Developmental Abnormalities in the Rat
Induced by Heat Shock

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WITH THREE PLATES

INTRODUCTION

Having repeated some older experiments, Brachet (1949) has shown that amphibian gastrulae submitted to a heat shock either stop their development or exhibit numerous specific malformations. Cytochemical and biochemical analyses made by several workers have left little doubt that RNA is particularly sensitive to heating (Brachet, 1949; Mookerjee, 1953; Hasegawa, 1955). Furthermore, it has recently been shown (Rosenbaum, 1960) that in amphibian eggs blocked at gastrulation by exposure to very high temperatures, catheptic activity increases.

As far as mammals are concerned, high temperatures have been applied to pregnant females but not directly to the embryos, so that the results cannot be easily compared with those obtained in other classes of vertebrates. Several authors have succeeded in obtaining resorption, abortion or rarely slight abnormalities (Hsu, 1948; MacFarlane et al., 1957; Brinsmade & Rübsaamen, 1957; Svetlov & Korsakova, 1959) which differ greatly from those obtained in amphibians.

If the direct application of temperature to the mammalian embryo were possible, it would, in our opinion, give a deeper insight into the embryonic development of mammals. We tried, therefore, to work out a simple method of applying high temperature directly to uterine horn, while the temperature of the maternal body remained unchanged. This paper deals with the effects of locally applied high temperature on different phases of rat development.

Most attention will be given to externally visible malformations.

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MATERIAL AND METHODS

Albino rats, which have been randomly interbred for more than 10 years in our laboratory, were used for the present experiment. No spontaneous congenital malformations have been observed.

Gestation was considered to have begun at 5 a.m. (Mulnard, 1955) of the morning on which sperm was found in the vaginal smear of females allowed to breed on the preceding night.

For our purpose the pregnant females were anaesthetized either with ether or with nembutal, and the ventral abdominal wall was opened by a large incision. After observation of both uterine horns one of them was brought to the surface of the incision. In the mesometrium of this horn a little incision was made which permitted a strip of gauze to be introduced underneath the horn. Care was taken to avoid compression of uterine blood vessels and handling was kept to a minimum. The incision in the abdominal wall was partly closed so that almost all the implantation sites of one uterine horn remained outside the body.

The animal was afterwards turned over and put on a thin wooden board with a hole in the middle, through which the operated uterine horn was to be introduced into the liquid. The board was placed over a glass container previously filled up with a slightly hypotonic physiological saline. The temperature of the saline was held constant to within 1°C. during the experiment. Every 10 min. some new hot saline was poured into the container to replace the same quantity of liquid. The reason for maintaining a constant temperature in this way was to prevent the saline from becoming hypertonic by evaporation, and to preserve its transparency. However, the saline did become slightly turbid due to the leakage of blood of the uterine horn. The temperature of the saline was 37–38°C. for the control series and 40–41°C. for the experiments. It must be pointed out that in all the experiments one uterine horn was not touched, its foetuses serving as controls.

After 40 min. to 1 hr. the operated horn was rapidly restored to its usual position in the abdominal cavity. The females, which were treated between the 8th and the 12th day of pregnancy were killed on the 15th, and those operated upon later were killed on the 20th day of gestation. Embryos were fixed in Bouin's mixture and examined under low magnification for externally visible malformations. The majority of them were serially sectioned and stained with haemalum and eosin for microscopic study. A few females were killed between 2 and 24 hr. following treatment and the embryos sectioned and stained with Unna-Brachet mixture for cytological examination.

RESULTS

Control series

On day 9½ twenty-six females were treated as indicated for controls. The uterine horn was kept in saline at 37°C. for 1 hr. Table 1 shows that the incidence of resorption was not significantly higher than that in the other horn.
There were no visible malformations. It can be concluded that the handling of one uterine horn does not disturb subsequent development.

