The effect of 6-aminonicotinamide on limb development

By JOHN C. McLACHLAN

From the Department of Biology as Applied to Medicine,
Middlesex Hospital Medical School, London

SUMMARY

The morphological effect of the nicotinamide analogue 6-aminonicotinamide on the development of the embryonic chick limb was studied, with special reference to muscle, cartilage, feather germ formation, and ossification. No evidence was found to support theories of control of differentiation by nicotinamide-like substances, although the effect of nicotinamide analogues has often been quoted as evidence for such theories.

However, the effect of 6-AN in shortening the limb provides an opportunity to study the interaction of various tissues in normal growth and development, and the results are used to shed further light on the processes involved in muscle splitting, and feather germ formation.

INTRODUCTION

Some nicotinamide analogues, notably 3-acetylpyridine (3-AP) and 6-aminonicotinamide (6-AN), are effective as teratogens of the developing chick, with marked effects on limb development in particular (Landauer, 1957); and, as a result, implications for the biochemistry of development have been drawn by a number of authors (Caplan, 1970, 1971, 1972a, b, 1977; Caplan & Koutroupas, 1973; McMahon, 1974; Schubert & Lacorbiere, 1976). However, the in vivo effects of these chemicals on the various tissues of the developing chick limb, on which such approaches should be firmly based, have not been completely or quantitatively described, and this has led to some misleading oversimplifications. For instance, it has been stated that '3-AP and 6-AN specifically alter the sequence of mesodermal differentiation in chick embryos' (Schubert & Lacorbiere, 1976), and that 3-AP causes 'severe muscle hypoplasia while having little or no effect on bone formation' (Caplan, 1972b). We have previously described the effect of 3-AP and shown that it does not act by specifically altering the pathways of differentiation, but by a deleterious effect on a number of tissues, particularly nerves (McLachlan, Bateman & Wolpert, 1976); here I provide a more complete description of the effect of 6-AN on limb development than has previously been available, with particular attention to

1 Author's present address: Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.
muscle, cartilage, bone, and feather germs, and conclude that there is no evidence in support of an in vivo mode of action for 6-AN involving developmental switching. However, I also suggest that careful use of these teratogens based on a closer knowledge of their action may help to elucidate certain specific problems in limb development, and in particular, I draw implications for the processes of muscle splitting and feather germ formation.

MATERIALS AND METHODS

A total of 240 White Leghorn eggs were windowed at 3 days of incubation, resealed with Sellotape, and returned to the incubator. Embryos were treated at 3, 4, 6, or 8 days of incubation with either 5, 10, or 20 µg of 6-AN (Sigma) in 50 µl of distilled water, a number being reserved as controls. This range of treatment times encompasses the period in which the major mesodermal types in the limb are differentiating: by day 8 these types are all separate and further development reflects growth only.

Embryos were removed for examination as close as possible to stage 36. (All stages after Hamburger & Hamilton, 1951.) One hundred and sixty-four of the embryos were briefly drained and weighed; and the wings removed at the shoulder. The length of these wings was measured from the point of the elbow to the wing tip, as in fresh specimens this offers a more recognizable starting point than attachment to the body wall. These wings were then fixed in 5% trichloracetic acid (TCA), dehydrated through an alcohol series which included a period of staining with 0.1% Alcian green in acid alcohol, and finally cleared in methyl salicylate. The humerus, ulna, and all the elements of digit III were measured with the aid of a graticule eyepiece on a dissecting microscope.

While fixed in TCA, these wings were drawn with the aid of a camera lucida. The feather germs of the row along the posterior edge of the limb were counted from the acute angle formed by the point of the elbow to the obtuse angle formed at the wrist (between the arrows on Fig. 1).

Fourteen limbs treated at 4 and 6 days with 10 or 20 µg of 6-AN were fixed in half-strength Karnovsky’s solution, dehydrated, and embedded in Araldite. Sections were cut to 1 µm thickness and stained with toluidine blue. These sections were used to study the effect of 6-AN on general histological features.

To study the effect on muscle pattern, eight wings from embryos treated at 4 days of incubation with 20 µg of 6-AN were fixed in Bouin’s fluid and double-embedded in wax, together with seven controls. These were serially sectioned at 7 µm intervals transversely to the long axis of the limb from the wing tip to the elbow, and stained with Van Gieson’s stain.

