Further Investigations on the Cytotoxic and Morphogenetic Effects of Some Nitrogen Mustard Derivatives

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with Five Plates

Previous experiments with TEM (triethanomelamine) on chick and mouse embryos (Jurand, 1958, 1959) have shown that some embryonic regions are more sensitive than others to its cytotoxic activity. TEM appeared to be specifically active against mesodermal structures, particularly against the somitic mesoderm, being relatively less active against other tissues. In amphibian embryos, however, the neural tube cells were the most sensitive (Waddington, 1958).

N-(p-amino-phenyl)-nitrogen mustard (the parent compound) and its acetyl derivative, which have been investigated on chick embryos (Jurand, 1960), have been shown to be specifically toxic to the mesodermal and neural cells. The affected regions were injured primarily at the cellular level, due to the cytotoxic properties of these compounds. The changes at the cellular level were followed by abnormal development of the somites and neural tube.

The present investigations represent further comparative studies on the activity of closely related nitrogen mustard derivatives in chick and mouse embryos, and aim at confirming localized susceptibility to these compounds. In addition to the parent compound and its acetyl derivative, a fluoro-acetyl derivative ('fluorine derivative') was used, viz.

\[
\text{FCH}_2-\text{CO}-\text{NH}-\text{C}_6\text{H}_4-\text{N(CH}_2-\text{CH}_2-\text{Cl)}_2.
\]

This compound is considered to be more readily decomposed by hydrolytic enzymes to the parent compound in Walker rat carcinoma cells than is the acetyl derivative and it therefore was expected to be more selective as a carcinostatic factor (Danielli, 1960).

Material and Methods

Experiments on chick embryos were performed with the fluorine derivative only, as the other two compounds had already been examined using this material and the results have been reported (Jurand, 1960).

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Chick embryos at stage 4 (Hamburger & Hamilton, 1951) were explanted from eggs and cultured *in vitro* according to the method described by New (1955). As the compound is hardly soluble, it was suspended in an appropriate volume of 0-9 per cent. saline solution. One part of this suspension was added to nine parts of liquid albumen, so that the final concentrations of the suspensions were: $10^{-4}$, $2 \times 10^{-4}$, $4 \times 10^{-4}$, and $8 \times 10^{-4}$ (w/v). The suspensions were administered in amounts of 0-5 ml. round the culture rings used for cultivation of the explanted embryos. Re-incubation took place for 22-24 hours, i.e. until the control embryos had reached 12 or 13 according to Hamburger & Hamilton (1951). In these experiments 109 experimental and 56 control chick embryos were used.

The material was prepared for histological examination by standard methods described previously (Jurand, 1958, 1960). Some of the experimental and control embryos were examined in the electron microscope in order to see the details of the necrotic changes in the neural tube. These embryos were fixed with 1 per cent. buffered osmic tetroxide solution (Palade, 1952), embedded in methacrylate, sectioned with an ultramicrotome, and viewed in a Siemens Elmiscop I.

In order to compare the results obtained from the experiments on chick embryos treated with the fluorine derivative and those reported previously, the specificities of the compounds were examined also on 10-, 13-, and 15-day-old mouse embryos mainly of the JC and to a lesser extent of the JU strain. Two- to five-months-old females with vaginal plugs were injected subcutaneously under slight anaesthesia with the newly prepared solution or suspension of the drugs. (The parent compound is fairly soluble, but the other two had to be applied in the form of suspensions.) The doses were calculated in mg. per kg. of the actual body-weight as the differences between molecular weights of the compounds concerned are small, and were divided into three equal parts which were injected on three consecutive days, either the 6th, 7th, and 8th days or the 7th, 8th, and 9th days of pregnancy, the day on which the plug was found being regarded as the 1st day. The experimental animals were weighed before each injection and were injected at the same time each day.

The animals were killed by cervical dislocation on the 10th, 13th, or 15th day of pregnancy. After preliminary fixation with Carnoy's fluid (6:3:1) in the intact uterus for about 3 hours, the embryos were dissected and fixed again for another 12 hours in the same fixative. For histological purposes the embryos were dehydrated with absolute alcohol for 6 hours, then cleared in methyl benzoate and embedded in wax. The orientation of the embryos for sectioning was done according to the group of organs to be examined. Sections 6-7 μ thick were stained with methyl green-pyronin. Some embryos were prepared as whole mounts after staining with Mayer's haematoxylin.

