Comparative Investigations of the Action of Two Nitrogen Mustard Derivatives on the Early Stages of Development of Chick Embryos

by A. JURAND

From the Institute of Animal Genetics, Edinburgh

WITH TWO PLATES

Cytotoxic compounds act by combining with the biochemical constituents of cells. Because of the complexity of living matter, the cytotoxic activity is highly complicated in nature and is therefore far from being thoroughly understood. In order to analyse the cytotoxicity of any chemical compound, many biological variables concerned in determining the mode of action of the compound and its selectivity for any particular range of cells have to be taken into account (Danielli, 1952, 1954).

Cells of the early embryonic stages are a suitable material for cytotoxic investigations. Although not completely differentiated, they soon arrange themselves into a few embryonic tissues originating directly from the three fundamental germ layers. These tissues consist of cells which may be regarded as the precursors of all the cells of the adult organ. It is interesting to inquire whether they show in these early stages a specific selectivity to cytotoxic compounds which is similar to the selectivity of tumour cells, and which may later be derived indirectly from different germ layers.

One of many biological variables which should be taken into account in cytotoxic investigations is the presence or absence of particular enzymes causing the cells to be more sensitive or more resistant to the cytotoxic drugs concerned. So far, very little information is available on the enzymatic constitution of the germ layers (Steinbach & Moog, 1955). The experiments reported here aimed at comparing the cytotoxic activity of two related derivatives of nitrogen mustard, one of which is an acetyl derivative of the other. Their formulas are as follows:

\[
\begin{align*}
\text{H}_2\text{N-C}_6\text{H}_4\text{N(CH}_2\text{CH}_2\text{Cl)}_2 & \quad \text{H}_2\text{C-CO-NH-C}_6\text{H}_4\text{N(CH}_2\text{CH}_2\text{Cl)}_2 \\
\text{N-(p-amino-phenyl)-2,2'-dichloro-diethylamine ('parent compound')} & \quad \text{N-(p-acetyl-amino-phenyl)-2,2'-dichloro-diethylamine ('acetyl derivative')} \\
\end{align*}
\]

It has been shown that the acetyl derivative is relatively less toxic for rats, and

that it is readily decomposed into its parent compound in tissues where hydrolytic peptidase is available (Danielli, 1954). This takes place particularly in cells of the Walker rat carcinoma, and the acetylation allows a much greater degree of selectivity in the cancerostatic properties. It seemed interesting to find out whether similar decomposition takes place in embryonic tissues and, if so, where and to what extent.

**MATERIAL AND METHODS**

Chick embryos at the definitive-streak stage (stage 4 according to Hamburger & Hamilton, 1951) explanted from eggs and cultured *in vitro* (New, 1955) were used. The chemical compounds were both obtained from Boots Drugs Co. Ltd., and were applied in a solution of liquid egg albumen containing 10 per cent. of sterile saline (0.9 per cent.). The concentrations of the two compounds are shown in the following table.

**Table 1**

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration</th>
<th>Number of embryos treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent compound</td>
<td>Acetyl derivative</td>
</tr>
<tr>
<td>1</td>
<td>$4 \times 10^{-6}$</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>$8 \times 10^{-6}$</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>$1.6 \times 10^{-5}$</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>$3.2 \times 10^{-5}$</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>$6.4 \times 10^{-5}$</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>$10^{-4}$</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>1.28 $\times 10^{-4}$</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>1.5 $\times 10^{-4}$</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>2 $\times 10^{-4}$</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>5 $\times 10^{-4}$</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>$10^{-3}$</td>
<td>4</td>
</tr>
</tbody>
</table>

In all the experiments the solutions were administered in amounts of 0.5 c.c. put round the plastic ring used. In the control experiments liquid egg albumen containing 10 per cent. of physiological saline was used. Reincubation of both control and experimental embryos lasted about 24 hours, until the control embryos had developed 15–19 somites, i.e. to stages 12–13 according to Hamburger & Hamilton (Plate 1, fig. 1).

Carnoy's liquid was used as a fixative. For macroscopical examination fixed embryos were stained with Carmalum or Mayer's haematoxylin and prepared as whole mounts. For histological and cytological purposes embedding was performed by standard methods. Transverse or longitudinal sections 6 µ thick were stained with methyl green-pyronine or in Feulgen reagent.

**RESULTS**

At concentrations lower than $6.4 \times 10^{-5}$ (nos. 1–4 in Table 1) neither the parent compound nor the acetyl derivative affected the external appearance or...
the microscopical structure of the treated embryos. Higher concentrations (nos. 5–9) of either compound proved to be cytotoxically effective. There was no significant difference between the compounds in degree of cytotoxicity. At the last two concentrations (nos. 10 and 11) both compounds appeared to be highly toxic, causing detachment, shrinkage, and necrosis of the blastoderms.