Gross pattern of the muscles was observed in dissections of the right wings of 22 embryos treated at 6 days of incubation with 10 or 20 µg of 6-AN, plus 10 controls. These wings had previously been preserved in 5% TCA. Two muscles in particular, flexor carpi ulnaris (FCU) and flexor digitorum profundus
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Table 1. Effect of 6-AN on whole embryos and wings

<table>
<thead>
<tr>
<th>dose (μg)</th>
<th>Time of treatment (days)</th>
<th>Survival</th>
<th>Wet weight (g)</th>
<th>Wing length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 (st. 20–22)</td>
<td>4 (st. 22–24)</td>
<td>6 (st. 28–29)</td>
<td>8 (st. 33–34)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12/13</td>
<td>3·6 (0·42)</td>
<td>9·93 (1·8)</td>
</tr>
<tr>
<td>5</td>
<td>0/10</td>
<td>10/17</td>
<td>2·32 (0·29)</td>
<td>7·80 (1·80)</td>
</tr>
<tr>
<td>10</td>
<td>0/10</td>
<td>8/14</td>
<td>2·37 (0·25)</td>
<td>7·63 (1·38)</td>
</tr>
<tr>
<td>20</td>
<td>0/10</td>
<td>0/10</td>
<td>—</td>
<td>5·80 (1·15)</td>
</tr>
</tbody>
</table>

Results expressed as mean values followed by standard deviations in parentheses.

(FDP) were dissected free and drawn with the aid of a camera lucida. Their lengths along their long axis and breadths across the widest parts were measured from the drawings.

Ossification was studied in embryos treated with 10 μg of 6-AN at 4 or 6 days of incubation. Here the right wings and legs were examined, since ossification only occurs in certain elements during the period of study, and of these the tibia is the longest, and most obviously affected by 6-AN. Five specimens in each category were examined, with five controls. Limbs were fixed in 95% alcohol and stained with Alizarin Red (Humason, 1962). The ossification collar of the humerus and the tibia, together with the total length of the element, were measured with a graticule eyepiece on a dissecting microscope.

RESULTS

Embryos treated with 6-AN are reduced in size and wet weight by an amount dependent on the dose and time of treatment. The wings are reduced in length (see Fig. 1 and Table 1). There is usually brachycephaly present, the lower jaw being more affected than the upper. Feather germ development also appears to be affected, the germs being smaller than usual. Blood clots are frequently present, the whole embryo sometimes having a reddish appearance.

Histologically, blood cells were frequently observed throughout the limb
outside the boundaries of blood vessels. Nerves were unaffected, both in appearance and distribution. Tendons and muscles were also normal in appearance and distribution. Only cartilage showed discernible abnormalities. However, the major tissues of the limb require consideration at greater length.

**Effect on muscle**

(a) **Gross effects**

The length, breadth, and cross-sectional area of the muscles FCU and FDP are shown in Table 2. The length is clearly reduced in a manner related to the
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Fig. 2. Transverse 1 μm sections from the mid radius-ulna region of the wing. (a) Control. (b) Treated at 6 days of incubation with 10 μg of 6-AN. R, Radius; U, ulna; FDP, flexor digitorum profundus; FCU, flexor carpi ulnaris.

Table 2. Effect on muscles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Dose (μg)</th>
<th>Length of ulna (mm)</th>
<th>Length of muscle (mm)</th>
<th>Breadth of muscle (mm)</th>
<th>Area of muscle (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCU</td>
<td>0</td>
<td>4.08 (0.27)</td>
<td>4.47 (0.44)</td>
<td>0.78 (0.13)</td>
<td>0.28 (0.04)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.47 (0.41)</td>
<td>3.41 (0.75)</td>
<td>0.81 (0.10)</td>
<td>0.28 (0.04)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.49 (0.57)</td>
<td>2.29 (0.49)</td>
<td>0.81 (0.11)</td>
<td>—</td>
</tr>
<tr>
<td>FDP</td>
<td>0</td>
<td>4.08 (0.37)</td>
<td>2.95 (0.20)</td>
<td>0.59 (0.07)</td>
<td>0.11 (0.07)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.47 (0.41)</td>
<td>2.09 (0.43)</td>
<td>0.63 (0.07)</td>
<td>0.12 (0.07)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.49 (0.57)</td>
<td>1.59 (0.20)</td>
<td>0.59 (0.07)</td>
<td>—</td>
</tr>
</tbody>
</table>

Results (expressed as mean values followed by standard deviations in parentheses) refer to embryos treated at 6 days of incubation. The length of the ulna is based on cleared fixed specimens; the length and breadth of muscles, on drawings of fixed dissected muscles; and the areas, on measurements of drawings of transverse Araldite sections (all as described in Materials and Methods). FCU, flexor carpi ulnaris; FDP, flexor digitorum profundus.

dose, while the breadth and cross-sectional area are quite unaffected. The muscle morphology and pattern of the muscles in dissection are also quite normal apart from this shortening along the long axis.

(b) Effects at histological level

Both in the Araldite sections, and in wax sections taken serially down the whole length of the wing, the muscle pattern was normal, although compressed into a smaller length (Fig. 2).