For purposes of comparison control mouse embryos of ages from 8 to 15 days' post conception were used. Pregnant control females were anaesthetized on the injection days without being injected.
The preliminary external examination of the embryos after they were excised from the uterus included an assessment of the degree of retardation of development and identification of the external characteristics, such as general body-shape, the line of the vertebral column and spinal cord, the size and shape of the eyes, the presence of liver hernia, the appearance of the limbs or limb-buds, &c. The central nervous system, eyes, mesodermal structures, and, where this seemed necessary, certain other structures were subjected to histological and cytological examination.

The stages of development of the mouse embryos were compared with data available in the literature (Grüneberg, 1943; Snell, 1941) and with the control material.

Table 1 gives the numerical data of the animal material used in all experiments.

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>Number of pregnant females</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl derivative</td>
<td>108</td>
<td>627</td>
</tr>
<tr>
<td>Fluorine derivative</td>
<td>160</td>
<td>896</td>
</tr>
<tr>
<td>Control</td>
<td>234</td>
<td>1,416</td>
</tr>
<tr>
<td>Control</td>
<td>66</td>
<td>528</td>
</tr>
<tr>
<td>Total</td>
<td>568</td>
<td>3,467</td>
</tr>
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</table>

RESULTS

The effect of the fluorine derivative on chick embryos

The effective dosage of this compound was determined after some preliminary experiments and appeared to be of a range similar to that of the parent compound and its acetyl derivative. Concentrations lower than $2 \times 10^{-4}$ proved to have very little or no visible influence on development, whereas those higher than $8 \times 10^{-4}$ were lethal for all experimental embryos. Hence there was a comparatively narrow range of concentrations which could be used for these experiments.

The lowest effective concentration, $2 \times 10^{-4}$, did not markedly retard the general development of the embryos. The number of somites averaged not less than the number of somites in the control embryos, but in 19 out of 24 cases the neural tube showed selective reactivity, remaining unclosed either in parts or along its entire length. In the first case the affected parts of the neural tube were the rhombencephalon, the region on the level of the 5th to the 9th somite and the caudal part of the neural plate. The latter remained unclosed so that the sinus rhomboidalis persisted longer than in the control embryos. In embryos of this group the anterior neuropore also failed to close.

In the case of total non-closure of the neural plate or neural groove, which
occurred sometimes after treatment with a concentration of $2 \times 10^{-4}$ and always after $4 \times 10^{-4}$, general development was usually retarded. In cross-sections the neural plate appeared to be open and in most cases completely flat, forming a medullary plate instead of a neural tube. It was flat especially in the head region where it failed to show any depression in the median line (Plate 1, figs. 1, 2). In other cases the medullary plate, unclosed along its entire length, formed rather a V-shaped groove, also in the head region (Plate 1, fig. 3).

When intermediate concentrations were used there was no particular abnormalities in other tissues, except that, because of general under-development, the number of somites was, on average, less than that in control embryos, so that the experimental embryos were found to be only at stage 10 or 11 according to Hamburger & Hamilton (1951). The specificity of the fluorine derivative in these concentrations for the developing neural tube seemed to be more pronounced than in the case of the acetyl derivative.

At the cellular level, in the unclosed medullary plates, frequent abnormalities, confined exclusively to the neural cells, were observed. There were found many giant cells (i.e. hypertrophied cells each with a single nucleus) and cells with pycnotic and necrotically disintegrated nuclei (Plate 1, fig. 4). All these changes occurred alongside normal looking cells (which were in the majority) and those undergoing mitotic division. In some cases, however, mitosis was abnormal, e.g. tripolar metaphase (Plate 1, fig. 5).

After application of still higher concentrations, e.g. $6 \times 10^{-4}$ and $8 \times 10^{-4}$, the neural tissue appeared to be completely destroyed. It consisted entirely of necrotic and deformed cells with very dense, shrunken nuclei staining dark greenish-blue with methyl green. The neural plate was in these cases practically non-existent; its necrotic cells formed only an open, more or less shallow groove (Plate 1, fig. 6). Other tissues of these embryos were also affected, although much less than the neural tube cells. The next most severely affected tissue appeared to be the somitic mesoderm.