**Macroscopical observations**

After the lowest effective doses (nos. 5 and 6) the experimental embryos were found to be at stages 10 or 11, whereas the controls were at stages 12 or 13. Retardation of development became more evident as the concentration increased, being greater in some regions of the embryo than in others. The most severely affected organs appeared to be the paraxial mesoderm, the somites, and the neural tube.

Macroscopical observations indicated that the axial mesodermal structures were the most sensitive parts of the embryo. When solution no. 6 was used, both the parent compound and the acetyl derivative produced embryos with severely affected somites. A mild degree of change was characterized by complete emptiness of the somites, due to the absence of the somite core, and a considerable widening of the myocoele, which made the somites appear to consist of ring-shaped accumulations of mesodermal tissue. Along with this abnormality there was observed a marked shortening of the longitudinal axis of the body as compared to the control embryos. This was due more to the changes in the somite region than to those in the head part (Plate 1, figs. 2, 3).

Further changes, observed after treatment with concentrations nos. 7 and 8 of both compounds, were severe disturbances in the shape and arrangement of the somites, which were much fewer in number and far less dense in structure than those in the control embryos (Plate 1, figs. 4, 5). After higher concentrations, and especially after concentration no. 9, there were found embryos with almost completely disintegrated somites, or with only a few vestigial groups of mesodermal cells in the somite region bearing little resemblance to somites in their arrangement (Plate 1, fig. 6).

In all effective concentrations both compounds frequently caused the paraxial mesoderm to move away from the axial line in the region of the sinus rhomboidalis caudal to the somite region, leaving free areas on both sides. In some embryos there were characteristic symmetrical patterns of defective structure in the paraxial mesoderm (Plate 1, figs. 6, 7, 8, 9).

The neural tube was affected by both substances in the same manner. After concentrations nos. 7, 8, and 9 the brain roof usually remained unclosed (asyntaxia dorsalis), while the rest of the neural tube was unclosed in some parts or, in extreme cases, throughout its entire length (Plate 1, figs. 7, 9).

There were some differences in appearance between heads of embryos treated with the parent compound and those treated with the acetyl derivative. After doses nos. 5 and 6 of the parent compound the heads, although nearly normally
developed, appeared to be much narrower than those of embryos treated with the corresponding solutions of the acetyl derivative, which did not differ very much from the heads of control embryos. Although deprived of some somites and with partially unclosed neural tubes, the heads of these embryos treated with the acetyl derivative appeared almost normal and had mesenchyme cavities filled with the normal quantity of head mesenchyme (Plate 1, figs. 4, 7).

**Microscopical observations**

After treatment with both compounds, in cytotoxically effective concentrations chosen from the range used in preliminary experiments, the neural tube (i.e. brain and spinal cord), the somites, and, to some extent, the mesenchyme showed pronounced changes in their cytological structure.

The neural tube, after treatment with both compounds in the effective concentrations, showed degenerating cells of various types depending on the concentration used. In embryos treated with concentrations nos. 5–7, both compounds caused an increase in the number of neural tube cells containing nuclei 2–3 times larger than normal, with similarly enlarged nucleoli (Plate 1, figs. 10, 11). Nuclear enlargement, whether slight or very pronounced, appeared to be the first sign of the cytotoxic activity of these compounds. The enlarged nuclei showed poor stainability with methyl green, as can be seen in Plate 1, fig. 11, in which the enlarged nuclei appear to be almost colourless. Experiments using the Feulgen reaction proved, however, that the DNA content of these nuclei did not differ from that of control cells, except that it was more dilute due to the nuclear enlargement.

In embryos treated with the more concentrated solutions (nos. 8 and 9) there were, besides the changes in the size of the cell components, many neural tube cells showing necrotic changes such as karyorrhexis, pycnosis, and nuclear disintegration. Nevertheless, in the immediate neighbourhood of the degenerating cells there were found cells in different phases of cell-division. In the cytoplasm of the degenerating cells there were often found large and fine pyronine-positive granules stained like the nucleoli (Plate 2, figs. 12, 13).

Simultaneously with the changes on the cellular level the neural tube tissue showed increased retardation of its organogenetic development, particularly as regards its closure, as the degenerative changes in the cells became more pronounced.

Microscopical investigations of the somite region in embryos treated with both compounds showed that, in general, these structures are extremely sensitive to increases in the concentrations of the cytotoxic agents. Comparing figs. 14, 15, 16, and 17 of Plate 2 (demonstrating the histological structure of corresponding somites in longitudinal section) one can see the associated stages of degeneration in these important embryonic organs following treatment with different concentrations of the compounds. The more severely affected somites fail to show any differentiation into sclerotome, dermatome, and myotome. In such cases they
represent merely unorganized cell aggregations showing almost no metamerism and are sometimes fused into irregular lumps of mesodermal tissue.