Even in the worst affected embryos, muscle fusion had occurred as normal (Fig. 3).
Fig. 3. Fused muscle straps from 11-day-old embryo treated at 4 days with 20 µg of 6-AN.

Effect on cartilage

(a) Gross effects

The pattern of the cartilage elements in the wing was unaffected, all elements being recognizably present. Their relative orientations were occasionally disturbed, digit III for example often pointing anteriad after treatment.

Figure 5 shows the reduction in length for the measured elements for a typical group treated at 8 days of incubation.

All of these elements are affected by the treatment, although there is considerable variation in the results. In general, the effect is related to the dose.

(b) Effects at histological level

Transverse sections through the mid radius–ulna region were normal in cell density and appearance (Fig. 2). However, longitudinal sections through the cartilage elements revealed patches lying at the core of each element in the epiphyseal region which were weakly stained with toluidine blue, a stain which reveals mucopolysaccharides by metachromasia. Closer examination showed that these pale staining areas contained disrupted cells lying in an almost matrix-free environment. This is a similar result to that described by Seegmiller, Overman & Meredith (1972), which they subsequently described as being due to a specific effect on the matrix-producing cells (Seegmiller, 1977), and to the results described by Caplan (1972a). Outside these areas cells appeared to have hypertrophied and died in the normal manner (see Fig. 4).
Fig. 4. (a) Longitudinal section through elbow region of 11-day-old embryo treated at 4 days with 20 μg of 6-AN. The clear patch in the centre represents the area affected by 6-AN. (b) Diaphysis of control ulna showing hypertrophied cells. (c) Diaphysis of embryo treated as in (a). The cells have hypertrophied as normal.
Since ossification is a process which in the normal chick begins in specified places and proceeds to grow from the centre to the ends of the cartilage elements at a controlled rate (Holder, 1978), I considered that it would be of interest to note the effect of shortening the cartilage elements with 6-AN on this process. The results are displayed in Table 3. Firstly, ossification does occur, and in the appropriate region of the elements. Secondly, the ossification collar does not grow at the same absolute rate as in the control, for if it did, and the cartilage element was reduced in size, then the ossification collar would occupy a greater proportion of the element; in other words, the ratio $L_c/L_e$ would be increased (where $L_c$ is the length of the collar and $L_e$ is the length of the element). It can be seen from Table 3 that this is not the case. Nor is the ossification collar a constant proportion of the element length, for then the ratio $L_c/L_e$ would be a constant. Rather the collar is retarded even compared to the reduced element. In addition, ossification has not yet occurred at all in some of the cases that are most seriously affected.

Effect on ossification

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Table 3. Effect on ossification

<table>
<thead>
<tr>
<th>Element</th>
<th>Time of treatment (days)</th>
<th>Length (mm)</th>
<th>Ossification collar (mm)</th>
<th>$L_c/L_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humerus</td>
<td>0</td>
<td>4.41 (0.23)</td>
<td>2.05 (0.10)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.00 (0.45)</td>
<td>1.27 (0.36)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.05 (0.28)</td>
<td>0.50 (0.07)</td>
<td>0.25</td>
</tr>
<tr>
<td>Tibia</td>
<td>0</td>
<td>6.87 (0.70)</td>
<td>4.24 (0.37)</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.48 (1.11)</td>
<td>1.76 (0.71)</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.57 (0.10)</td>
<td>0.28 (0.04)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Results (expressed as mean values followed by standard deviations in parentheses) refer to embryos treated with 10 $\mu$g 6-AN.

![Graph](image)

Fig. 6. Effect on the number of feather germs of treatment at different times with 10 $\mu$g of 6-AN. These times are plotted along the x-axis in accord with their effect on the length of the ulna, and this length is shown in brackets beneath each time.

Effect on feather germs

Since the length of the wing has been reduced, it is also of interest to discover what effect this has on the normally stable number of feather germs present. The results are displayed in Fig. 6. Since feather germs are normally laid down in a proximo-distal sequence, it might be argued that the number of feather germs present is affected simply by a retardation of the developmental programme, especially since the feather germs are slightly reduced in size. To eliminate this possibility, measurements were confined to the region of the wing between the acute angle formed by the elbow, and the obtuse angle formed at the wrist, since in all cases feather germs have advanced beyond this region at
the time of examination. It should be emphasized that in normal embryos, once the feather germs of a particular row have reached the wrist, their number remains invariant no matter how much the wing subsequently grows.

It can be seen from the figure that the number of germs decreases as the length of the wing is increasingly affected. This reduction in number is discernible even in Fig. 1.

The apparent reduction in size of the germs may result from the continued failure of the limb to elongate in the normal manner after feather germ formation, so that each germ is deprived of the normal 'nutrient territory' which is necessary for its growth.