**Necrotic changes as seen by the electron microscope**

The neural tube cells of control embryos at stages 12–13 of development appear in electron micrographs to consist of comparatively large nuclei surrounded by a thin layer of cytoplasm. The electron density of the internal content of the nuclei is slightly different from that of the cytoplasm. In some cases it is less, in others higher, presumably depending on what stage the cell is in its life-cycle. The nucleolus, with a diameter averaging one-fifth to one-quarter of that of nucleus, is much denser than is the nucleus, and contains many small vacuoles. There are also cells with more than one nucleolus and, in some cases, cells with small fragments of nucleolar material alongside the main nucleolus. The nuclear membrane has a double-layered structure. In the cytoplasm there are abundant, often elongated mitochondria with the usual lamellar structure, and yolk granules of all types, as described by Bellairs (1958), although at this stage
they are present in small numbers only, as most have undergone conversion into cytoplasmic constituents (Plate 2, fig. 7).

After treatment with the fluorine derivative there occurred many different necrotic changes which could be distinguished more easily by means of the electron microscope. From histological examination of the experimental embryos it is seen that the first symptom of cytotoxic activity is the enlargement of the cells; in extreme cases they may be referred to as giant cells. These cells may be up to 3 times larger than normal cells. The electron density of all their parts remains similar to that of the control cells. In the cytoplasm, however, signs of necrotic changes can be found, in particular pronounced vacuolization, side by side with large granules which appear to be thick-walled vacuoles, presumably identical with the pyronine-positive granules known from light microscope examination of these embryos. The mitochondria also appear to be enlarged (Plate 2, fig. 8).

Simultaneously with the enlargement of the nuclei, the nucleoli usually also become enlarged in relation to the nuclei, as is shown in Plate 2, fig. 8. In some cases, however, there occurred cells with enlarged nucleoli which showed no distinct change in the size of the nuclei (Plate 2, fig. 8).

Other necrotic changes that follow treatment with higher doses appear mostly in nuclei. The first sign is the dissolution of the nucleolus and the simultaneous gradual vacuolization of the structure of the nucleus which ceases to show its normal fine granular appearance. Underneath the nuclear membrane there appear dense masses which are known to be deeply stainable with nuclear stains. This stage of necrosis with more or less advanced changes in the cytoplasm, is assumed to be karyorrhexis (Plate 2, fig. 10).

In the cytoplasm of these cells further necrotic changes are observed which lead to advanced vacuolar destruction of the cells, resulting eventually in complete disintegration of both nuclei and cytoplasm. Such disintegrated tissue fragments, if examined in the electron microscope, are difficult to recognize as having cellular structure at all. They consist merely of very dense solid masses of homogeneous structureless material which presumably represent remains of nuclei, and complex yolk granules in various, probably abnormal, stages of delayed digestion (Plate 3, fig. 11).

True pycnosis of the nuclei is one of the stages in necrosis. It appears morphologically in various patterns as a less common response to the cytotoxic activity. In such cases the nuclei consist always of denser material, often surrounded by a more homogeneous envelope underneath the nuclear membrane. In general, the nuclei are smaller in these cells, but their cytoplasm is comparatively well preserved (Plate 3, fig. 12).

The effects of the parent compound, acetyl and fluorine derivatives on mouse embryos

Natural prenatal mortality in stages later than the 10th day of gestation
occurs very rarely in the strains of mice used in these experiments. In 34 control females only about 1 per cent. of dead embryos were found on the 15th day of pregnancy. Like TEM, the compounds used in the present experiments cause a high incidence of prenatal mortality if used in doses above a certain threshold. They differ considerably, however, in the level of their threshold dose, and, as in previous experiments on TEM, the embryonic LD$_{50}$ was therefore determined for each of the compounds. The embryonic LD$_{50}$ is the total dose which, if administered subcutaneously in three equal parts on the 7th, 8th, and 9th days of pregnancy, kills by the 15th day of pregnancy about 50 per cent. of implanted embryos. Similarly, the lethal doses (LD$_{50}$) for adult mice were determined.

Table 2 gives the embryonic LD$_{50}$ as compared with the LD$_{50}$ of the compounds under investigation.

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>LD$_{50}$</th>
<th>Embryonic LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent compound</td>
<td>15 mg. per kg.</td>
<td>12 mg. per kg.</td>
</tr>
<tr>
<td>Acetyl derivative</td>
<td>65 &quot;</td>
<td>36 &quot;</td>
</tr>
<tr>
<td>Fluorine derivative</td>
<td>80 &quot;</td>
<td>45 &quot;</td>
</tr>
</tbody>
</table>

The parent compound (108 pregnant females). Compared with the other two derivatives, the parent compound shows, in mice, a comparatively small difference between the embryonic LD$_{50}$ and the dose resulting in the prenatal mortality of all embryos, and therefore the doses used in these experiments were restricted to 9, 10-5, 12, and 13-5 mg. per kg. All doses higher than the last named resulted in complete mortality of embryos, or caused the death of the pregnant females 24 hours or more after the last injection. In all cases death was preceded by acute diarrhoea, due to extensive inflammation of the intestinal tract.