On the other hand, on day 15 this simple procedure causes an increased incidence of resorption. The embryos, being already very heavy, do not probably permit the normal blood circulation during the experiment. It must be recalled that the experimental uterus horn with embryos is hanging on the opened abdominal wall. When the embryos are small there is no interference with circulation, but when they grow larger they probably disturb the circulation by their weight.

General survey of results

Table 2 shows the results of all the experimental series. It can be seen from the table that different numbers of animals were used in different series. In

<table>
<thead>
<tr>
<th>Day of gestation</th>
<th>Number of animals</th>
<th>Normal</th>
<th>Malformed</th>
<th>Resorbed</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>26</td>
<td>113</td>
<td>15 (12%)</td>
<td>89</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>6</td>
<td>26 (81%)</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

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On day 7½ the embryos withstood treatment in the hot saline even for 90 min. without any malformation occurring. From day 8½ 40 min. were sufficient to produce abnormalities specific for each phase of development.

Resorption

It is well known that normally a certain percentage of mammalian embryos undergo resorption. The data obtained in one strain of mice make an overall resorption rate of 15.7 per cent. (McLaren & Michie, 1956), but the incidence of death is often much higher (Boyd & Hamilton, 1952). In our experiment the resorption rate after implantation was alone taken into account, because the operations were carried out from the 8th day of pregnancy when implantation had already taken place. If on the day of treatment no implantation sites were observed, although several days earlier sperm had been found in the
vaginal smear, the female was excluded from further consideration. For the same reason animals whose untouched control horn was without an embryo on the day of autopsy were discarded. Such cases were probably due to operational stress. Thus our analyses will be based solely on those animals whose embryos in the control horn were normal at autopsy.

The incidence of resorption in the control horn was about 10 per cent. if the animals were killed on the 15th day of pregnancy and a little more (15–20 per cent.) when they were killed on the 20th day.

From Table 2 and Text-fig. 1 two conclusions can be drawn. The percentage of resorption rises slowly from the 8th to the 12th day, with a peak on the latter day, and then there is a significant drop. The high resorption rate in later series is, as mentioned above, unspecific. Another result worth mentioning is a positive correlation between the duration of heat shock and the number of resorptions. The embryos of the 8th day were very resistant, while those of the 9th day were not.

**Malformations**

True malformations were recognized in animals treated on or after day 8½, treatment on day 1 causing no subsequent defects. The percentage of malformations was calculated to include all embryos, even from those which were in resorption or which had already resorbed but whose implantation site had previously been observed. The duration of heat shock did not influence either the number or the severity of the malformation produced.

<table>
<thead>
<tr>
<th>Eye</th>
<th>Brain</th>
<th>Face</th>
<th>Palate</th>
<th>Foot</th>
<th>Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>8½</td>
<td>Anophthalmy</td>
<td>Smaller prosencephalon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microphthalmy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9½</td>
<td>Anophthalmy</td>
<td>Anencephaly, smaller prosencephalon</td>
<td></td>
<td>Without snout, pr. maxill.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microphthalmy</td>
<td></td>
<td></td>
<td>not fused</td>
<td></td>
</tr>
<tr>
<td>10½</td>
<td>Microphthalmy</td>
<td>Smaller prosencephalon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11½</td>
<td>Rosettes in retina</td>
<td>Rosettes in prosencephalon</td>
<td></td>
<td></td>
<td>Short</td>
</tr>
<tr>
<td>12½</td>
<td>Thin wall of lat. ventr., rosettes in prosencephalon</td>
<td></td>
<td></td>
<td>Forefeet</td>
<td>only 4 toes</td>
</tr>
<tr>
<td>13½</td>
<td>Total size smaller, rosettes in prosencephalon</td>
<td></td>
<td>Cleft palate</td>
<td>Micromely</td>
<td>syndactyly ectrodactyly</td>
</tr>
</tbody>
</table>

Table 3 shows the different malformations obtained after treatment on days 8½ to 13½.
\[8\frac{1}{2}\] days