DISCUSSION

After treatment with 6-AN there is death of cartilage cells and failure of matrix production, and it may be concluded that this is the cause of limb micromelia. Since cartilage cells which survive treatment with 6-AN enlarge and die in the normal manner, and since the appearance of treated cartilage is that of core areas in which cells show failure of matrix production and cell death, I also conclude that 6-AN acts by adversely affecting cells at their matrix-producing stage. These conclusions are in accord with the basic findings of Caplan (1972a) and Seegmiller et al. (1972, 1977) on the action of 6-AN on cartilage cells. It is clear that as 6-AN is still effective at 8 days of incubation when the differentiating tissues are already separated and growth occurs by division, matrix-production, and cell hypertrophy rather than by recruitment of cells from an undifferentiated mesenchyme, that it acts on newly differentiated chondrocytes rather than on the process of differentiation from the mesenchyme (Caplan, 1972a).

If nicotinamide analogues did act by altering the process of differentiation of muscle and cartilage cells, then they might be expected to affect the complex pattern of muscle and cartilage. It has been claimed that this is, in the early stages of development at least, ordered by nicotinamide-like substances diffusing from the blood vessels in localized regions (Caplan & Koutroupas, 1973). But in fact the pattern of muscle and cartilage, both with 6-AN and 3-AP treatment (McLachlan et al. 1976), is not at all affected.

6-AN has been reported to lead myoblast-type cells to express certain features of cartilage type in vitro (Schubert & Lacorbiere, 1976). There is no evidence that this in fact occurs in the limb. Muscles are normal in pattern, morphology, and cross-sectional area: only their length is reduced harmoniously with the cartilage. Even at doses which are very markedly teratogenic, 6-AN does not even prevent muscle fusion, in contrast to the in vitro situation. However, these authors expose their cells to 1 mM 6-AN while I add 50 µl of at maximum 3 mM 6-AN to each egg of average contents of 55 ml. Since the embryo alone, exclusive of all membranes and extra-embryonic fluids, occupies by measurement 150 µl at 4 days of incubation, the dilution factor lies between 4× and
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1000 x, and I therefore suspect that my doses are effectively more dilute. It is clear that 6-AN does not exert its teratogenic effect in ovo on the muscles, and that no support can be drawn from its action for a role for nicotinamide in development.

Since the pattern of muscles in treated limbs is normal, it can also be concluded that this pattern is not dependent on the normal elongation of the limb providing shearing forces to split up the pre-muscle masses (Shellswell, 1977). However, the muscles are shortened by an amount related to the shortening of the limb, and this suggests that muscle elongation at later stages is dependent on elongation of the cartilage, perhaps by a tension effect. In this way, the final volume and proportions of the muscles are not specified in advance, but are dependent on interaction with other limb tissues.

It has also been reported that 6-AN slows ossification (Seegmiller, 1972). The possibility exists that this is dependent only on the shortness of the cartilage elements, the ossification collars being reduced in proportion. However, my results do not support this. Since ossification does not occur in certain cases at times when it is due, it is possible that 6-AN exerts its effect on ossification by delaying its onset. Perhaps the passage of cartilage and osteoblast cells through their temporal sequence is delayed; or the death of a crucial sub-population of cells delays the onset of osteogenesis until those that follow can replace them.

It has been suggested (Sengel, 1976) that development of the feather germs is controlled by a spacing mechanism such that each territory of sufficient size will initiate a feather germ when appropriately induced. This predicts that if the size of the limb is reduced, so will be the number of germs. An alternative theory is that the number of germs is specified in advance by, say, a gradient of positional information, so that if the limb shortens, the number of feather germs remains constant. However, it is clear that in this case the number of germs is reduced in the smaller field, and this provides support for the mechanism proposed by Sengel.

This contrasts with the situation found in the formation of the somites, where, if a smaller embryo is created by removal of cells, the number of somites formed remains constant, with each somite being reduced in size (Cooke, 1977).

In conclusion, this study demonstrates that 6-AN does not act by specifically altering the pathways of differentiation, but by deleterious effects on cells already differentiated. This, taken with our study of 3-AP, calls into question such implications as have been drawn for the control of differentiation from the in vivo action of these teratogens. Since it has also recently been demonstrated that, far from differentiating in situ from a population of multi-potent cells, muscle cells are derived by invasion from the somites (Christ, Jacob & Jacob, 1977; Chevallier, Kieny, Mauger & Sengel, 1977), perhaps the time has come to re-assess theories which suggest that nicotinamide-like substances control the decision of limb cells to form muscle or cartilage.

However, a useful role still remains for these teratogens. By causing specific
defects of the limb, they provide useful tools for studying such aspects of limb development as muscle splitting, feather germ formation, and ossification.

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REFERENCES


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