After effective doses, injected in three equal parts either on the 6th, 7th, and 8th day or on the 7th, 8th, and 9th day of pregnancy, no selective effects in particular embryonic regions were recorded. The only effect was retardation of development, amounting, on the average, to about 1 day in 10-day-old embryos and up to 3 days in 13-day-old embryos, according to the dose used.

In 36 females injected with the embryonic LD$_{50}$ or with 13-5 mg. per kg. on the 7th, 8th, and 9th day of gestation the 145 surviving embryos showed general retardation of development by 2–3 days. Among these embryos were some with unilateral microphthalmia (12 per cent.) and liver hernia (8 per cent.). Both these abnormalities were macroscopically and histologically of the same kind as those described previously after treatment with TEM (Jurand, 1959). Microscopical examination of the retarded 10- and 13-day-old foetuses did not reveal any specificity of the parent compound. In less-retarded individuals no tissues appeared to deviate from their normal histological performance, whereas in those more retarded all the tissues were injured to the same extent, showing severe necrotic changes.

Acetyl and fluorine derivatives (394 pregnant females). The ranges of the
effective doses per kg., divided into three equal parts and injected on three consecutive days, were 24–45 mg. for the acetyl derivative and 30–54 mg. for the fluorine derivative. The results will be reported together as there were no qualitative differences between the effects of these two compounds on mice.

In 10-day-old embryos, after injection with low effective doses on the 6th, 7th, and 8th days of gestation, both compounds slowed down the rate of development by 1–2 days in rough proportion to the dose used. The most sensitive region appeared to be the medullary plate which remained open in the majority of embryos of this age group (Plate 4, fig. 13). The rest of the body did not show any abnormalities. After higher doses, however, besides the necrotic changes in the open medullary plate, the mesodermal structures showed scattered necrotic changes (Plate 4, fig. 14).

In 13-day-old embryos the susceptibility of the neural tube tissue was still more pronounced, particularly after injections on the 6th, 7th, and 8th days of gestation. The total doses used in these experiments were 36 or 45 mg. per kg. of the acetyl derivative and 45 or 54 mg. per kg. of the fluorine derivative; in other words, the doses were equal to, or 20–25 per cent. higher than, the respective embryonic LD$_{50}$ of these compounds.

In the surviving embryos, apart from general underdevelopment by up to 3 days, necrotic changes which were confined, after lower doses, almost exclusively to the neural tube tissue, were observed after the use of both compounds. In the majority of surviving embryos of this group the neural tube was found to be closed. This was presumably due to the fact that all the more severely injured embryos with unclosed neural tubes had died before the 13th day of gestation, i.e. before the day of fixation. The extent of necrosis of the neural tube tissue showed a considerable individual variation. In some cases the necrosis was confined to the deeper part of the organ, affecting up to half of it, but in extreme cases the entire cross-section showed necrotic damage (Plate 4, figs. 15, 16, 17, 18).

In cases of confinement of necrosis to the deeper part of the neural tube, although the upper part contained also some randomly scattered necrotically changed cells, such as giant, karyorrhectic, and pycnotic cells, it consisted, in general, of healthy-looking cells, some of which were even in the process of mitotic division. The deeper part of the neural tube of these embryos consisted entirely of necrotic, apparently dead tissue, with deformed nuclei that stained deeply with methyl green. As can be seen in figs. 16, 17, and 18 of Plate 4, there was always present within the lumen of this part of the neural tube a kind of cellular debris which stained mainly red with pyronin. In addition, the damaged part showed in many cases complicated convolutions, whereas the healthy upper part did not deviate in its structure from the axial line (Plate 4, fig. 19).

In this group there were few embryos with necrosis of the deeper part of the neural tube, which was closed as far as the trunk region and open towards its caudal end (Plate 4, fig. 20).
In cases in which the entire neural tube consisted of thoroughly necrotic cells, which occurred particularly after higher doses, extensive changes in some mesodermal structures, e.g. in the somitic mesoderm and mesenchyme cells, were also found. In such embryos the cells in the deepest part of the neural tube had already disappeared, so that in this region the continuity of the neural tube was broken (Plate 4, fig. 18).