The eye is almost always affected. From seven cases six had anophthalmy while only one had a smaller prosencephalon.

\[9\frac{1}{2}\] days

As we had for this day fifty-five cases of malformation it is preferable to give a table with frequency of different malformations (Table 4). As regards

**Table 4**

*Frequency of different malformations following the treatment on day 9½*

<table>
<thead>
<tr>
<th>Day of autopsy</th>
<th>15</th>
<th>20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microphthalmy</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Deformity of the head</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anophthalmy only</td>
<td>8</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Anophthalmy</td>
<td>27</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>Deformity of the head</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extreme defects of brain and face</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>7</td>
<td>55</td>
</tr>
</tbody>
</table>

the eye it is very often damaged. There are different abnormalities ranging from total absence of the organ to only small deformities of optic stalk and lens. On the presented table we called deformity of the head the malformations of the prosencephalon. It is very often smaller than usually, so that the lateral ventricles are small too, and even absent. In some rare cases only some clusters of nerve cells represented the prosencephalon. When the maxillary processes were not fused, and when the snout was not formed, we called this kind of malformation the extreme defects of the face. In all these cases no eyes were formed. At autopsy on the 20th day post-conception these cases presented malformation often called anencephaly. It must be nevertheless pointed out that the same defects were already present at autopsy on the 15th day and that the anencephaly is not only the consequence of the degeneration of once well developed brain (Plate 1, figs. A–D, Plate 2, figs. F, G).

\[10\frac{1}{2}\] days

Three embryos have smaller prosencephalon. One of them has relatively well developed eyes but oriented in a wrong direction (Plate 3, fig. J). Another embryo was without the lens on one side, while on the other side the lens was present but very small.

\[11\frac{1}{2}\] days

Two embryos have short tails, and one of them shows on sections some deformities of the brain. It was easy to find typical rosettes in the lateral walls
EXPLANATION OF PLATES

PLATE 1

Experiment on day 9½, autopsy on day 15

FIG. A. Important malformations of the face and the head.
FIG. B. Normal embryo.
FIG. C. Abnormality of the forehead.

Experiment on day 9½, autopsy on day 20

FIG. D. Normal (left) and anencephalic embryo (right).

Experiment on day 13½, autopsy on day 20

FIG. E. Deformities of the fore and hind feet.

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(Facing page 450)
FIG. F. Frontal section through the forehead of normal (left) and malformed embryo (right). Important malformation of the brain (see Fig. D).

FIG. G. The same as Fig. F, but killed on the 15th day of gestation (see Fig. A).

FIG. H. Section of a control embryo without any cytological changes (compare with Fig. I).

FIG. I. Section of an embryo, 6 hr. after operation. The medullary plate shows more necrotic cells than the mesoderm (see explanation in text).

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**PLATE 3**

*Experiment on day 10 1/2, autopsy on day 15*

Fig. J. Frontal section. Microphthalmia, the optic stalk and the lens are very small and malformed (wrong orientation).

*Treatment on day 12 1/2, autopsy on day 20*

Fig. K. Frontal section of an eye with malformed retina (two rosettes behind a normally configurated lens).

Fig. L. Frontal section through the telencephalon with many rosettes.

*Experiment on day 13 1/2, autopsy on day 20*

Fig. M. Section of the hind foot (compare with Fig. E). Note a mass of blood without the skeletogenous tissues of the digits.

Fig. N. Frontal section of a fetus with meningocele.

Fig. O. Section of a cleft palate.

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of telencephalon, which were already many times described after X-rays irradiation (Hicks et al., 1957).

12\frac{1}{2} days

Two embryos have microphthalm and one of them only four toes on the forefeet. The latter malformation was visible on three other embryos. Histological analysis has been able to show that the brain and the eye are damaged, too. The wall of lateral ventricles was thinner and the rosettes were present (Plate 3, fig. L). In one embryo the ventricles were very large. The retina is malformed or has some rosettes (Plate 3, fig. K).