In the experimental embryos examined the somite cells showed changes similar to those in the neural tube cells, although they did not undergo the extreme degenerative processes observed commonly in the latter. The enlargement of the somite cells and of their internal constituents was usually associated with a more or less marked disintegrating effect in their organogenetic appearance.

Other mesodermal structures (heart and extraembryonic mesoderm) did not show any other cytological changes than those described in the somites. Heart anlage, along with the general retardation of development, was usually also retarded. The blood islands showed a normal structure and contained similar numbers of dividing cells to those found in the control embryos.

The head mesenchyme also underwent degenerative changes, which were more noticeable after treatment with the parent compound than after the acetyl derivative. The differences are shown in Plate 2, figs. 18, 19, 20. After treatment with the parent compound the head mesenchyme tissue became less dense and its cells appeared slightly larger, with enlarged nuclei and nucleoli. Their cytoplasm was often vacuolized and their external shape was in most cases spherical, without the usual amoeboid character. These morphological changes suggest loss of the ability of the cells to form pseudopodia and, therefore, defective motility (Plate 2, figs. 21, 22). In extreme cases, after administration of the parent substance in the highest effective concentration (no. 9), the head mesenchyme was almost completely absent and the head mesenchyme cavities were shrunken.

Treatment with the acetyl derivative in similar concentrations did not have any distinct influence on the number or cytological appearance of the head mesenchyme cells. Even after treatment with the highest concentrations they remained similar to the control embryos.

The endodermal cells of the fore-gut and archenteron roof appeared not to be easily influenced by either compound. A comparison of the severely affected neural tube cells with the endodermal cells of the same embryo demonstrated the greater resistance of the latter.

Similarly, the notochord cells were more resistant to the cytotoxic activity of both compounds than were the neural tube and mesodermal cells.

DISCUSSION

According to data given by Danielli (1954, 1959) the LD$_{50}$ (rats) of the parent compound is 6–8 mg. per kg., whereas that of the acetyl derivative is 48–50 mg. per kg. This means that in rats the former compound is roughly 7 times more toxic than the latter.
In the experiments reported here it was found, however, that there was no difference between the two compounds in the effective and toxic doses. This fact seems to suggest that the cytotoxic mechanism of these compounds in embryonic tissues is in some way different from that in the adult rat. If it is true that the acetyl derivative acts cytotoxicly after being enzymatically activated, due to hydrolysis by some kind of peptidase, and that it does not act as a whole molecule, it can be assumed that in chick embryos it is decomposed in all the cells except those of the head mesenchyme.

In all the affected tissues degenerative changes were observed. After the lower concentrations the affected cells underwent either slight or severe cytoplasmic, nuclear, and nucleolar enlargement, sometimes reaching as much as 3 times their normal size. At higher doses they showed, in addition, karyorrhexis, pycnosis, and nuclear fragmentation. Similar degenerative effects have been recorded after nitrogen mustard in chicks (Karnofsky, 1950), after triethanomelamine in amphibian embryos (Waddington, 1958) and in chick and mouse embryos (Jurand, 1958, 1959), and after many different antimetabolites and antagonists (Waddington, Feldman, & Perry, 1955; Schultz, 1959).

Changes in somite differentiation have been observed in chick embryos after treatment with amino-acid analogues (Rothfels, 1954; Herrmann, Königsberg-Rothfels, & Curry, 1955) after purine antagonists (Waddington, Feldman, & Perry, 1955), and triethanomelamine (Jurand, 1958, 1959). There is a suggestion that all these changes, induced in such different ways, are due to some not fully understood disturbances in protein synthesis which give rise to the abnormal appearance and the loss of differentiation capacity of the affected tissue.

After treatment with either of these compounds cells at various mitotic stages are always found in the immediate neighbourhood of cells in the process of degeneration. This suggests that neither the parent compound nor its acetyl derivative can be regarded as having antimitotic properties.

The difference between the marked sensitivity of the neural tube, mesodermal structures, and head mesenchyme cells on the one hand, and the resistance of the entoderm and notochord on the other, is striking. There is no clear explanation of this difference. It is known that nitrogen mustard and its derivatives, as well as other related compounds, sometimes have an effect in the case of neoplastic diseases of blood-forming organs that are of mesodermal origin; but in the embryos neural tissue appears to be as sensitive as the axial mesoderm, and there is no evidence for any strong effect on the blood-islands.

The only difference found between the two compounds in the experiments reported here was that the parent compound affects the head mesenchyme cells whereas the acetyl derivative appears to have less effect on this tissue. A possible explanation is that it is due to the low content of hydrolytic protease in the head mesenchyme cells, but, so far, no proof of this has been obtained by cytochemical or histochemical investigations.
SUMMARY

1. N-(p-amino-phenyl)-2,2’-dichloro-diethylamine and its acetyl derivative have the same degree of toxicity for early stages of chick embryo development.