A comparison of the effects of the above derivatives on the neural tube after different doses and in different embryos leads to the conclusion that there exists a gradient in the sensitivity of the neural tube to these compounds. The maximum sensitivity is in the deepest part, and it decreases in the opposite direction. This seems to be the reason why the continuity of the neural tube is broken in cases of severe necrosis, as the deepest part, being affected first, undergoes complete necrosis and resorption earlier.

In some cases regeneration of the neural tube presumably took place, so that no, or less severe, necrotic changes were actually present, but the organ showed some structural defects (Plate 4, fig. 21).

In other embryos, after the same doses, the brain and the medullary parts of the neural tube had a typically hydrocephalic appearance. In these cases the widened neural tube, when histologically examined, was seen to have extremely thin walls, in some regions only a single cell thick, showing at the same time scattered necrotic cells (Plate 4, fig. 22).

In a number of embryos of this group, particularly in those with more advanced necrosis of the neural tube, a very distinct difference could be seen between the susceptibilities of the two main components of the developing eye to the cytotoxic activity of the two derivatives. The optic cup was changed necrotically simultaneously with necrotic changes in the deepest part of the neural tube, whereas the lens primordium, which was by this time induced and invaginated, appeared to consist of healthy-looking cells, similar to those in corresponding control embryos (Plate 5, figs. 23, 24).

In the experimental embryos the mesoblast of the limb-buds also showed necrotic changes. In Plate 5, figs. 25 and 26, there are shown dorso-ventral sections through the limb-bud of an experimental embryo and corresponding control. The internal mesoderm of the experimental limb-bud shows extensive necrotic changes, whereas the epidermis and the 2- to 3-celled layer of the adjacent mesoderm are well preserved and do not differ from the corresponding layers of the control limb-bud.

In 15-day-old embryos, which survived the treatment with the embryonic \( \text{LD}_{50} \), and in those receiving slightly higher doses (up to 10 per cent. more) injected either on the 6th, 7th, and 8th days or on the 7th, 8th, and 9th days of the gestation, there were found the following morphogenetic malformations: (1) kinking and convolution of the axial organs, (2) unilateral and bilateral underdevelopment of the eye ball resulting in microphthalmia, (3) liver hernia, and (4) shortening of the limbs.
All these anomalies occurred after the use of both acetyl and fluorine derivatives but with higher frequency with the latter. All but the last were identical with those observed after treatment with TEM (Jurand, 1959). Table 3 shows the frequency of the above malformations together with those after the parent compound expressed in percentages:

**Table 3**

<table>
<thead>
<tr>
<th>Type of malformation*</th>
<th>Parent compound</th>
<th>Acetyl derivative</th>
<th>Fluorine derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinking and convolution of the axial organs</td>
<td>—</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Unilateral and bilateral microphthalmia</td>
<td>12</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Liver hernia</td>
<td>8</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Shortening of limbs</td>
<td>—</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td>Number of embryos investigated</td>
<td>145</td>
<td>242</td>
<td>267</td>
</tr>
</tbody>
</table>

* In many cases embryos in these groups showed more than one of above malformations and therefore were recorded more than once.

**DISCUSSION**

The present investigations have shown that the two derivatives of amino-phenyl nitrogen mustard, unlike the parent compound, first cause abnormalities in the process of neural tube closure if applied in the lowest effective dose at early stages of embryonic development. The appearance of this anomaly is parallel with or is followed by the first, still mild, necrotic changes in the cells of the neural plate, where giant cells and cells in the process of karyorrhexis or pycnosis are randomly scattered between normal cells.

After higher doses the neural plate tissue undergoes more profound necrosis with distinct localization, whereas the remaining structures of the embryo appear to be still normal or show only some minor necrotic changes. In extreme cases, when the dose used is still higher, all embryonic tissues undergo necrosis, resulting in early prenatal mortality and resorption of mouse embryos. Histologically these embryos, if fixed while still alive, resemble embryos treated with the parent compound.

A detailed comparison of all the experimental material of both chick and mouse embryos suggests that the embryonic germ-layers and the embryonic tissues derived from them can be arranged in the following approximate order of decreasing susceptibility to the compounds under investigation: medullary plate or neural tube > mesoderm and mesenchyme > entoderm > ectoderm.