13\frac{1}{2} days

Although the damage is especially great on the foot the brain shows changes which are very similar to those after X-rays. Six embryos have damaged feet without toes, or lacking only some toes. The foot can be smaller than usual, so that as well as syndactyly, micromely was observed. In one case the hindfeet were swollen and like boxing gloves (Plate 1, fig. E, Plate 3, fig. M). The swollen form is due to the accumulation of blood in a probably previously degenerated region (Giroud et al., 1955). In three cases cleft palate was observed and only in one meningocele (Plate 3, figs. N, O).

The rosettes were present in the dorsal, lateral and inferior parts of lateral cerebral vesicles and the corpus callosum seems to be thinner. Although it is difficult to appreciate the size of the brain without a quantitative measurement it seems that at least a slight reduction in total size is rather a rule than an exception. Syndactyly occurred even later, but we preferred not to analyse embryos treated later because the percentage of resorption increases in the controls, which is a sign probably of a bad vascular circulation.

It should be borne in mind that other possible abnormalities of internal organs cannot be excluded.

Immediate effect

Different cytological changes were observed on serial sections 2 to 24 hr. after heat shock.

Some granules stainable with pyronine were found both inside and outside the cells 2 hr. after treatment. Six hours later we could observe a range of diverse cytological changes. Some egg cylinders remained unchanged, while others showed plenty of granules stained either with pyronine or methyl green. These cytological alterations were observed either in the ectoderm, or in the ectoderm and mesoderm, but never in the mesoderm alone. The entoderm does not seem to be affected (Plate 2, figs. H, I). These observations suggest the possibility of the germ layers being selectively sensitive. In extra embryonic parts there were small changes only if the embryo itself was already affected. Sometimes a few decidua cells with cytological changes were seen.
All the observations described represent, from the pathological point of view, a process of necrosis which is similar to that observed following X-rays. We have so far too few observations to be able to single out the reason for this selective sensitivity.

**DISCUSSION**

It was shown that heat shock locally applied could produce resorption and malformations similar to those obtained by X-rays (Russel & Russel, 1954).

In order to give an adequate explanation of the results obtained some points must be discussed separately.

**Reliability of the method**

There are many shortcomings in the technique used in our experiment. One of them must be especially pointed out. During the experiment, when the uterine horn is kept in the hot saline, it is almost impossible to see exactly through the slightly turbid medium how many embryos are in the water. It is possible that one or more of them are pulled back to their normal position, together with a part of the uterine horn. Very often the embryos at one or both of the uterine extremities remain normal, having probably been pulled out of the saline. For this reason the percentage of resorptions can be calculated only approximately.

The later stages of pregnancy cannot be examined by this method, because the control series show a high incidence of resorption in comparison with the corresponding untouched uterine horn.

In spite of this, the peak of death incidence on day 11½ seems to be real. Similar results were obtained when pregnant rats were exposed to a hot environment (Svetlov & Korsakova, 1959). It seems likely that these results are due to the same cause. The formation of the rat placenta is proceeding very actively on the 11th and 12th days of gestation (Everett, 1935). It must therefore be concluded that besides producing many cytological changes in the embryo, heat shock also acts on the rapidly growing placenta, which seems to be particularly vulnerable to damage. All the workers who have exposed pregnant females to hot environments observed resorption but hardly ever any malformation. For producing malformations our method seems preferable to the others used so far.

While other workers (Brinsmade & Rübsaamen, 1955) have obtained rare and unspecific malformations, we succeeded in obtaining external deformities typical of each stage treated. This method, which is easily applicable, might throw more light on a number of problems in mammalian causal embryology.

**Periods of development sensitive to heat shock**

Comparing the effect of X-rays with our data and taking into account only the malformations of the head and feet, an almost complete similarity can be
observed. In both cases the forebrain is damaged during the 9th and 10th day, while the feet are damaged on the 13th and 14th day (Hicks, 1953; Wilson et al., 1953).

