating advice during the course of the investigation.

We also thank Professor P. D. Nieuwkoop for critically reading the manuscript and Dr J. N. Miller for improving the English text.

We are grateful to Dr A. Heltai for the generous gift of the two folic acid analogues.

Finally, we wish to acknowledge the assistance of A. Brinkman, Chem. Cand., and of Miss G. B. van Drie, who took care of the animals during the experiment.

REFERENCES


(Manuscript received 3 February 1966)