Surviving 15-day-old mouse embryos, if examined histologically, are found to be largely those in which the neural tube injuries have been healed and which show only abnormalities caused by injury to the mesodermal structures similar to that following treatment with TEM (Jurand, 1959). This fact suggests that neural tube injury is much more critical for the future development of the embryo than is injury of the mesodermal structures.

Localized cell necrosis confined to particular embryonic regions has been
reported by many authors working with cytotoxic substances, and with amino-acid and purine analogues. Recently Schultz (1959), for example, reported that the somitic mesoderm of early chick embryos, as well as other tissues, shows extensive pycnotic changes after treatment with $\Omega$-brom-allyl-glycine (BAG) due to its leucine requirement.

The restriction of injury to the neural plate, which remains open and flat both in the chick and in the mouse embryos described in this report, recalls the results of Brachet (1958, 1959) and of Brachet & Delange-Cornil (1959), which were obtained after $\beta$-mercaptoethanol treatment of amphibian eggs. This compound was found to inhibit the movements of the presumptive medullary plate cells at the gastrula-neurula stage, which results in a flat medullary plate instead of neural tube without any visible influence on other systems. These authors attribute their findings to the reducing property of $\beta$-mercaptoethanol, which maintains the $-\text{SH}$ groups in the neural plate cells in the reduced form, thus causing a disturbance in metabolism. It seems reasonable to assume that nitrogen mustard derivatives, after being decomposed by hydrolysing enzymes to their parent compound in tissues containing such enzymes in higher concentration, interfere with the biochemical balance, although they probably do so at a different point in the metabolic process. It is widely accepted that the alkylating agents disturb the mechanism of DNA synthesis (Bodenstein, 1954) and as a result they disturb the synthesis of specific proteins (Danielli, 1954) indispensable for the morphogenetic development of the sensitive organ.

It is true that the spontaneous failure of neural tube closure called platyneury occurs sometimes in normal chick embryos but this phenomenon is always associated with anomalies in somite formation (Grünwald, 1935). In the present investigations no such anomalies in the somites of experimental embryos with neural plate non-closure were found after fluorine derivative, and the frequency of the injury was so high, even when the lowest effective concentration was used, that this effect cannot be compared with spontaneous platyneury. Moreover, there were no control embryos similar to the experimental ones. Last, but not least, spontaneous platyneury in mouse embryos is not known and yet the acetyl and fluorine derivatives, if used in proper dosage, prevent the closure of the neural tube.

In addition to causing injury to the medullary plate, both the acetyl derivative, as reported previously (Jurand, 1960), and the fluorine compound (particularly in higher dosage) have an adverse effect on the mesoderm. In the present investigations the fluorine derivative caused necrosis of the mesoblast in the limb-buds, leaving unharmed the outer layer, which is 2–3 cells thick, and the limb-bud epidermis, as well as the apical ectodermal ridge. This fact suggests that the epidermis and the underlying thin mesoblast layer are less susceptible than the deeper mesodermal cells. What causes the difference between the outermost and the internal cells of the mesoblast is not known. Possibly there are some biochemical similarities between the external layer of the mesoblast and the
epidermal cells due to their close proximity anatomically, but it may also be that the outermost layer of the mesoblast has some relation to the so-called 'refractile' layer of the mesoblast, which seems to play an epidermis-like role in limb differentiation. This problem has recently been the subject of discussion (Bell, Saunders, & Zwilling, 1959; Grünberg, 1960).

As far as the process of necrobiosis is concerned, it is well known that it is basically a natural process occurring in practically all living tissues, both in those in the course of differentiation and growth and those of adult organisms. The different stages of necrosis resulting from the cytotoxic activity of the compounds under investigation seem to be of the same nature as those described by many authors in normal tissues, like those in the regressing tissues of tadpoles' tails, or developing tissues (Leuchtenberger, 1950; Glücksmann, 1951; and others). It seems probable that cell death, regardless of its cause, follows the same pathway and results in the same cytological abnormalities of the nucleus, the cytoplasm, or both.

When the present data are looked at from a more general point of view some confirmation can be found of certain principles of teratogeny put forward by Wilson (1960). His second principle states that in some cases different agents produce characteristic patterns of defects due to similar action upon specific phases of metabolism in embryonic tissues. In other words, it seems that these characteristic patterns may result from the similar susceptibility of certain embryonic tissues to different agents, e.g. TEM, acetyl and fluorine derivatives, in respect of their activity against mesodermal structures. This might be the reason that in 15-day-old mouse embryos the teratological effects following the use of compounds under investigation are similar to those after triethanomelamine.