2. In concentrations of 6.4 x 10^-5 to 2 x 10^-4 they cause degeneration of the neural tube and somite cells. The first sign of degeneration is the enlargement of the affected cells. More severe damage (karyorrhexis, pycnosis, and nuclear disintegration) leads to pronounced disturbances in the differentiation of the affected organs.

3. The parent substance causes degeneration of the head mesenchyme, whereas its acetyl derivative is much less effective in this tissue.

4. Both compounds have comparatively little effect on the endodermal structures, the notochord, the heart, or the lateral mesoderm.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to the British Empire Cancer Campaign for the grant which enabled the prosecution of this work. Grateful thanks are due to Professor C. H. Waddington under whose direction this work was done and who was so kind as to read and discuss the manuscript. Last, but not least, the author thanks Miss A. P. Gray for the editorial advice and Miss A. R. Whightman for printing the photographs.

REFERENCES


—— (1959). Personal communication.


A. JURAND

Plate I
A. JURAND

Plate 2
DERIVATIVES ON CHICK EMBRYOS


EXPLANATION OF PLATES

**PLATE 1**

**Fig. 1.** Macroscopical view of a control embryo of 15-somite stages. ×20.

**Fig. 2.** An embryo treated with the parent compound (concentration 6·4×10⁻⁵). Note a marked shortening of the body-length. ×20.

**Fig. 3.** The somite region of the same embryo as Fig. 2. Note ring-shaped somites. ×50.

**Fig. 4.** An embryo treated with the acetyl derivative (1·28×10⁻⁴). Note the decreased number and the less dense structure of somites. ×20.

**Fig. 5.** An embryo treated with the parent compound (1·28×10⁻⁴). Note irregular arrangement of somites. ×20.

**Fig. 6.** An embryo treated with the acetyl derivative (2×10⁻⁴). Note nearly complete lack of somites. ×20.

**Fig. 7.** An embryo treated with the parent compound (6·4×10⁻⁵) showing comparatively normal development, but narrow head and unclosed neural tube in its caudal end. ×20.

**Fig. 8.** An embryo treated with acetyl derivative (1·5×10⁻⁴). Note nearly complete lack of somites, destructive changes in the paraxial mesoderm, unclosed neural tube, but almost normally developed head. ×20.

**Fig. 9.** An embryo treated with the parent compound (1·5×10⁻⁴). Note unclosed neural tube, vestigial somites, and the symmetrical pattern of the destructive changes in the caudal mesoderm. ×20.

**Fig. 10.** An embryo treated with the parent compound (15×10⁻⁴). Note nearly normal condition, ×80.

**Fig. 11.** Neural tube cells of an embryo treated with the parent compound (10⁻⁴). ×500.

**PLATE 2**

**Fig. 12.** Neural tube cells of an embryo treated with the parent compound (1·5×10⁻⁴). *Py*, pycnosis; *Kr*, karyorrhexis; *Nd*, nuclear disintegration; *Gr*, pyronin positive granules in the cytoplasm; *M*, dividing cell. ×830.

**Fig. 13.** Neural tube cells of an embryo treated with the acetyl derivative (1·5×10⁻⁴). *Py*, pycnosis; *Kr*, karyorrhexis; *Nd*, nuclear disintegration; *M*, dividing cell. ×830.

**Fig. 14.** Longitudinal section through somites of a control embryo. ×150.

**Fig. 15.** Longitudinal section through the somites of an embryo treated with the parent compound (6·4×10⁻⁵). ×150.

**Fig. 16.** Longitudinal section through somites of an embryo treated with the acetyl derivative (1·28×10⁻⁴). ×150.

**Fig. 17.** Longitudinal section through the somite region of an embryo treated with acetyl derivative (2×10⁻⁴). Note the vestigial structure of the somites. ×150.

**Fig. 18.** Transversal section through the head of a control embryo. ×80.

**Fig. 19.** Transversal section through the head of an embryo treated with the parent compound (1·5×10⁻⁴). Note the unclosed neural tube and loose mesenchyme tissue. ×80.

**Fig. 20.** Transverse section through the head of an embryo treated with the acetyl derivative (1·5×10⁻⁴). Note nearly normal condition. ×80.

**Fig. 21.** Head mesenchyme cells of a control embryo. ×600.

**Fig. 22.** Head mesenchyme cells of an embryo treated with the parent compound. *V*, vacuolization of the cytoplasm; *Nd*, nuclear disintegration. Note the spherical shape of the cells. ×600.

*Manuscript received 21:ix:59*