Other teratogens cannot be easily timed, since their action is often chronic. In the terminology of Kalter & Warkany (1959) heat treatment applied as in our experiments can be referred to as an 'acute teratogen'. Large doses of vitamin A (Giroud & Martinet, 1956), although their action may be chronic, have also the same critical period as regards brain defects, but lead very rarely to syndactyly. By contrast, 8-azaguanine (Nishimura & Nimura, 1958), which also leads to abnormalities of the extremities, has no clear-cut periods of action.

All these responses of the embryo can easily be understood if the morphological and cytochemical data are taken into consideration. It is well known that the mesoderm starts to form on day 8½, the very day when heat treatment leads to brain malformations. The neural plate is formed during this stage and Mulnard (1955) was able to show that the activity of alkaline phosphatase suddenly increases in the presumptive neuroctoblast. The basophilia of this layer is also very strong. The formation of extremities in the rat begins on the 12th day of gestation and on the 13th day RNA and phosphatase increase in the anterior buds (Milaire, 1956). Heat shock leads to abnormalities on the 13th day.

Sensitive periods for the agent used in our experiment correspond to the periods of active metabolism and synthesis of differentiating presumptive organ regions. Naturally, much more remains to be done regarding other presumptive regions.

The specificity of heat shock

In order to exclude possible unspecific effects of heat shock some experiments must be recalled and presented together with our control series.

The careful handling of an uterine horn up to the 15th day of gestation does not disturb the normal development. Even later it is only the resorption rate which increases. According to Brent & Franklin (1960), uterine vascular clamping on the 9th day of gestation, lasting 1 hr., has no deleterious effect. Longer clamping leads to some abnormalities. This experiment allows the conclusion that on the 9th day, the rat embryo, even if it is completely isolated from the maternal circulation for 1 hr., remains unaffected. Also, in pregnant female mice subjected to hypoxia or fasting, a treatment longer than 1 hr. is needed to produce malformations (Runner & Dagg, 1960). In our experiments, however, heat treatment of no more than 40 min. produced specific malformations.

These results strongly suggest that, at least on the 9th day of pregnancy, heat shock has some direct action on embryonic cells, which undergo necrosis.

Cytological analysis leads to the same conclusion. The embryonic cells are easily damaged, while the extra-embryonic cells and the cells of uterine origin
are damaged very rarely, and, if so, it never occurs without simultaneous pronounced changes of the embryonic cells.

It is, however, difficult to state what is the real cause of the changes observed. Finally it must be stated that heat shock may perhaps interfere in some way with placental function, because we have no data which exclude this possibility.

Contrary to the data of Stilwel (1957) dealing with the cells in vitro, heat shock does not affect mitoses, for the majority of cell divisions seem to be normal.

The hypothesis of a specific action of heat shock is further substantiated by the quality of brain malformations. Different teratogens can lead to brain or head malformations. Thus large doses of vitamin A (Giroud & Martinet, 1957) lead to anencephaly, which is the consequence of the degeneration of a previously well developed brain. Actinomycin D (Tuchmann-Duplessis & Mercier-Parot, 1959) or the lack of panthotenic acid (Giroud et al., 1957) mainly produce exencephaly, while cold applied to pregnant hamsters (Smith, 1957) has a rather inconsistent effect leading to hydrocephalus, anencephaly and herniation of the brain.

Hydrocephalus can also be caused by injections of trypan blue (Wilson, 1954). In considering hypoxia and the lack of folic acid (Ingalls et al., 1952; Giroud et al., 1952) as teratogens it should be pointed out that they effect the brain very rarely. The former can lead to hydrocephalus and the latter to exencephaly with hydrocephalus.