In conclusion, it should be emphasized that the influence of the acetyl and fluorine derivatives may be regarded as specific with regard to their morphogenetic effect on the neural plate non-closure; but the necrotic changes in the attacked organs are in general the same as after other radiomimetic agents. Some of the affected cells break down soon after treatment and others, arrested at the interphase, continue to grow and to reach giant proportions.

**SUMMARY**

The cytotoxic and morphogenetic activity of N-(p-amino-phenyl)-2, 2'-dichlorodiethylamine and its acetyl and fluoro-acetyl derivatives was studied on chick and mouse embryos.

In chick embryos the fluorine derivative showed a specific affinity for the medullary plate, causing inhibition of neural tube closure at lower concentrations and complete destruction of the medullary plate at higher concentrations.

Electron microscope observations of the affected neural cells showed some examples of necrotic changes after treatment with the fluorine derivative in chick embryos.
The parent compound and its acetyl and fluorine derivatives slow down the developmental rate of the mouse embryos. Here also, the tissue most sensitive to acetyl and fluorine derivatives appeared to be the neural tube, particularly its deepest part. The extent to which it is affected depends on the dose of the cytotoxic agent, so that a gradient in the sensitivity of the neural tube seems likely.

After higher doses the cytotoxic influence extends to other embryonic tissues which can be arranged as follows according to decreasing sensitivity: medullary plate or neural tube > mesoderm and mesenchyme > entoderm > ectoderm.

Older (i.e. 15-day-old) mouse embryos show malformations due to injury to the mesodermal structures, presumably because those with neural tube injuries either fail to survive or regenerate them. In the last case the injury to the mesodermal structures result in anomalies like convolution and kinking of axial organs, liver hernia, microphthalmia, &c.

**RÉSUMÉ**

*Nouvelles recherches sur les effets cytotoxiques et morphogénétiques de quelques dérivés de l'ypérite nitrée*

On a étudié, sur des embryons de poulet et de souris, l'activité cytotoxique et morphogénétique de la N-(p-amino-phényl)-2,2'-dichlorodiéthylamine et de ses dérivés acétylé et fluoro-acétylé.

Sur l'embryon de poulet, le dérivé fluoré a montré une affinité spécifique pour la plaque médullaire, inhibant la fermeture du tube nerveux aux concentrations les plus faibles, et détruisant complètement la plaque médullaire aux concentrations élevées.

Observées au microscope électronique, les cellules neurales atteintes montraient quelques exemples de modifications nécrotiques après un traitement par le dérivé fluoré, chez l'embryon de poulet.

Le composé initial et ses dérivés fluoré et acétylé ralentissent le rythme de développement des embryons de souris. Ici encore, le tube nerveux apparaît comme étant le tissu le plus sensible aux dérivés fluoré et acétylé et, en particulier, dans sa région la plus profonde. L'extension des lésions dépend de la dose du facteur cytotoxique, de sorte que l'existence d'un gradient de sensibilité du tube nerveux paraît vraisemblable.

Aux doses élevées, l'influence cytotoxique s'étend aux autres tissus embryonnaires, qui peuvent être rangés comme suit selon leur sensibilité décroissante: plaque médullaire ou tube nerveux > mésoderme et mésenchyme > endoderme > ectoderme.

Les embryons de souris plus âgés (15° jour) présentent des malformations dues aux lésions des structures mésodermiques, sans doute parce que ceux dont le tube nerveux a subi des lésions ou bien ne survivent pas, ou bien les réparent. Dans ce dernier cas, les lésions des structures mésodermiques provoquent des
anomalies telles que: enroulement et plissement des organes axiaux, hernie hépatique, microphthalmie, &c.

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REFERENCES


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Plate 1
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Plate 3
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Plate 4
EXPLANATION OF PLATES

Abbreviations: cd, cellular debris; dm, dense masses of necrotic chromatin; ec, eye cup; g, giant cells; lp, lens primordium; mi, mitochondria; ne, nucleus; ncl, nucleolus; nn, nuclear necrosis; no, normal cell; ns, necrosis; pg, pyronin positive granules; pn, pycnotic nucleus; v, vacuolization of cytoplasm; y, yolk granules.