Finally we must compare the effect of mustard gas and X-rays observed on the same strain of rats in our laboratory. Mustard gas can produce exencephaly only after the 12th day of pregnancy (Müller, unpublished results), while the effect of X-rays is very similar. As was clearly shown by Wilson et al. (1953) by the 5th post-irradiation day some areas on the surface of the head begin to bulge outward. Hicks (1953) observed anencephaly at term which was probably a direct effect of degenerative changes in the herniated brain.

In our laboratory the same abnormalities following X-rays were observed at autopsy on the 15th day post-conception (Škreb, 1961). However, after heat shock the brain did not bulge outward in any instance. As early as a few days after treatment the telencephalon was more or less defective, or in some cases completely absent.

According to Hicks (1953) some embryos treated by X-rays 'had already become anencephalic' when removed on the 15th day of gestation. Malformations obtained after treatment on later days of gestation are even more like those following X-rays. Short, deformed tail in our experiments were already mentioned by Hicks et al. (1957) after X-rays. Formation of so called rosettes (a cluster of cells around a center) is, according to Hicks et al. (1957), 'a characteristic reaction in the embryonic nervous system occurring after many injuries to the primitive neural cells'.

According to cytological observation immediately after heat shock we must presume the death of certain groups of cells as the main cause of malformations.
It seems likely that the same groups of cells are sensitive to heat shock and to the X-rays, because the effects are so similar. Naturally we cannot find the reason and the whole problem of differential sensitivity to the heat shock remains open. It would be interesting to work with the isolated mammalian embryos to exclude the eventually indirect effect via placenta and the uterus.

The eye, the palate and the feet, which were also malformed in our experiments, showed similar changes after different teratogens.

If we can presume a direct action of heat on embryonic cells in regard to malformations, it seems that the high temperature leads to resorption by two simultaneous ways: by interfering with the growing placenta and by killing the embryonic cells. The hypothesis that resorption is rather an unspecific and probably indirect effect of heat, has been claimed several times by other workers. Hsu (1948) showed that a hot environment produced only resorption without any abnormalities, which was later verified by Svetlov & Korsakova (1959). MacFarlane et al. (1957) were able to diminish the high percentage of resorption by application of certain hormones. Shah (1956) was able to show that unheated blastocysts transplanted to heated pseudopregnant does underwent resorption, whereas heated embryos in an unheated female developed normally.

Brinsmade & Rübsaamen (1957) were the only investigators who were rarely able to get malformations following heat treatment of the pregnant does. It must be pointed out that they obtained the increase of the body temperature by injections of milk and not by a hot environment. They observed only three visible malformations (one encephalocele, two microcephaly) and very little resorption (seven of seventy embryos from thirteen females). Without further verification on a larger scale it would be very difficult to explain their results.

At the present time it would also be difficult to say why one cannot obtain malformations when the maternal body is heated, but can do so very easily when heat is applied locally.

**SUMMARY**

1. A simple method for applying heat shock directly to the uterus of pregnant rats is described.
2. In an experimental series a temperature of 40–41°C. was maintained for 40 min. A number of malformed foetuses were observed, and the resorption rate was high.
3. In a control series animals were operated upon in the same manner as the experimental ones, but the temperature was kept at 37–38°C. No malformations were found.
4. Deformities of the head and extremities were produced by the heat shock.
5. These malformations are compared with those produced by other teratogens, and the differences are discussed.
RESUME

Malformations induites par un choc thermique

1. Les auteurs ont élaboré une méthode très simple afin d'appliquer un choc thermique directement sur l'utérus des râtes gestantes.

2. Pour les séries expérimentales la température a atteint 40° à 41°C pendant une durée de 40 minutes. Plusieurs malformations ont été trouvées et le taux de résorption a été élevé.

3. Les témoins ont été opérés de la même manière mais la température a été seulement de 37° à 38°C pendant une heure. On n'a pas trouvé de malformation.

4. En ce qui concerne les malformations, on a trouvé les déformations de la tête et des extrémités.

5. La qualité des malformations obtenues a été comparée avec différents effets d'autres agents teratogènes. L'interprétation possible a été discutée.

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