PLATE 1

Fig. 1. Transverse section through the head of a control chick embryo (stage 12). x 80.
Fig. 2. Transverse section through the head of a chick embryo treated with fluorine derivative in concentration 2 x 10^-4. Note the flat neural plate. x 80.
Fig. 3. Transverse section through the head of a chick embryo treated with fluorine derivative (4 x 10^-4). Note a few necrotic cells at the bottom of the groove. x 80.
Fig. 4. Portion of an open neural plate of a chick embryo treated with fluorine derivative (4 x 10^-4). x 420.
Fig. 5. Tripolar metaphase in the neural plate of a chick after treatment with fluorine derivative. x 700.
Fig. 6. Transverse section through the head of a chick embryo treated with fluorine derivative (8 x 10^-4). Note complete necrosis of neural cells. x 80.

PLATE 2

Fig. 7. Electron micrograph of a control neural tube cell (chick). mi, mitochondria; nc, nucleus; ncl, nucleolus. x 8750.
Fig. 8. Electron micrograph of a giant cell in the chick neural plate after treatment with fluorine derivative (4 x 10^-4). x 8750.
Fig. 9. A chick neural tube cell with an enlarged nucleolus (ncl)-fluorine derivative (4 x 10^-4). x 8750.
Fig. 10. A chick neural tube cell in the stage of karyorrhexis after treatment with fluorine derivative (6 x 10^-4). Note the dense masses of necrotic chromatin (dm) at the periphery of the nucleus. x 8750.

PLATE 3

Fig. 11. Severely damaged neural tube tissue after treatment with fluorine derivative (8 x 10^-4). Note an advanced stage of nuclear necrosis (nn) in comparison with slightly less injured pycnotic nucleus (pn) in the lower left side of the photograph and yolk granules (y) in different stages of preservation. x 6600.
Fig. 12. Neural tube cells in chick after treatment with fluorine derivative (6 x 10^-4). In the middle there is a cell with a dense pycnotic nucleus (pn), beside a normal cell (no) with well-preserved nucleoli (ncl). x 10000.

PLATE 4

Fig. 13. Transverse section through the head of a 10-day-old mouse embryo after treatment with fluorine derivative (total dose 36 mg. per kg.). x 60.
Fig. 14. Transverse section through the head of a 10-day-old mouse embryo after treatment with acetyl derivative (total dose 45 mg. per kg.) x 60.
Fig. 15. Transverse section through the neural tube of a control 13-day-old mouse embryo. x 115.
Fig. 16. Transverse section through the neural tube of a 13-day-old mouse embryo treated with fluorine derivative (total dose 36 mg. per kg.). Note the necrotic changes (ns) at the bottom of the neural tube and the cellular debris (cd). x 115.
Fig. 17. Transverse section through the neural tube of a 13-day-old mouse embryo treated with fluorine derivative (total dose 42 mg. per kg.). Necrotic changes (ns) occupy more than half of the neural tube. x 115.
Fig. 18. Similar section after treatment with acetyl derivative (total dose 45 mg. per kg.). Note extensive necrosis (ns) in all tissues. x 115.
Fig. 19. Longitudinal section through a 13-day-old embryo treated with fluorine derivative (total dose 45 mg. per kg.). Note the comparatively well-preserved upper part of the neural tube and necrosis (ns) of the lower part, with convolutions. x 60.
Fig. 20. Transverse section through a 13-day-old mouse embryo after treatment with acetyl derivative (total dose 42 mg. per kg.). Note partial necrosis (ns) of the deeper part of the neural tube in the
trunk region and the open neural plate in the tail region. Retardation in the development amounted in this case to about 3 days. ×100.

**Fig. 21.** Transverse section through the neural tube which regenerated after injury by fluorine derivative (total dose 36 mg. per kg.). ×115.

**Fig. 22.** Transverse section through the hydrocephalic neural tube after treatment with fluorine derivative (total dose 42 mg. per kg.).

**Plate 5**

**Fig. 23.** Section through the developing eye of a control 11-day-old mouse embryo. ×330.

**Fig. 24.** Section through the developing eye of a 13-day-old mouse embryo treated with fluorine derivative (total dose 42 mg. per kg.). Note well-preserved lens primordium (lp) and many necrotic changes (ns) in the eye-cup. ×330.

**Fig. 25.** Dorso-ventral section through the limb-bud of an 11-day-old control mouse embryo. ×125.

**Fig. 26.** Dorso-ventral section through the limb-bud of a mouse embryo treated with fluorine derivative (total dose 48 mg. per kg.). Note the well-preserved epidermis and the adjacent layer of the mesoderm in comparison with necrosis (ns) of the internal part of the mesoblast. ×125.

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A. JURAND

Plate 5